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REGULAR PAPER

Chloroplast DNA phylogeography of *Betula maximowicziana*, a long-lived pioneer tree species and noble hardwood in Japan

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Abstract *Betula maximowicziana* is an ecologically and economically important tree species in Japan. In order to examine the phylogeographical pattern of the species in detail, maternally inherited chloroplast (cp) DNA variations of 25 natural populations of *Betula maximowicziana* and a total of 12 populations of three related species were evaluated by PCR-RFLP analysis. Two main haplotypic groups of *B. maximowicziana* populations (northern and southern) were detected, with the main boundary passing through the Tohoku region in northeastern Japan; in addition there was high genetic differentiation among the 25 populations studied ($G_{ST} = 0.950$, $G'_{ST} = 0.977$). The phylogeographical pattern exhibited by *B. maximowicziana* was much more similar to that of alpine plants than to that of beech and oak. Comparison of the patterns of genetic structure obtained from the cpDNA with previously and newly acquired data on bi-parentally inherited nuclear DNA indicates that the nuclear genome was transferred via pollen from the northern haplotypic group to the southern group more frequently than it moved in the opposite direction. Although common haplotypes were detected among *B. maximowicziana* and the two related species examined, these haplotypes were not shared sympatrically, suggesting very rare hybridization among the species currently occurring in their natural populations.

Keywords *Betula maximowicziana* · Chloroplast DNA · Haplotype sharing · Introgression · Nuclear DNA · Phylogeography

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Introduction

Recent advances in genetic analysis techniques have greatly enhanced our ability to detect genetic structure in the nuclear genomes of plant species (Heuertz et al. 2004a; Tsuda and Ide 2005; Magri et al. 2006; Hiraoka and Tomaru 2009). However, their nuclear genomes are not only subject to recombination but are also biparentally inherited, and are thus affected by both pollen and seed flows from contributory lineages. This can complicate attempts to decipher a species' phylogeographical pattern, evolutionary history and gene flow patterns. In contrast, the chloroplast (cp) genome in angiosperms is usually maternally inherited, so phylogeographical patterns revealed by cp DNA-based markers are attributable solely to seed flow among populations. Furthermore, since mutation rates in the cp genome are low, cp markers are not affected by recombination (Palmé et al. 2003a; Heuertz et al. 2004a). Therefore, since seed flow among populations is usually considered to be more restricted than pollen flow, cpDNA-based markers generally exhibit higher levels of population differentiation than nuclear markers (Petit et al. 1993; Palmé et al. 2003a), and thus their use facilitates the

reconstruction of ancestral lineages (Palmé et al. 2003a). Moreover, since the dynamics of gene transfer via seed and via pollen are different (e.g., Liepelt et al. 2002), comparison of genetic structures detected by maternal cp and biparental nuclear marker analyses allows detailed assessment of the differences between wide-scale seed and pollen flow dynamics.

Betula maximowicziana (Monarch birch) belongs to the Betulaceae, and is found in the cool temperate zone of Japan, from the eastern part of Honshu Island to Hokkaido (Ohwi 1965). The species is considered to be endemic and restricted to Japan. *B. maximowicziana* is a diploid ($2n = 28$), monoecious, wind-pollinated species that produces wind-dispersed seeds. It is a pioneer tree species that grows rapidly in open sites, such as gaps, and is often found in even-aged stands (Osumi and Sakurai 1997). *B. maximowicziana* plays an important role in the stability and sustainability of forest ecosystems in the cool temperate zone of Japan, not only as a major pioneer tree species but also as a long-lived dominant species (Watanabe 1989; see also Tsuda and Ide 2005 for a discussion of its ecological importance). *B. maximowicziana* also yields high quality wood, so its use as a commercial crop from natural forests is being promoted, and its seeds or seedlings are currently being commercially distributed and/or translocated with little or no regard for their provenance. Therefore, mainly for conservation purposes, the species' phylogeographical structure has been examined in several studies using nuclear DNA markers such as random amplified polymorphic DNA (RAPD; Tsuda et al. 2004) and simple sequence repeat (SSR) loci (Tsuda and Ide 2005). In the latter study, evidence from 23 populations was obtained indicating that they could be divided into two distinct groups, one located in the northern part of the species' range and the other in the south. The two groups probably originated from populations that occupied different refugia during past glacial periods (Tsuda and Ide 2005). However, the groups identified were not clearly differentiated, partly because of the limitations of nuclear DNA for such analyses as a result of its bi-parental inheritance and extensive gene flow among populations, especially via pollen. In the current study, we examined maternal cpDNA variation in *B. maximowicziana* in the hope that it may reveal aspects of phylogeographical patterns that are difficult or impossible to detect using nuclear DNA markers. Our aim was to elucidate the phylogeography of the species at a higher resolution than has been achieved previously.

