RAPD Analysis of Genetic Variation Within and Among Four Natural Populations of *Betula maximowicziana*

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Summary

Betula maximowicziana is a long-lived pioneer tree species in cool temperate forests that plays an important role in the forest ecosystem and has high economic value. Random amplified polymorphic DNA (RAPD) markers were used to evaluate the genetic variation of four natural populations of *B. maximowicziana* (three in central Honshu and the other in Hokkaido) to obtain fundamental information on this natural resource. Sixty-one reproductive amplified bands were obtained with 23 primers. Of these 61 bands, 22 were monomorphic and 39 were polymorphic. The level of genetic variation within each population may be very similar, because the population rankings according to number of polymorphic loci, Shannon's indices and intra-population genetic variance revealed no definite patterns. Global analysis of AMOVA (analysis of molecular variance) showed that genetic variation among populations accounted for 15.6% of the total variation, with the remainder (84.4%) occurring within population. These results demonstrate that genetic differentiation among the four populations is moderate. Hierarchical AMOVA analysis showed that variation among regions (Hokkaido and central Honshu) accounted for 10.4% of the total genetic variation, suggesting that regional genetic differentiation is relatively high. Significant correlations between pairwise Φ_{PT} values and geographic distance were detected, and results of both a neighbor-joining dendrogram based on pairwise Φ_{PT} values, and principal coordinate analysis (PCO) based on a Euclidean metric revealed that the Furano population in Hokkaido was genetically different from the three populations in central Honshu. The data obtained in this study should have important implications for the conservation and management of regional genetic variation of *B. maximowicziana.*

Key words: Betula maximowicziana, natural resource, RAPD, regional genetic variation.

Introduction

One of the Japanese birches, Monarch birch (*Betula maximowicziana*), is distributed in the cool temperate zone from the eastern part of Honshu Island to Hokkaido in Japan (OHWI, 1965). *B. maximowicziana* is a monoecious species with an outcrossing breeding system involving wind-pollination and wind dispersal of seeds. *B. maximowicziana* is a pioneer tree species that grows rapidly in open sites, such as gaps, and often establishes even-aged stands (OSUMI and SAKURAI, 1997). Thus, this species plays important roles in the stability and sustainability of forest ecosystems in the cool temperate zone, not only as a major pioneer tree species but also as a long-lived dominant species (WATANABE, 1989). Furthermore, in plantations of *Cryptomeria japonica*, natural germination or recruitment of *B. maximowicziana* is promoted by artificial disturbance of the soil surface such as clear cutting, site preparation and plantation (HASEGAWA and TAIRA, 2000). These recruited birches can make valuable contributions to forest growth parameters, and may both enhance the public welfare of the forests and provide significant additional natural resources, especially where plantations of *C. japonica* have been unsuccessful for instance in snowy regions (HASEGAWA, 1998; HASEGAWA and TAIRA, 2000).

B. maximowicziana also yields high quality wood, so its use as a commercial crop from natural forests is being promoted nowadays. The decline of natural resources in hardwood tree species, including *B. maximowicziana*, was predicted in Hokkaido more than 20 years ago (ADACHI *et al.,* 1980). However, little is known about the genetic variation of this species, despite its ecological and economic importance, and the fact that knowledge about genetic variation provides an important baseline for conservation and forest management (GEBUREK, 1997). Moreover, *B. maximowicziana* seeds or seedlings are being commercially circulated with little or no regard for their provenance now. Thus, there is an urgent need to obtain genetic information on natural populations of *B. maximowicziana,* before fragmentation of the species occurs through further cutting and plantation, since excessive cutting and plantation can affect the genetic variation in natural populations.

