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# Genetic Variation and Structure of *Rhododendron brachycarpum* D. Don, a Rare and Endangered Tree Species in Korea

By S. W. LEE[1]) Y. M. KIM, W. W. KIM, S. S. JANG and J. M. CHUNG

Korea Forest Research Institute, 44-3 Omokchun-dong, Kwonsun-ku, Suwon 441-350, Rep. of Korea

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**Summary**

Rare and endangered plant species are commonly hypothesized to have little genetic variation because of inbreeding, genetic drifts, genetic bottlenecks and/or other factors. We investigated genetic variation in *Rhododendron brachycarpum* D. Don, a rare and endangered evergreen tree species in Korea, by examining allozyme variation at 13 loci in 200 individuals distributed among 6 populations. As expected, low level of genetic diversity was observed ($A$ = 1.3, $P$ = 29.5 %, $H_e$ = 0.075). While a single allele was the most common in almost all the polymorphic loci, two allele frequencies were completely or nearly intermediate at the locus *Pgi-2* in all populations, suggesting a selection effect on the *Pgi-2*. The Uleung Island population (*R. brachycarpum* var. *roseum*) had a unique genetic structure and was most distinctive from the inland populations.

*Key words*: *Rhododendron brachycarpum*, rare and endangered species, genetic variation, allozyme, selection.

## Introduction

The genus *Rhododendron* is a large taxonomic group, including evergreen and deciduous woody species and is primarily distributed in the Northern Hemisphere. In Korea, up to 12 different rhododendron species have been recorded (LEE, 1989). *Rhododendron brachycarpum* D. Don is an insect-pollinated, alpine evergreen tree species, native to central and southern Korea, the Kuril Islands, and mountainous northern and central parts of Japan. The species can reach a maximum height of about 4m, withstand wind and dry mineral soil better than any other rhododendrons occurring in Korea, and can live in temperature under –30 °C ~ –40 °C. In Korea, *R. brachycarpum* has been traditionally used as a medicinal plant and has been designated as a rare and endangered plant.

Rhododendrons have been the subject of extensive genetic studies in recent years (KREBS, 1996; ESCARVAGE et al., 1998; NAITO et al., 1999; DE RIEK et al., 1999; PORNON and ESCARAVAGE, 1999; PORNON et al., 2000; MILNE and ABBOT, 2000; Ng and CORLETT, 2000; KAMEYAMA et al., 2001), but there have been no published studies of population genetics in *R. brachycarpum*. The objectives of this study were: (1) to estimate levels of genetic diversity in six native populations of *R. brachycarpum* in Korea employing allozyme markers; (2) to measure the distribution of genetic variation within and among populations; and (3) to assess the implications for conservation of this species in Korea.

## Materials and Methods

### *Plant materials*

From the late June to the mid-July of 2001, foliage tissues were collected from six natural stands located throughout the native range of *R. brachycarpum* (*Fig. 1*) in Korea. Within each stand, 31–36 trees were selected for foliage collection with a minimum distance of 30 m between trees in order to decrease the risk of relatedness. In several stands such as HONGCHEON and JIRI, however, the scarcity of suitable trees dictated sampling in close proximity (within 30 m) until the goal of 30 trees was reached. Foliages collected were placed in ice chests, and transported to the laboratory within 48 h, where they were stored at 4 °C.