In addition, it has become increasingly clear that many plant species or species complexes share cpDNA haplotypes (e.g., *Quercus*, Petit et al. 2002; Okaura et al. 2007; *Salix*, Palmé et al. 2003b; *Betula*, Palme et al. 2004; Maliouchenko et al. 2007 and *Fraxinus*, Heuertz et al. 2006). In particular, Palme et al. (2004) and Maliouchenko et al. (2007) found evidence of extensive sharing of chloroplast haplotypes due to introgression in three European species of *Betula*: *B. pendula*, *B. pubescens* and *B. nana*. Since such extensive sharing of haplotypes among species disrupts species-specific phylogeographical patterns (Palme et al. 2004), we also need to consider the possible occurrence of haplotype sharing and introgression in *B. maximowicziana* and other Japanese birches in order to determine whether the phylogeographical pattern in *B. maximowicziana* detected by cpDNA is species-specific or influenced by extensive introgression among related species. For this reason, the cpDNA variations of three other birch species (*B. ermani*, *B. platyphylla* and *B. grossa*) were also assessed. According to the classification and taxonomy presented by de Jong (1993)—which has been used frequently in recent phylogenetic and taxonomic studies of *Betula*—*B. maximowicziana*, *B. ermanii*, *B. platyphylla* and *B. grossa* are members of the subgenera *Betulaster*, *Neurobetula*, *Betula* and *Betulenta*, respectively; thus, each of the four species examined here belongs to a different subgenus. Eleven *Betula* species occur naturally in Japan, but these three were selected for chloroplast DNA variation comparisons with *B. maximowicziana* because they are widely distributed, while the other species have much more restricted distributions. *B. ermanii* is a tetraploid ($2n = 56$) species found in the subalpine zone from Shikoku to Hokkaido; it usually inhabits sites at altitudes higher than those of other *Betula* species. *B. platyphylla*, like *B. maximowicziana*, is a diploid species ($2n = 28$) distributed from the eastern part of Honshu to Hokkaido. *B. grossa* is a hexaploid species ($2n = 84$) that is distributed from Kyushu to the northern part of Honshu Island and is not found in Hokkaido. Although each birch species has a unique life-history and a specific niche, these species often coexist in the same stands. A chemotaxonomic analysis by Keinänen et al. (1999) indicated that the four species examined here are relatively divergent from each other, but their degree of molecular genetic divergence and their apparent phylogeny depends on the sequence region and genome examined (Järvinen et al. 2004; Nagamitsu et al. 2006). Thus, the phylogenies derived from molecular data relating to nuclear and cpDNA in previous studies are not consistent (see e.g., Järvinen et al. 2004; Nagamitsu et al. 2006).

In this study, the cpDNA phylogeography of *B. maximowicziana* was assessed by PCR-RFLP analysis in an attempt to obtain clearer evidence of a major genetic dichotomy between the northern and southern groups. The species' past colonization and gene flow patterns were also assessed using the data obtained from both cp and nuclear DNA analyses. As well as presenting the results of these analyses, the phylogeographical structures of *B. maximowicziana* and other tree species in Japan are compared

herein, and related to their apparent persistence or disappearance in the northern region during the last glacial period.

Materials and methods

Plant sampling

DNA samples from 16 randomly selected individuals from each of 25 natural populations covering most of the range of *B. maximowicziana* were used in the analysis (Fig. 1); 23 of these were sampled in our previous study (Tsuda and Ide 2005) and 2 (Erimo; H6 and Nanae; H7) were sampled in this study. Thus, samples from 400 individuals were used in total.

To analyze the extent and patterns of haplotype sharing between *B. maximowicziana* and other Japanese birches, samples were also collected from eight individuals representing three to five populations of each of the species *B. ermanii*, *B. grossa*, and *B. platyphylla*. These three species were sampled from the same locations, or close to the locations, where *B. maximowicziana* was sampled.

DNA extraction and PCR-RFLP analysis

For the 2 newly sampled populations of *B. maximowicziana* and the 11 populations representing the other three species, total genomic DNA was extracted from fresh cambium or bud samples using a DNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). For the other 23 populations, DNA extracted during our previous study (Tsuda and Ide 2005) was used. Three primer pairs—AS, CD and TF developed by Taberlet et al. (1991), Demesure et al. (1995) and Dumolin-Lapegue et al. (1997), respectively—and two restriction enzymes (*Hif*I and *Taq*I) were used for the initial screening of the primer-enzyme combinations. This was conducted using 24 individuals sampled from geographically distant populations of *B. maximowicziana* and small samples from the other three species. Since TF exhibited low levels of variation and *Hif*I and *Taq*I seemed to detect the same indel mutations in the TF region in this screening experiment, only the TF-*Taq*I combination was used for further analysis of this region.