Molecular markers are good tools for the detection of genetic variation within and among populations. Range-wide surveys of genetic variation, based on molecular markers, have now been performed on thousands of species (PETIT *et al.,* 1998). Allozymes, especially, have been examined in many species and provide useful information related to evolution and population genetics (HAMRICK *et al.,* 1992). However, in a previous allozyme analysis of *B. maximowicziana,* in which 13 enzymes were examined, only two loci were detected as useful markers and polymorphism at these two loci was relatively low (KADO *et al.,* 2002). To evaluate genetic variation, as many loci as possible should ideally be used. Random amplified polymorphic DNA (RAPD) markers (WILLIAMS *et al.,* 1990) allow us to obtain large amounts of data on genetic variation within and among populations without detailed prior knowledge of DNA sequences (HOLSINGER *et al.,* 2002). Furthermore, the practically unlimited number of primers that can be used provide information on variation across the whole genome (RAMSER *et al.,* 1996; GEBUREK, 1997). Moreover, RAPDs have several advantages over allozyme, sequencing and restriction fragment length polymorphism (RFLP) analyses, including speed, low cost, and ease of collection and storage of material (RAMSER *et al.,* 1996; SCHIERENBECK *et al.,* 1997; SAITO *et al.,* 2002). Due to these advantages, RAPD markers have been used for genome mapping, identifying cultivars, breeding and analyzing genetic variation within and among populations (both intra- and interspecies), although RAPDs are dominant markers. In this study, genetic variations within and among *B. maximowicziana* populations were evaluated using RAPDs to obtain fundamental

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Table 1. – Location and number of samples collected for each of the four populations of *Betula maximowicziana.*

Population	Latitude (N)	Lomgitude (E)	Altitude (m)	number of samples
Chichibu (CCB)	35°56'	138°49'	1100 - 1350	20
Mitomi (MTM)	35°52'	138°42'	1650 - 1850	24
Hayakawa (HYK)	35°20'	138°17'	1450 - 1550	24
Furano (FRN)	43°28'	142°46'	400 - 650	25

genetic information for conservation and management of this natural genetic resource.

Materials and Methods

Study site and plant material

Four natural populations of *B. maximowicziana* were sampled (*Table 1*). The Chichibu (CCB) population in University Forest in Chichibu, The University of Tokyo, in Saitama prefecture, and the Mitomi (MTM) and Hayakawa (HYK) populations in Yamanashi prefecture are all located in central Honshu, while the Furano (FRN) population, is located in University Forest in Hokkaido, The University of Tokyo (*Figure 1*). The three populations in central Honshu are in mixed forest stands with deciduous broad-leaved trees and conifers such as *Acer mono, Betula grossa, Fagus crenata, Fagus japonica, Quercus crispula, Abies veitchii* and *Tusga diversifolia.* The FRN population is also in a mixed forest with species such as *Abies sachalinensis, A. mono, Betula ermanii, Picea jezoensis, Q. crispula* and *Tilia japonica.*

Fresh branches were sampled randomly from 18–25 individuals for each population. In total, 94 samples were collected (*Table 1*), and the branches were stored at –80 °C prior to DNA extraction.

DNA extraction and PCR procedures

Total genomic DNA was extracted from fresh cambium using a DNeasy Plant mini kit (Qiagen Co. Ltd. Tokyo). PCR reactions were carried out in 10 µL volumes containing 10 ng of genomic DNA, 200 µM each of dATP, dCTP, dGTP and dTTP, 1x buffer, 2.5 mM $MgCl_2$, 0.5 units of Stoffel Fragment DNA Taq polymerase (ABI) and 0.5 µM of each primer. Samples ware amplified in a DNA thermal cycler (Takara Co. Ltd. Tokyo) programmed to provide a denaturation step at 94 °C for 5 min, followed by 45 cycles of 94 °C for 10 s, 36 °C for 30 s, 72 °C for 1 min and a final extension step at 72 °C for 7 min. The amplified products were separated on 2% agarose gels in 1x TBE buffer (Takara Co. Ltd. Tokyo) and subjected to electrophoresis. Gels were then stained with ethidium bromide and photographed on a UV transilluminator.