[1]) To whom correspondence should be addressed. LEE Seok-Woo: Tel: 82-31-290-1154, Fax: 82-31-290-1040, e-mail: swlee66@hotmail.com

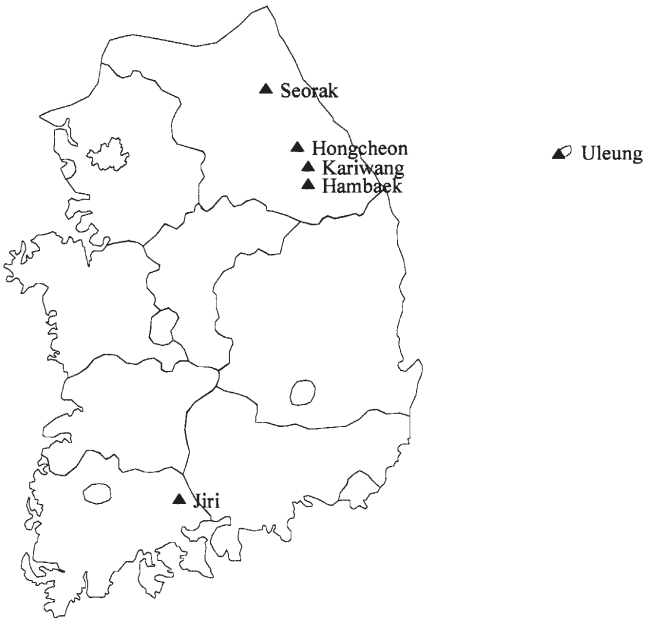


*Figure 1.* – Locations of the six study populations of *R. brachycarpum* in Korea.

*Enzyme extraction and allozyme procedure*

Enzymes were extracted between 1 and 7 d after collection. Leaves were cut finely, and crushed with a mortar and pestle in an extraction buffer. In preliminary trials, enzyme activity showed the best results in the CHELIAK and PITEL (1984) extraction buffer. The enzyme extract was absorbed onto 4 mm × 10 mm wicks cut from Whatmann 3MM chromatography paper, which were stored at –70 °C until needed for analysis.

Using techniques of starch-gel electrophoresis based on CONKLE et al. (1982), 15 enzyme systems were surveyed to delineate suitable systems. Nine enzyme systems showing consistent and clear band patterns were selected to conduct the study (*Table 1*). Where several zones of activity were observed for a single enzyme, hyphenated numerals following the enzyme abbreviation were used for identification.

*Table 1.* – List of enzyme systems tested.

| Enzyme | Abbreviation | E.C. No. | Buffer* | No. of loci scored |
|---|---|---|---|---|
| Acid phosphatase | ACP | 3.1.3.2 | B | -** |
| Aconitase | ACO | 4.2.1.3 | E | - |
| Aspartate aminotransferase | AAT | 2.6.1.1 | B | 1 |
| Esterase | EST | 3.1.1.- | A | - |
| Glutamate dehydrogenase | GDH | 1.4.1.3 | B | 1 |
| Glucose 6-phosphate dehydrogenase | G6PD | 1.1.1.49 | B | 2 |
| Formate dehydrogenase | FDH | 1.2.1.2 | B | - |
| Isocitrate dehydrogenase | IDH | 1.1.1.42 | E | 1 |
| Leucine aminopeptidase | LAP | 3.4.11.1 | A | 1 |
| Malate dehydrogenase | MDH | 1.1.1.37 | E | 2 |
| Menadion reductase | MNR | 1.6.99.- | A | 1 |
| 6-Phosphogluconate dehydrogenase | 6PGD | 1.1.1.44 | B | 2 |
| Phosphoglucose isomerase | PGI | 5.3.1.9 | A | 2 |
| Phosphoglucomutase | PGM | 2.7.5.1 | B | - |
| Shikimate dehydrogenase | SKDH | 1.1.1.25 | E | - |

*A = a lithium borate electrode buffer (pH 8.3) used with a Tris citrate gel buffer (pH 8.3); B = a sodium borate electrode buffer (pH 8.0) used with a Tris citrate gel buffer (pH 8.8), C = a morpholine electrode and gel buffer (pH 8.0). For more details, refer to CONKLE et al. (1982).
**- = tested but not included for scoring due to the lack of enzyme activity and/or smeared band patterns.