The targeted sequences in each sample were amplified by PCR in 15 μL reaction mixtures containing 1–10 ng genomic DNA, 2.0 μM of each dinucleotide, 1× buffer,

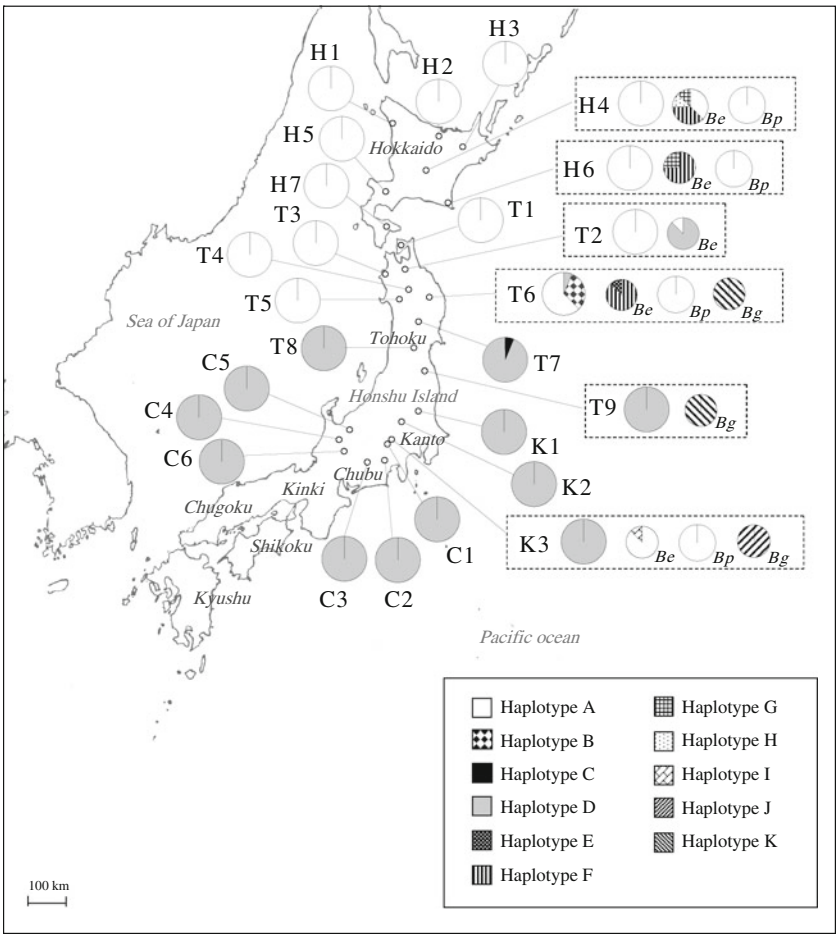

Fig. 1 Locations and distributions of detected chloroplast haplotypes of the 25 *Betula maximowicziana* populations and 11 populations of three related species. *Dashed boxes* Locations at which representatives of more than two species were examined. For these populations, *Be*, *Bp* and *Bg* indicate the haplotypes of *B. ermanii*, *B. platyphylla* and *B. grossa*, respectively. No such abbreviations are used for *B. maximowicziana*. The code letters *C*, *K*, *H* and *T* followed by numerals refer to the *B. maximowicziana* populations examined in Chubu, Kanto, Hokkaido and Tohoku

2.5 mM $MgCl_2$, 0.5 units LA *Taq* polymerase (Takara), and 0.2 μM of each primer in a DNA thermal cycler (Takara). The amplification program consisted of a denaturation step at 94°C for 5 min, followed by 45 cycles of 94°C for 30 s, annealing temperature for 30 s, 72°C for 4 min and finally 72°C for 5 min as a final extension step. The annealing temperatures (50, 55 and 54°C for the TF, CD and AS primers, respectively) and extension time were identical to those applied by Palmé and Vendramin (2002), but unlike the latter authors we did not use a touch-down PCR procedure. Each PCR product was cut with two restriction enzymes, *Hif*I and *Taq*I (Promega, Madison, WI) in incubations at 37°C for *Hif*I and 65°C for *Taq*I lasting 1.5–3 h in reaction mixtures with a total volume of 6.0 μL, containing 4.8 μL PCR product, 0.39 μL H_2O, 1× buffer (Promega), 0.06 μL BSA (10 mg/mL) and 1.5 U of the respective enzyme. The resulting products were separated electrophoretically on 3.0–4.5% agarose gels in 1× TAE buffer. The gels were then stained with ethidium bromide, photographed on a UV transilluminator and the fragment variation was evaluated.