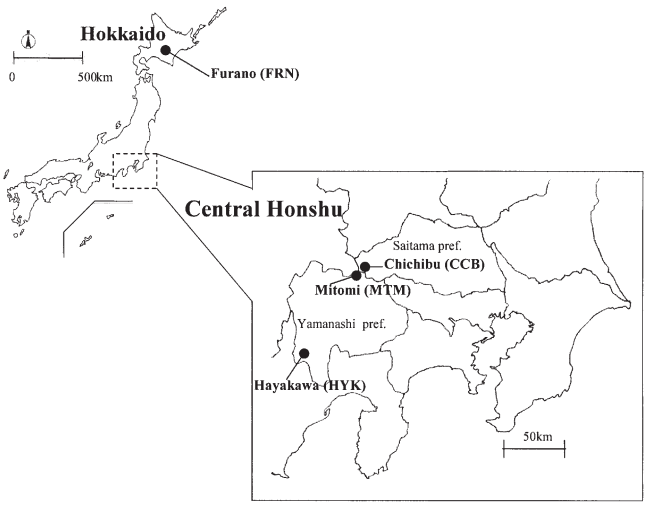

Figure 1. – Sampling site of *Betula maximowicziana* in Yamanashi, Saitama pref. and Hokkaido.

Primer screening

Two hundred and seventeen primers derived from an Operon 10-mer kit (Qiagen Co. Ltd. Tokyo) were screened in small samples to check the reproducibility and polymorphisms of the RAPD fragments they yielded using more than three runs for each primer.

Data analysis

RAPD phenotypes for each primer were scored as present (1) or absent (0) for each individual. We only compared these 1/0 data, and did not analyze them assuming Hardy-Weinberg equilibrium following the approach of LYNCH and MILLIGAN (1994), because we did not know whether the populations used in this study were in Hardy-Weinberg equilibrium. Genetic variation within the populations was estimated in terms of Shannon's indices (LEWONTIN, 1972) using the program POPGENE version 1.31 (YEH *et al.,* 1999), from the formula:

$$-\Sigma_{pi} \log_{2 pi}$$

where *pi* is the frequency of amplified bands among individuals of each population. Shannon's index is suitable for analyzing RAPD data because of its insensitivity to the bias that can be introduced into data by the inability to detect heterozygous individuals (DAWSON *et al.,* 1995; GILLIES *et al.,* 1997; PARANI and PARIDA, 1997; GUSTAFSON *et al.,* 1999; MAKI and HORIE, 1999; OIKI *et al.,* 2001; AGA *et al.,* 2003). Genetic variation was also evaluated by analysis of molecular variance (AMOVA) (EXCOFFIER *et al.,* 1992) using the program GenALEx (PEAKALL and SMOUSE, 2001). Although AMOVA was originally designed for haploid mitochondrial DNA data (EXCOFFIER *et al.,* 1992), HUFF *et al.* (1993) successfully applied it to RAPD data. Since then, AMOVA has been used in many RAPD analyses (e.g. BARTISH *et al.,* 1999; JORDANO and GODOY, 2000). The AMOVA was based on the Euclidean metric of EXCOFFIER *et al.* (1992). The formula is:

$$E = \{\varepsilon^2_{xy}\} = n\,[1 - 2n_{xy} / 2n]$$

where $2n_{xy}$ is the number of markers shared by two individuals (x and y) and n is the total number of polymorphic markers (HUFF *et al.,* 1993). This Euclidean metric describes the number of RAPD markers that were not shared by two individuals, because only two RAPD states were recognized: 1 or 0. The molecular variance within each population was calculated as

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an indicator of intra-population genetic variation. Estimates of the partitioning of the genetic variation among the four populations and among individuals within the populations were initially derived from a global analysis without considering differences in region. However, in hierarchical analysis with AMOVA, the genetic variation was partitioned among regions (Hokkaido and central Honshu), as well as among populations within regions and among individuals within populations (CCB, HYK and MTM). The significance of the variance components was tested by calculating their probabilities, based on 999 random permutations using the program GENALEx (PEAKALL and SMOUSE, 2001). The indicator of population genetic differentiation, pairwise Φ_{PT} from Φ-statistics (EXCOFFIER *et al.,* 1992; PEAKALL *et al.,* 1995) of AMOVA was calculated, and correlations between pairwise Φ_{PT} values and geographic distance were tested by regression analyses. The Euclidean metric was also used as the basis for principal coordinate analysis (PCO) (GOWER, 1966), from which graphical relationships between individuals were derived using the program GENALEx (PEAKALL and SMOUSE, 2001). In addition, pairwise Φ_{PT} values were used to generate a neighbor-joining dendrogram using the program Populations 1.2.28 (LANGELLA, 2002).