*Estimating genetic parameters*

We used the BIOSYS-1 (SWOFFORD and SELANDER, 1989) computer program to estimate genetic diversity (*A*, the number of alleles per locus; $P_{99}$, and $P_{95}$, the proportion of polymorphic loci at the 99 %, and 95 % level, respectively; $H_o$ and $H_e$, the observed and unbiased expected heterozygosities), WRIGHT's (1965) *F* statistics ($F_{IS}$, $F_{IT}$, and $F_{ST}$), NEI's (1978) genetic distance and identity, and UPGMA-derived dendrogram according to SNEATH and SOKAL (1973). The degree of genetic isolation among populations was estimated by *Nm*, the number of migrants per generation. *Nm* was calculated from WRIGHT's $F_{ST}$ (WRIGHT, 1951), and from the number and frequency of private alleles, the unique alleles found in only one population (BARTON and SLATKIN, 1986) using the GENEPOP program (RAYMOND and ROUSSET, 1995a). Deviations of genotype distributions from the Hardy-Weinberg expectations were tested by exact tests (ROUSSET and RAYMOND, 1995). The exact *P* values were estimated by the Markov chain method (RAYMOND and ROUSSET, 1995b) using GENEPOP software. We used the computer program BOTTLENECK (CORNUET and LUIKART, 1996) to determine whether effective population numbers had been restricted in the recent past. The infinite allele model (KIMURA and CROW, 1964) was chosen because empirically it tends to fit allozyme data better than alternatives (LUIKART and CORNUET, 1998). The Wilcoxon sign-rank test was preferred to the sign test because the former has higher power and can be used with as few as four polymorphic loci (PIRY et al., 1999). A Mantel test, using 500 random replicates, was performed using the Mantel version 2.0 software (LIEDLOFF, 1999), to test for significance of correlation between geographical distance and genetic distance among populations.

## Results

Thirteen loci were consistently resolved and used for statistical analysis (*Table 1*). Seven of 13 loci were monomorphic in all populations sampled (*Gdh, 6Pgd-1, 6Pgd-2, Mdh-1, Mdh-2, G6pd-1,* and *G6pd-2*), and 6 loci were polymorphic (*Table 2*), i.e., a polymorphic locus is one in which any variants were observed in any population. In most polymorphic loci, a single allele was the most common in all populations. Alternately, at the locus *Pgi-2*, allele (*a* and *b*) frequencies were completely or nearly intermediate in all populations except for the Uleung Island population. In three populations (JIRI, SEORAK and HONGCHEON), only heterozygote ($Pgi\text{-}2_{ab}$) was detected, while in the Uleung Island population, more homozygous genotype ($Pgi\text{-}2_{aa}$) was found and consequently the frequency of allele a was higher than that of any other populations (*Table 2*). Only one population had a unique allele at one locus, i.e., alleles found in only one population (HONGCHEON population at locus *Aat-1*).

The percentage of polymorphic loci ($P_{99}$) ranged from 23.1 % to 38.5 % with a mean of 29.5 %, while $P_{95}$ varied between 15.4 % and 30.8 % with a mean of 23.1 %; alleles per polymorphic locus (*A*) ranged from 1.2 to 1.4 with a mean of 1.3; observed heterozygosity ($H_o$) ranged from 0.050 to 0.139 with a mean of 0.104; and expected heterozygosity ($H_e$) ranged from 0.044 to 0.085 with a mean of 0.075 (*Table 3*).

In each population, the observed heterozygosity was higher than expected heterozygosity, which suggests an excess of heterozygotes. The heterozygote excess is also reflected in a population mean of –0.420 for WRIGHT's $F_{IS}$ (*Table 4*). Likewise,

the value of WRIGHT's $F_{IT}$ was negative (–0.399), indicating a heterozygote excess at the species level. These results are in good agreement with the exact test for deviation from the H-W expectations. Eight of 23 tests for polymorphic loci indicated a significant deviation from the H-W proportions. In 6 of 8 significant tests, populations had an excess of heterozygotes (*Table 2*). At the locus *Pgi-2*, all of the populations had a significant excess of heterozygotes except for the Uleung Island population. The genotype frequencies at the *Pgi-2* in the Uleung Island population were, however, nearly deviated from the H-W expectations ($p$ = 0.054). A multi-population exact test for the H-W proportions at the *Pgi-2* locus also showed a significant deviation.