Analysis of PCR–RFLP variation

The gene diversity (H) of each population and the population differentiation (G_{ST}) of each species were calculated. In addition, the population differentiation of *B. ermanii* was evaluated in terms of both unordered alleles (G_{ST}) and ordered alleles (N_{ST}), as described by Pons and Petit (1996), using the Permut software (developed by Petit, http://www.pierroton.inra.fr/genetics/labo/Software/Permut/index.html); 1,000 random haplotype permutations among populations were used to test whether the N_{ST} values were significantly higher than the G_{ST} values. According to Pons and Petit (1996), the presence of phylogeographical structure is indicated when the N_{ST} values obtained are significantly higher than the G_{ST} values. This test was performed only for *B. ermanii* because extremely little, or no, intra-population variation was detected in *B. maximowicziana*, *B. grossa* and *B. platyphylla* (see Results). Genetic variation between *B. maximowicziana* and *B. ermanii* was evaluated hierarchically by analysis of molecular variance (AMOVA: Excoffier et al. 1992) using GenAlEx ver. 6 software (Peakall and Smouse 2006: hereafter, GenAlEx). The significance of genetic variation, subdivided into between species, among populations within species, and among individuals within populations, was evaluated by a permutation test ($n = 999$) using GenAlEx. In addition, AMOVA was performed to evaluate the genetic variation among populations and among individuals within populations for the two species *B. maximowicziana* and *B. ermanii*. In this case, the significance of genetic variation was tested among populations using GenAlEx.

A haplotype network was constructed and edited following the statistical parsimony procedure implemented in TCS ver.1.06 software (Clement et al. 2000).

Comparison of within-haplotypic group genetic structures of *B. maximowicziana* using previous nuclear SSR data

The wide-scale genetic structure of *B. maximowicziana* was re-analyzed in detail using cpDNA data obtained in this study and information on variations at 11 nuclear SSR loci acquired from the two newly sampled and analyzed populations (32 individuals from the Nanae population and 45 individuals from the Erimo population) and the previous SSR dataset covering 1,014 individuals in 23 populations. The characteristics of these 11 loci, and the procedures used for the PCR genotyping, are described in Tsuda and Ide (2005). Using the genotype data for the 1,091 individuals in total, representing 25 populations, the average subpopulation gene diversity parameter H_S and the population differentiation parameters G_{ST} and F_{ST} (Weir and Cockerham 1984) were calculated using FSTAT ver. 2.9.3.2 software (Goudet 2001: hereafter, FSTAT). The significance of F_{ST} was tested by comparison to 95 and 99% confidence intervals derived from 1,000 bootstrap permutations implemented in FSTAT. The results of this study revealed two divergent cpDNA haplotypic population groups in *B. maximowicziana* (see Results). The genetic differentiation at the nuclear SSR loci associated with the two cpDNA haplotype population groups was evaluated hierarchically by AMOVA using Arlequin ver. 3.1 (Excoffier et al. 1992, 2007). To evaluate the genetic diversity within each cpDNA population group in more detail, the genetic diversity of nuclear SSR loci within each group was compared in terms of the parameters allelic richness (El Mousadik and Petit 1996), expected heterozygosity (H_E) and F_{ST}. The differences between groups, for individual parameters, were examined using a permutation test in FSTAT.

Standardization of population differentiation and pollen/seed migration ratios

Since absolute values of the population differentiation parameters F_{ST} or G_{ST} depend on the level of genetic variation in the examined material (Hedrick 2005), in this study, we also calculated standardized values of G_{ST}, G'_{ST} (Hedrick 2005), which always range from 0 to 1, using values of intra-population gene diversity (H_S), total gene diversity (H_T) and G_{ST}. Thus, the measure allows comparisons between loci with different levels of genetic variation and allows us to examine genetic differentiation of organisms with different effective population sizes (Hedrick 2005).