Results and Discussion

Sixty-one RAPD markers, generated from 23 out of the 217 primers screened, were selected because their banding patterns were unambiguous and reproducible. The designations and sequences of these 23 primers, together with the size of the monomorphic and polymorphic fragments they generated, are shown in *Table 2*. Out of the 61 RAPD markers selected, 22 were monomorphic in all samples of the four populations examined and 39 were polymorphic in at least one population (*Table 2*). However, even the 22 monomorphic RAPD markers in this study are potentially valuable because they may reveal polymorphism in other regions in future studies.

The number of polymorphic loci and Shannon's index for each population are shown in *Table 3*. The number of polymorphic loci ranged from 29 (FRN) to 31 (CCB and MTM) and averaged 30.3. Shannon's indices ranged from 0.360 (FRN) to 0.460 (MTM), averaging 0.405. In comparison, the range and mean value of the Shannon's indices were 0.349–0.612 and 0.547, respectively, for 12 populations of the South American conifer *Fitzroya cupressoides* (ALLNUT *et al.,* 1999), 0.592–0.733 and 0.651 for eight populations of *Populus tremuloides* (YEH *et al.,* 1995) and 0.301–0.367 and 0.332 for six populations of the endangered tropical tree *Plathymenia reticulate* (LACERDA *et al.,* 2001). Compared with these previous studies, the within-population genetic variation of *B. maximowicziana* appears to be moderate. Molecular variance within the populations we sampled ranged from 4.870 (MTM) to 5.187 (FRN), averaging 5.017. LACERDA *et al.* (2001) detected a strong correspondence between Shannon's indices and molecular variance within populations, i.e. populations with the highest Shannon's indices had the highest molecular variance and vice versa. In contrast, while the numbers of polymorphic loci and Shannon's indices in our study indicated a tendency for the genetic variation of the FRN population to be a little lower than that of the populations in central Honshu, the within-population molecular variance of FRN was slightly higher than that of the Honshu populations

Table 2. – Designations, sequences and size of monomorphic and polymorphic fragments generated by the primers.

Primer	Sequence (5' to 3')	Monomorphic fragment size (bp)	Polymorphic fragment size (bp)
OPA-10	GTGATCGCAG	-	410 and 470
OPA-17	GACCGCTTGT	460	800, 850 and 920
OPA-18	AGGTGACCGT	-	590, 640 and 700
OPC-08	TGGACCGGTG	520	700
OPD-19	CTGGGGACTT	260	580
OPF-14	TGCTGCAGGT	550	480 and 640
OPG-05	CTGAGACGGA	380	630, 760 and 850
OPH-04	GGAAGTCGCC	460	510
OPH-14	ACCAGGTTGG	-	750
OPH-15	AATGGCGCAG	400 and 530	800
OPH-18	GAATCGGCCA	-	620, 810 and 910
OPI-03	CAGAAGCCCA	380, 490 and 580	840
OPI-05	TGTTCCACGG	-	590
OPI-11	ACATGCCGTG	-	530
OPI-18	TGCCCAGCCT	350 and 530	580, 660 and 780
OPP-14	CCAGCCGAAC	750 and 860	450
OPS-07	TCCGATGCTG	410	620 and 730
OPS-19	GAGTCAGCAG	290	470
OPV-06	ACGCCCAGGT	400 and 450	700
OPW-02	ACCCCGCCAA	450 and 640	760 and 810
OPW-10	TCGCATCCCT	-	550 and 630
OPX-14	ACAGGTGCTG	450	580
OPX-16	CTCTGTTCGG	-	380 and 550

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Table 3. – Number of polymorphic loci, Shannon's index I (LEWONTIN, 1972) and variance within each of the four population.