*Table 2.* – Allele frequencies for 6 polymorphic loci in 6 natural populations of *R. brachycarpum*.

| Locus/allele | Seorak | Hongcheon | Kariwang | Hambaek | Jiri | Uleung |
|---|---|---|---|---|---|---|
| AAT-1/a | 1 | 0.943 | 1 | 1 | 1 | 1 |
| AAT-1/b | 0 | 0.057 | 0 | 0 | 0 | 0 |
| IDH/a | 0.323$^-$ | 0.243 | 0.250$^+$ | 0.047 | 0.132$^+$ | 0 |
| IDH/b | 0.677 | 0.757 | 0.750 | 0.953 | 0.868 | 1 |
| LAP/a | 0.081 | 0.043 | 0 | 0 | 0 | 0 |
| LAP/b | 0.919 | 0.957 | 0.903 | 0.906 | 1 | 1 |
| LAP/c | 0 | 0 | 0.097 | 0.094 | 0 | 0 |
| MNR/a | 1 | 1 | 0.972 | 0.938 | 0.985 | 0.984 |
| MNR/b | 0 | 0 | 0.028 | 0.063 | 0.015 | 0.016 |
| PGI-1/a | 0 | 0 | 0 | 0.109 | 0.059 | 0.078 |
| PGI-1/b | 1 | 1 | 1 | 0.891 | 0.941 | 0.922 |
| PGI-2/a | 0.500$^-$ | 0.500$^-$ | 0.403$^-$ | 0.516$^-$ | 0.500$^-$ | 0.734 |
| PGI-2/b | 0.500 | 0.500 | 0.597 | 0.484 | 0.500 | 0.266 |

+ = heterozygote deficit compared to Hardy-Weinberg expectations,
- = heterozygote excess compared to H-W expectations.

*Table 3.* – Genetic diversity estimates for 6 *R. brachycarpum* populations. Standard errors appear in parentheses.

| Population | $A$ | $P_{95}$ | $P_{99}$ | $H_o$ | $H_e$ |
|---|---|---|---|---|---|
| Seorak | 1.2 (0.1) | 23.1 | 23.1 | 0.139 (0.087) | 0.085 (0.050) |
| Hongcheon | 1.3 (0.1) | 23.1 | 30.8 | 0.130 (0.082) | 0.083 (0.046) |
| Kariwang | 1.3 (0.1) | 23.1 | 30.8 | 0.094 (0.062) | 0.085 (0.046) |
| Hambaek | 1.4 (0.1) | 30.8 | 38.5 | 0.123 (0.074) | 0.084 (0.041) |
| Jiri | 1.3 (0.1) | 23.1 | 30.8 | 0.090 (0.076) | 0.068 (0.041) |
| Uleung | 1.2 (0.1) | 15.4 | 23.1 | 0.050 (0.041) | 0.044 (0.031) |
| Mean | 1.3 | 23.1 | 29.5 | 0.104 | 0.075 |

$A$ = average number of alleles per locus including monomorphic loci;
$P_{95}$ = proportion of polymorphic loci at 95% level (A locus is considered if the frequency of most common allele does not exceed 0.95);
$P_{99}$ = proportion of polymorphic loci at 99% level; $H_o$ = observed heterozygosity; $H_e$ = expected heterozygosity (unbiased estimate).