Table 1 Location, sample size and distribution of chloroplast haplotypes in each of the 25 populations of *Betula maximowicziana*

Region	Population name	Code	Sample size	Longitude (E)	Latitude (N)	Altitude (m)	Haplotypes				Gene diversity (*H*)
							A	B	C	D	
Hokkaido	Shosanbetsu	H1	16	141°49′	44°27	100–150	16	0	0	0	0.000
Hokkaido	Okoppe	H2	16	143°04′	44°19′	200–250	16	0	0	0	0.000
Hokkaido	Oketo	H3	16	143°29′	43°36′	400–450	16	0	0	0	0.000
Hokkaido	Furano	H4	16	142°26′	43°17′	400	16	0	0	0	0.000
Hokkaido	Rankoshi	H5	16	140°33′	42°41′	400–450	16	0	0	0	0.000
Hokkaido	Erimo	H6	16	143°16′	42°09′	150-200	16	0	0	0	0.000
Hokkaido	Nanae	H7	16	140°45′	41°53′	600-700	16	0	0	0	0.000
Tohoku	Mutsu	T1	16	141°05′	41°18′	200–300	16	0	0	0	0.000
Tohoku	Towadako	T2	16	140°56′	40°35′	450–600	16	0	0	0	0.000
Tohoku	Fukaura	T3	16	140°04′	40°34′	250–450	16	0	0	0	0.000
Tohoku	Appi	T4	16	140°57′	39°59′	750–850	16	0	0	0	0.000
Tohoku	Tazawako	T5	16	140°46′	39°47′	600–700	16	0	0	0	0.000
Tohoku	Iwaizumi	T6	16	141°28′	39°43′	900–1050	10	5	0	1	0.542
Tohoku	Naruko	T7	16	140°41′	38°48′	500–550	0	0	1	15	0.125
Tohoku	Zao	T8	16	140°25′	38°09′	1250–1400	0	0	0	16	0.000
Tohoku	Katsurao	T9	16	140°41′	37°32′	800–950	0	0	0	16	0.000
Kanto	Shiobara	K1	16	139°49′	36°55′	900–1000	0	0	0	16	0.000
Kanto	Tone	K2	16	139°17′	36°39′	1200–1350	0	0	0	16	0.000
Kanto	Otaki	K3	16	138°49′	35°56′	1200–1350	0	0	0	16	0.000
Chubu	Mitomi	C1	16	138°41′	35°52′	1800–1900	0	0	0	16	0.000
Chubu	Hayakawa	C2	16	138°17′	35°19′	1450–1600	0	0	0	16	0.000
Chubu	Kiso	C3	16	137°35′	35°44′	1000	0	0	0	16	0.000
Chubu	Toga	C4	16	137°02′	36°23′	1000–1200	0	0	0	16	0.000
Chubu	Oyama	C5	16	137°19′	36°25′	1050–1200	0	0	0	16	0.000
Chubu	Shirakawa	C6	16	136°49′	36°08′	1100	0	0	0	16	0.000

The pollen/seed migration ratio, r (Ennos 1994), was calculated using the formula:

$$r = m_p/m_s = \{2(1/G_{STc} - 1) - (1/G_{STn} - 1)\}/(1 - 1/G_{STc})$$

where m_p is the pollen migration rate, m_s the seed migration rate, G_{STc} is the cytoplasmic G_{ST} and G_{STn} is the nuclear G_{ST}. In this study, standardized G'_{STc} and G'_{STn} values were calculated for all materials used in the analysis, from either newly acquired data or data presented in the cited studies.

Results

Chloroplast DNA variation in *B. maximowicziana* and three related species

In total, 11 haplotypes were detected among the four species. In the analysis of the 25 *B. maximowicziana* populations, a total of four haplotypes were detected (Fig. 1; Table 1), and a total of nine haplotypes were detected in the analyses of the three related species: seven, two and one for *B. ermanii*, *B. grossa* and *B. platyphylla*, respectively (Fig. 1; Table 2). The identified haplotypes are described in Table 3 and the genetic relationships among the haplotypes, based on estimated mutational steps, are shown in Fig. 2. The *B. maximowicziana* populations in the northern (northern Tohoku and Hokkaido) and southern (southern Tohoku, Kanto and Chubu) areas were dominated by haplotypes A and D, respectively. Haplotype B was rare and unique to the Iwaizumi (T6) population. Haplotype C was extremely rare and detected in only one individual in the Naruko (T7) population. Thus, the group of populations dominated by haplotype A (H1 to T6 populations) is hereafter referred to as the "northern group" and the populations dominated by haplotype D (T7 to C6 populations) as the "southern group". The Iwaizumi (T6) population is the only one that was found to include both the northern haplotype A and the southern haplotype D, although the frequency of haplotype D therein was low. Overall, for the 25 populations, the values of G_{ST} and G'_{ST} were 0.950 and 0.977, respectively, indicating that population differentiation was high.