Population	Number of polymorphic loci	Shannons's index	varience
CCB	31	0.410	5.124
MTM	31	0.460	4.870
HYK	30	0.392	4.888
FRN	29	0.360	5.187
mean	30.3	0.405	5.017

Table 4. – Analysis of molecular variance (AMOVA) for 93 individuals grouped in four populations from two regions. The degrees of freedom (d.f.), sum of squares (SS), mean squares (MS), variance components, the total variation contributed by each component (%) and its associated significance (n = 999 permutations) are shown.

Source of variation	d.f.	SS	MS	variance components	total variance (%)	*P* - value
Global						
Among populations	3	80.064	26.688	0.933	15.6	< 0.001
Among individuals within population	89	448.087	5.035	5.035	84.4	< 0.001
Hierarchical						
Among region	1	43.286	43.286	0.1038	10.4	< 0.001
Among populations within region	2	36.777	18.389	0.0942	9.4	< 0.001
Among individuals within population	89	448.087	5.035	0.802	80.2	< 0.001

(*Table 3*). Furthermore, the range of number of loci, Shannon's indices and the molecular variance within populations appeared to be relatively limited. These results suggest that the populations used in this study have very similar levels of within-population genetic variation.

According to the global AMOVA analysis there was significant partitioning of the genetic variation ($P < 0.001$), with 15.6% occurring among populations and within-population variation accounting for the remaining 84.4% (*Table 4*). The among-population value of the indicator of genetic differentiation, Φ_{PT}, for the four populations was 0.156, suggesting that genetic differentiation of *B. maximowicziana* was moderate and that within-population variation accounted for most of the genetic variation of *B. maximowicziana.* Similar tendencies for most genetic variation to be partitioned within-population have been found in several previous studies of tree species involving AMOVA analysis based on RAPDs. For example, 84% of the total genetic variation detected was within-population for four populations of *Abies* (three populations of *Abies alba* and a population of *A. nebrodensis*) (VICARIO *et al.,* 1995), 97.4% for eight populations of *Populus tremuloides* (YEH *et al.,* 1995), 63% for six populations of *Pseudotsuga menziesii* (AAGAARD *et al.,* 1998), 87.7% for six populations of *Plathymenia reticulate* (LACERDA *et al.,* 2001), 85.6% for 12 populations of *Fitzroya cupressoides* (ALLNUT *et al.,* 1999) and 80.2% for 37 populations of *Eucalyptus globulus* (NESBITT *et al.,* 1995). The findings of the cited studies, and the study reported here are consistent with conclusions published in reviews of isozyme data (HAMRICK and GODT, 1989; HAMRICK *et al.,* 1992), showing that long-lived, outcrossing tree species with a wide and continuous range generally retain most of their genetic variation within populations.

According to the hierarchical AMOVA analysis, the amount of genetic variation partitioned among regions and among populations was 10.4% and 9.4%, respectively, with the remainder

Table 5. – Pairwise Φ_{PT} (above diagonal) and geographic distance (bellow diagonal : km) between populations.

Population	1	2	3	4
1 CCB	-	0.120	0.080	0.172
2 MTM	12.5	-	0.119	0.219
3 HYK	85.0	70.0	-	0.190
4 FRN	870.0	880.5	951.5	-

__ each pairwise Φ_{PT} value was significant ($P < 0.001$).

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(80.2%) occurring within-population. NESBITT *et al.* (1995) found 3.5% of total genetic variation among regions in a study of RAPD variation of 31 *Eucalyptus globules* ssp. *globules* populations situated in Victoria and Tasmania, Australia. Compared with the findings of NESBITT *et al.* (1995), the genetic variation among regions we found (10.4%) is higher than might be expected, since gene flow among *B. maximowicziana* populations is thought to be relatively substantial as it is a widely distributed, outcrossing, wind-pollinated and wind-dispersed species. This somewhat high value of genetic variation partitioning among regions reflects the regional genetic differentiation between the FRN population and the other three populations.

The pairwise Φ_{PT} values and geographic distances between the four populations are shown in Table 5. The pairwise Φ_{PT} values ranged from 0.080 (CCB and HYK) to 0.219 (MTM and FRN) and each of the pairwise Φ_{PT} values was significant according to tests based on 999 random permutations ($P < 0.001$). In addition, significant correlation between pairwise Φ_{PT} and geographic distances was found in the regression test ($r = 0.904$, $P < 0.05$), suggesting that geographic distance influences genetic variation.