*Table 4.* – WRIGHT's *F*-statistics for 6 polymorphic loci in *R. brachycarpum*.

| Locus | $F_{IS}$ | $F_{IT}$ | $F_{ST}$ |
|---|---|---|---|
| AAT-1 | -0.061 | -0.010 | 0.048 |
| IDH | 0.013 | 0.108 | 0.097 |
| LAP | -0.092 | -0.042 | 0.046 |
| MNR | -0.044 | -0.021 | 0.023 |
| PGI-1 | 0.174 | 0.214 | 0.048 |
| PGI-2 | -0.848 | -0.773 | 0.041 |
| Mean | -0.420 | -0.339 | 0.057 |

$F_{IS}$ and $F_{IT}$ = deviations of genotype frequencies from Hardy-Weinberg expectations within each population and over all populations;
$F_{ST}$ = proportion of the total genetic diversity among populations.

The WRIGHT's $F_{ST}$ value was 0.057, which can be interpreted to mean that 94.3% of the total genetic variation was within populations and only 5.7% was among populations. *Nm* calculated from WRIGHT's $F_{ST}$ and by BARTON and SLATKIN's method after correction for size was 4.14 and 1.28 migrants per generation, respectively.

The estimates of genetic distance among populations ranged from 0.000 to 0.014 with an average of 0.004. To better visualize the results, a dendrogram produced by the UPGMA clustering technique is presented in *Fig. 2*. The dendrogram shows some decisive geographic patterns; the closer in geographic distance, the more closely related in genetic structure (*Fig. 1* and *2*). The Uleung Island population was most distinctive from the other populations. A Mantel test showed a positive correlation (r = 0.228) between genetic and geographic distances, but was not statistically significant.

## Discussion

Recent reviews of the literature on plant allozymes (HAMRICK and GODT, 1989; HAMRICK et al., 1992) and studies (LEE et al., 1997; LEE et al., 1998) have shown that endemic rare and narrowly distributed plant species tend to maintain lower levels of genetic variation than more widespread species. In the extreme cases, some rare and endangered plant species showed no polymorphism at allozyme level (WALLER et al., 1987). *Rhododendron brachycarpum* also revealed a low level of genetic diversity ($P_{95}$ = 23.1%, $P_{99}$ = 29.5%; $H_e$ = 0.075) as in the cases for other rare and endangered plants. For example, the $P$ and $H_e$ values averaged over 100 endemic and rare plant species were 26.3% and 0.063, respectively (HAMRICK and GODT, 1989). Similarly, those for 26 endemic rare woody plants were 26.3% and 0.056, respectively (HAMRICK et al., 1992).

Seedlings and germination of *R. brachycarpum* are highly dependent on ground conditions (LEE et al., 1982; HONG et al.,

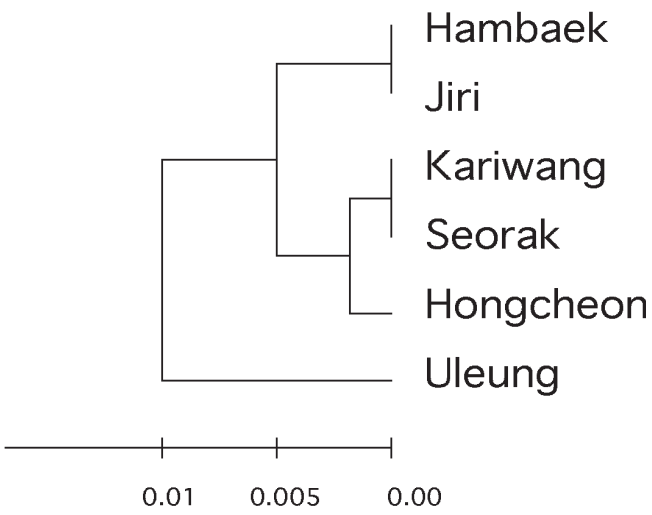


*Figure 2.* – UPGMA dendrogram based on NEI's genetic distance among 6 populations of *R. brachycarpum*.