Table 2 Location, sample size and distribution of chloroplast haplotypes in each of three to five populations of *Betula ermanii*, *B. platyphylla* and *B. grossa*

Species	Population name	Code	Sample size	Haplotypes									Gene diversity (*H*)
				A	D	E	F	G	H	I	J	K	
B. ermanii	Furano	H4	8	3	0	0	3	1	1	0	0	0	0.786
	Erimo	H6	8	0	0	0	6	2	0	0	0	0	0.429
	Towadako	T2	8	1	7	0	0	0	0	0	0	0	0.250
	Iwaizumi	T6	8	0	0	1	7	0	0	0	0	0	0.250
	Otaki	K3	8	7	0	0	0	0	0	1	0	0	0.250
B. platyphylla	Furano	H4	8	8	0	0	0	0	0	0	0	0	0.000
	Erimo	H6	8	8	0	0	0	0	0	0	0	0	0.000
	Iwaizumi	T6	8	8	0	0	0	0	0	0	0	0	0.000
	Otaki	K3	8	8	0	0	0	0	0	0	0	0	0.000
B. grossa	Iwaizumi	T6	8	0	0	0	0	0	0	0	0	8	0.000
	Katsurao	T9	8	0	0	0	0	0	0	0	0	8	0.000
	Otaki	K3	8	0	0	0	0	0	0	0	8	0	0.000

Table 3 Descriptions of the 11 identified chloroplast haplotypes

Haplotypes	CD			AS						TF	
	Hif I	*Taq* I-1	*Taq* I-2	*Hif* I-1	*Hif* I-2	*Taq* I-1	*Taq* I-2	*Taq* I-3	*Taq* I-4	*Hif* I	*Taq* I
A	1	1	0[a]	0	0	1	0	0	0	1	1
B	1	1	0	0	0	1	0	0	0	2	2
C	2	2	0	0	0	2	1	0	0	1	1
D	2	2	0	0	0	2	0	0	0	1	1
E	1	1	1	0	0	1	0	0	0	1	1
F	2	2	0	0	0	2	0	0	0	1	1
G	2	2	0	0	0	2	0	1	0	1	1
H	2	2	0	0	0	2	0	0	1	1	1
I	2	2	0	0	1	2	0	0	0	1	1
J	3	3	2	1	2	3	0	0	0	3	3
K	4	4	3	2	3	4	0	0	0	4	4

The variations are scored in multistates

[a] Complete absence of a restrict fragment from its expected position on a gel

For the related species examined, both intra-species and intra-population variations were detected only in *B. ermanii* ($H_T = 0.822$, $H_S = 0.393$). The N_{ST} value for *B. ermanii* was 0.378 and was not significantly larger than its G_{ST} value (=0.522). Further, although the G_{ST} value for *B. ermanii* was not very high, the standardized parameter G'_{ST}, taking variation within populations into consideration, was high (0.944), indicating that between-population differentiation was high in *B. ermanii*. The two haplotypes J and K were detected only in populations of *B. grossa*, and they diverged considerably from haplotypes (A–I) detected in *B. maximowicziana*, *B. ermanii* and *B. platyphylla*, differing in each case by at least eight mutational steps (Table 3). Although haplotype sharing among *B. maximowicziana*, *B. ermanii* and *B. platyphylla* was detected in this study (Fig. 1), the geographical distributions of the shared haplotypes differed among the species. In Otaki (K3), for example, *B. ermanii* population was dominated mostly by haplotype A, while the haplotype of all examined members of the *B. maximowicziana* population was haplotype D. In Towadako (T2), conversely, *B. ermanii* population was dominated mostly by haplotype D, while the haplotype of all examined members of the *B. maximowicziana* population was haplotype A.

The results of the hierarchical AMOVA of cpDNA data revealed that the proportions of genetic variance partitioned between the two species *B. maximowicziana* and *B.*

ermanii, and among populations within these species were 14.55 and 76.63%, respectively, with the remainder (8.82%) occurring within-populations (Table 4). Each value of partitioned genetic variance was significant ($P < 0.001$) and, thus, these AMOVA results indicate significant genetic differentiation of the cpDNA among the two species. Although genetic variation among populations was significant for both of the species examined in the intra-species AMOVA, the amount of genetic variation subdivided into 'among populations' and 'among individuals within populations' was clearly different for *B. maximowicziana* and *B. ermanii* (Table 4).