The first three principal coordinates derived from the principal coordinate analysis (PCO) based on the Euclidean metric for the sampled individuals of the four populations described 13.8, 10.5 and 8.9% of the total variance. Plots of the first two coordinates are shown in *Figure 2*. The plots of the three populations in central Honshu were closer to one another, or overlapped, than to the FRN population. The neighbor-joining dendrogram based on pairwise Φ_{PT} values among populations is shown in *Figure 3*. The results from the PCO and the neighbor-joining dendrogram revealed the same pattern, i.e. that the three populations in central Honshu (CCB, MTM and HYK) are genetically close to each other and differ from the FRN population, which is located about 870–950 km away from them. These results also agree with the findings of the hierarchical AMOVA analysis, indicating that the among-region genetic variation was somewhat high. Although a significant correlation between geographic distance and genetic distance was detected, other factors that may influence genetic differentiation like gene flow, wind-pollination, the wind-dispersal of *B. maximowicziana* seeds and the effects of the glacial episodes of the Holocene should also be considered. In addition, *B. maximowicziana* has a seed bank, which may play compensational roles in years immediately following poor seed years (OSUMI and SAKURAI, 1997), and thus influence genetic variation.

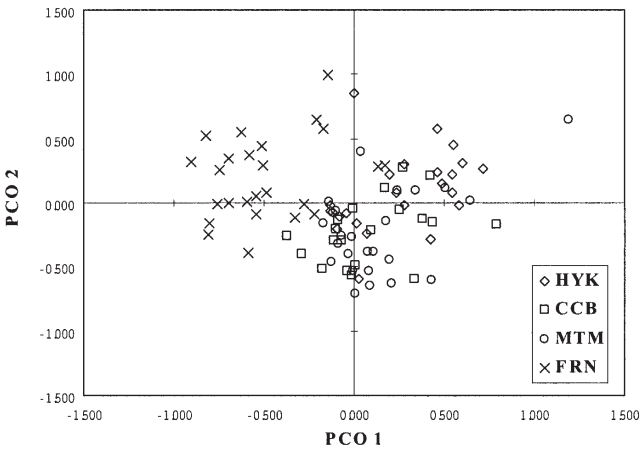

Figure 2. – Plot of the first two principal coordinates for each individual of four populations.

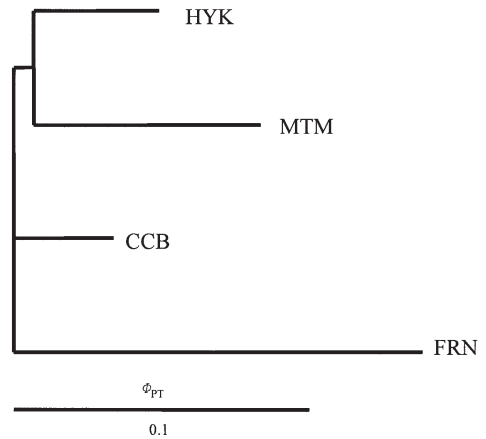

Figure 3. – Neighbour-joining dendrogram based on pairwise ΦPT among four populations of *B. maximowicziana*.

FRN population is geographically distant from the other three populations, and the Tsugaru Straits (known as the "Blakiston line") separates Hokkaido and Honshu. However, there were no unique markers that could distinguish these two regions. This might also be considered to be indicative of moderate genetic differentiation among populations, since RAPD markers that are unique to certain regions or populations isolated by geographic factors have been reported in other species (KANETANI *et al.,* 1994; MOSSELER *et al.,* 1992).

In conclusion, fundamental genetic information concerning natural populations of *B. maximowicziana* was obtained using RAPDs in this study and geographic or regional patterns in genetic variation among populations were detected. These genetic findings have important implications for the conservation and management of regional genetic variation of this valuable natural genetic resource. To obtain detailed genetic information facilitating the conservation and management of forest ecosystems containing *B. maximowicziana*, wide-ranging and fine-scale analysis using molecular markers will be required in future studies.

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