*Table 5.* – NEI's (1978) unbiased genetic distance (below diagonal) and genetic identity (above diagonal) among 6 populations of *R. brachycarpum*.

| Population | Hambaek | Kariwang | Jiri | Seorak | Hongcheon | Uleung |
|---|---|---|---|---|---|---|
| Hambaek | * | 0.996 | 1 | 0.993 | 0.996 | 0.996 |
| Kariwang | 0.004 | * | 0.998 | 0.999 | 1 | 0.986 |
| Jiri | 0 | 0.002 | * | 0.997 | 0.999 | 0.995 |
| Seorak | 0.007 | 0.001 | 0.003 | * | 1 | 0.987 |
| Hongcheon | 0.004 | 0 | 0.001 | 0 | * | 0.991 |
| Uleung | 0.004 | 0.014 | 0.005 | 0.013 | 0.009 | * |

1983); seedling density was extremely low on soil but high on moss mats and decayed woods. Although important for *R. brachycarpum* regeneration, decaying logs and moss mats are fairly rare in forests. Leaf litter is generally the most abundant seedbed available, but is not considered favorable for the germination and establishment of *R. brachycarpum*. So, the lack of suitable sites in forests may be one of the explanations for the rarity and limited distribution of this species. Consequently, the low level of genetic diversity investigated in here can be easily expected because rare and endangered species commonly have little genetic variation due to inbreeding, genetic bottlenecks and/or other factors (WALLER et al., 1987).

Populations that have experienced a recent reduction of their effective population size generally show a correlated reduction of allele numbers and gene diversity ($H_e$) at selectively neutral polymorphic loci, but allele number is reduced more rapidly than gene diversity (CORNUET and LUIKART, 1996; LUIKART and CORNUET, 1998). Thus, in a population recently reduced in size and/or a population that has only recently expanded after a reduction in size, i.e., a bottlenecked population, the Hardy-Weinberg equilibrium heterozygosity ($H_e$) is higher than the expected heterozygosity in an equilibrium population ($H_{eq}$), calculated from the observed number of alleles under the assumption of a constant-size (equilibrium) population (CORNUET and LUIKART, 1996; LUIKART and CORNUET, 1998). In a population at mutation-drift equilibrium, i.e., the effective size of which has remained constant in the recent past, there is approximately an equal probability that a locus shows a gene diversity excess or a gene diversity deficit. In the present study, the CORNUET and LUIKART test detected no excess of heterozygosity ($H_e > H_{eq}$) in any of the *R. brachycarpum* populations. Consequently, we can conclude that *R. brachycarpum* in Korea has not recently experienced a bottleneck leading to genetic drift and correspondingly, hypothesize that this species has had a small population since long time ago.

In alpine and arctic environments, outcrossing of entomophilous plants is restricted by low temperature, strong wind, and a short growth season, which restrict the activity and diversity of pollinators (WILCOCK and NEILAND, 2002). This may induce and promote selfing if the plant species is self-compatible. *Rhododendron aureum* Georgi – a species closely related to *R. brachycarpum*, is self-compatible although most of pollination is outcrossing (KUDO, 1993). Considering the *R. brachycarpum*'s taxonomic relationship with *R. aureum*, we can expect that *R. brachycarpum* is also self-compatible to some extent, and the selfing might explain the low level of genetic diversity maintained in this species.

Phosphoglucose isomerase (E.C.5.3.1.9) has a critical role in glycolysis, which is important in germination (DENNIS and TURPIN, 1990). The importance of glycolysis in seed germination may be one of the plausible explanations for the extreme excess of heterozygotes detected at the locus *Pgi-2* in this study. The heterozygous genotype of the *Pgi-2* may have the advantage in seed germination and seedling establishment over the homozygotes. Additional studies on comparing genotype frequencies of seedlings and mature trees are needed before more definitive conclusions can be reached.