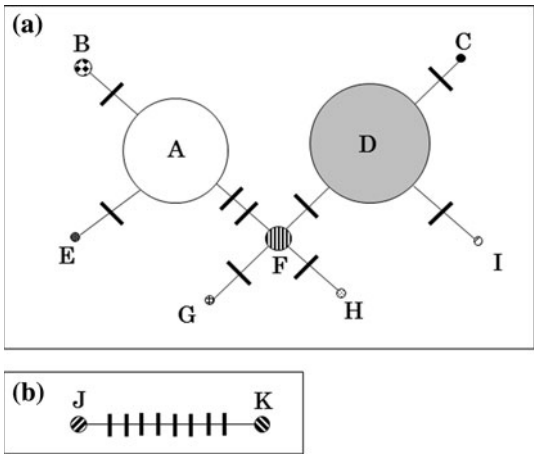

Fig. 2 Relationships between chloroplast haplotypes based on a statistical parsimony network. *Circle* sizes roughly reflect haplotype frequency, and *thick lines* in the network indicate mutational steps. The relationships between nine haplotypes (*A–I*) could be inferred (**a**). However, it was impossible to infer which of these haplotypes was most closely related to the remaining two haplotypes (*J* and *K*) in *B. grossa* (**b**), since no polymorphisms were shared between haplotypes *A–I* and *J–K*

Comparison of within-haplotypic group genetic structures of *B. maximowicziana* using previously acquired nuclear SSR data

The F_{ST} value obtained from the analysis of nuclear SSRs in the samples from the 1,091 individuals representing 25 populations was 0.060, with confidence intervals of 0.049–0.073 (95%) and 0.047–0.077 (99%), indicating that population genetic differentiation was relatively low. Accordingly, based on the H_S and G_{ST} values in each nuclear SSR locus, the G'_{ST} value was 0.100. The pollen/seed migration ratio *r* of *B. maximowicziana* was 387.5. The AMOVA of the nuclear SSR data revealed that the proportions of genetic variance partitioned between the two haplotypic groups of *B. maximowicziana* populations (northern and southern) and among populations within the groups were 2.32 and 4.69%, respectively, with the remainder (92.99%) occurring within-populations. Each value of partitioned genetic variance was significant ($P < 0.001$). None of the three parameters allelic richness, H_E and F_{ST} of nuclear SSR loci provided any indication of significant differences between the cpDNA population groups.

Discussion

Population differentiation of *B. maximowicziana*

The cpDNA variation detected here was clearly structured, with a single haplotype fixed in all of the populations except Iwaizumi (T6) and Naruko (T7). Therefore, population differentiation in the cpDNA of *B. maximowicziana* was high ($G'_{ST} = 0.977$) and higher than in its nuclear DNA ($G'_{ST} = 0.100$). This high G'_{ST} value for *B.*

Table 4 Analysis of molecular variance (AMOVA) for 440 individuals in 25 *B. maximowicziana* populations and 5 *B. ermanii* populations based on chloroplast DNA variations

Source of variation	*df*	SS	Variance components	Total variance (%)[a]	*P* value[b]
B. maximowicziana and *B. ermanii*					
Between species	1	5.702	0.048	14.55	<0.001
Among populations within species	28	105.533	0.252	76.63	<0.001
Among individuals within population	410	11.875	0.029	8.82	<0.001
B. maximowicziana					
Among populations	24	97.882	4.078	95.01	<0.001
Among individuals within population	375	5.000	0.013	4.99	
B. ermanii					
Among populations	4	7.650	1.912	52.20	<0.001
Among individuals within population	35	6.875	0.196	47.80	

df Degrees of freedom, *SS* sum of squares

[a] Total variation contributed by each component

[b] $n = 999$ permutations

maximowicziana cpDNA is similar to values reported for other Japanese broad-leaved tree species, such as *Fagus crenata* ($G_{ST} = 0.963$, $G'_{ST} = 0.996$; Tomaru et al. 1998). Okaura et al. (2007) also detected a high level of differentiation among populations ($G_{ST} = 0.853$, $G'_{ST} = 0.974$) of *Quercus mongolica* var. *crispula*. On the other hand, according to estimates based on data presented by Palmé et al. (2003a), cpDNA population differentiation was lower in *B. pendula* ($G_{ST} = 0.42$, $G'_{ST} = 0.696$) than in *B. maximowicziana* (which we investigated using the same primer–enzyme combinations as the cited authors; all the G'_{ST} values were calculated by Y.T.).