The UPGMA dendrogram showed that the Uleung Island population was most distinctive from the other populations. Several hypotheses can be made for this difference. Uleung Island is the most easterly habitat for *R. brachycarpum* in Korea, and the climate is very different from those of inland areas. In particular, air humidity is much higher than that in inland area and would be more conducive to seed germination and seedling establishment (LEE et al., 1982; HONG et al., 1983, 1984). Therefore, if this environmental factor functions as a major selection force, *R. brachycarpum* in the Uleung Island has been under a different selection regime. The distribution patterns of alleles and genotypes support this hypothesis. At the *Pgi-2* locus, the frequency of genotype $Pgi\text{-}2_{aa}$ in the Uleung Island population was much higher than that of the other populations and consequently the frequency of allele *a* was higher, while that of allele *b* was much lower than those of the inland populations (*Table 2*).

Alternately, *R. brachycarpum* in the Uleung island might be in the process of speciation. The geographic distance from the Uleung island to the closest inland spot is 137 km. This distance may be too far for pollinators to deliver genes from the inland *R. brachycarpum* populations. Consequently, the Uleung Island population may have evolved in a direction different from that of inland populations.

Given the low level of genetic variation and the lack of suitable sites in natural forests, *R. brachycarpum* is a prime candidate for the conservation studies. For plant species with low genetic diversity like the *R. brachycarpum*, large sample sizes are not necessary to capture the majority of the genetic variation within populations. However, the relatively wide range of genetic differentiation suggests that samples from multiple populations across its range in Korea are needed for *ex situ* conservation in order to preserve its genetic structure. The Uleung Island population is considered of high conservation value because not only it has the unique genetic structure, but also it is the eastern edge of *R. brachycarpum* distribution in Korea.

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# Genetic Structure in the Northernmost Marginal Population of Japanese Beech (*Fagus crenata* BLUME): Influence of the Founding Event on Genetic Structure

By M. TAKAHASHI[1*]), N. TOMARU[2]), M. UBUKATA[1]) and K. KOONO[1])

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**Abstract**

We examined the genetic variability and structure within the northernmost marginal population of Japanese beech (*Fagus crenata* BLUME), located in the Kuromatsunai lowland (Utasai), Hokkaido Island, Japan, with special regard to the influence of the founding process on its genetic structure. From palynological records, it appears that the population was established between 350 and 680 years ago. We investigated 119 trees, using 11 isozyme loci, which encode eight enzyme systems. The proportion of polymorphic loci, average number of alleles per locus, effective number of alleles per locus, expected heterozygosity and observed heterozygosity in the population were 0.64, 2.5, 1.30, 0.182 and 0.178, respectively. The average $F_{IS}$ value was 0.046. Significant linkage disequilibrium was found for two pairs of loci ($P < 0.05$), probably related to founder effects during and after establishment of the population. Genetic structure in the population was examined by Moran's *I*, standard normal deviate (SND), and the number of alleles in common (NAC). The genetic clustering in the population was weaker and less clear than in previously studied populations. The genetic structure commonly seen in populations of this species has probably not yet emerged here because of the small number of generations since it was founded.

*Key words: Fagus crenata,* founder effect, Japanese beech, genetic variation, linkage disequilibrium, marginal population, spatial autocorrelation, within-population genetic structure

[1]) Forest Tree Breeding Center, 3809-1 Kakozawa, Ishi, Juo, Ibaraki 319-1301 Japan. Tel: +(0)293-32-7000, Fax: +(0)293-32-7034

[2]) Laboratory of Forest Ecology and Physiology, Gradual school of Bioagricultural Science, Nagoya University, Nagoya, Aichi 464-8601 Japan. Tel: +(0)52-789-4048, Fax: +(0)52-789-5014

* Corresponding Author: MAKOTO TAKAHASHI, Tel: +(0)293-32-7000, Fax: +(0)293-32-7034, E-mail: makotot@affrc.go.jp