Comparative phylogeography with *B. maximowicziana*

The main boundary associated with the cpDNA haplotypes of *B. maximowicziana* passes through the Tohoku region in northeastern Japan**.** Although identifying likely refugia and colonization patterns of individual *Betula* species in Japan using pollen fossil data would be difficult due to the difficulty of distinguishing between *Betula* species in pollen analysis, the phylogeographical patterns exhibited by *B. maximowicziana* and described here can be discussed on the basis of a comparative phylogeographical approach, comparing them with those of other species. On the one hand, although *B. maximowicziana* occasionally coexists with *Fagus crenata* and *Q. mongolica* var. *crispula* in cool temperate forests in Japan, phylogeographical patterns of these two species are different from that of *B. maximowicziana*. For example, *F. crenata* populations can be broadly divided into two groups: one on the Sea of Japan side and the other on the Pacific Ocean side of Japan, with a boundary running through Honshu Island. This pattern is apparent not only in the nuclear, cp and mitochondrial (mt) DNA variations (e.g., Tomaru et al. 1998; Fujii et al. 2002; Okaura and Harada 2002; Hiraoka and Tomaru 2009) but also in the morphological variations of the leaves (Hagiwara 1977). In *Q. mongolica* var. *crispula*, two main cpDNA lineages of the species have been identified, although their main boundary is further south than that of *B. maximowicziana*, running across central Honshu, south of the Tohoku region (Okaura et al. 2007). These differing phylogeographical patterns between *B. maximowicziana* and the other two tree species might mirror differences in the life-history traits of the species and different past colonization episodes influenced by species-specific life history traits, in particular cold-tolerance, as discussed by Lascoux et al. (2004). Since *B. maximowicziana* is cold tolerant, the species is considered to have survived in more northerly regions during past ice ages than other tree species such as beech and oak. Indeed, Bhagwat and Willis (2008) have presented evidence of various species' ability to persist in northerly refugia based on the life-history traits of 12 European woody species of small-seeded, wind-dispersed angiosperms and gymnosperms—including *Alnus*, *Betula*, *Picea*, *Pinus* and *Salix* spp.

On the other hand, the phylogeographical pattern exhibited by *B. maximowicziana* is very similar to that of the alpine tree *Pinus pumila*, examined by Tani et al. (1996). *P. pumila* populations can be divided between the northern Tohoku and southern Tohoku regions largely on the basis of their allozymes (Tani et al. 1996). The similarity of these phylogeographical patterns between the cold-tolerant broadleaved tree, *Betula pendula* and the cold-tolerant conifer, *Picea abies* has also been discussed by Palmé et al. (2003a). Moreover, based on cpDNA lineages, similar genetic boundaries between the central Honshu and Tohoku regions have also been detected in five alpine plants: *Pedicularis chamissonis*, *Primula cuneifolia*, *Loiseleuria procumbens*, *Cardamine nipponica*, and *Anemone narcissiflora* (Fujii and Senni 2006). Fujii et al. (1997) and Fujii and Senni (2006) hypothesized that the most likely explanation for the phylogeographical pattern detected in alpine plants was that two major lineages had been transported southwards to the Japanese Archipelago during different glacial periods. The phylogeographical pattern exhibited by *B. maximiwicziana* might also be the result of multiple colonization events following different glacial episodes within the Japanese Archipelago, during repeated glacial and inter-glacial cycles. Alternatively, the two haplotypic groups detected here may represent different lineages originating from two distinct refugia established during a single glacial period. Although we cannot determine which of the two hypotheses better explains the observed phylogeographic pattern, both of these hypotheses suggest northerly persistence of *B. maximowicziana* during a glacial period, which is consistent with the species' aforementioned cold-tolerance. In addition, private haplotypes were detected in the Iwaizumi (T6; haplotype B) and Naruko (T7; haplotype C) populations, suggesting that the areas around these populations may have been "cryptic refugia (Provan and Bennett 2008)" during a past ice age.

There were three mutations between the northern (A) and southern (D) haplotype of *B. maximowiciziana* in this study. Although the exact mutation rate of cpDNA in the Betulaceae is unknown, the fragment pattern of the PCR-RFLP examined suggests that these three are probably point mutations. The northern and southern haplotypes may have diverged over a time scale of millions of years, based on the fact that the substitution rate of *rbc*L in the cpDNA in the Fagaceae is $2.36 \pm 0.79 \times 10^{-10}$ (Frascaria et al. 1993), as also discussed in Okaura and Harada (2002) and Okaura et al. (2007). *B. maximowicziana* is endemic to Japan and a number of *B. maximowicziana* fossils from the Miocene period (Tertiary) have been found there (e.g., see

the fossil lists in the homepages of the National Museum of Nature and Science, http://www.kahaku.go.jp/education/specimen_rent/kaseki.html, and the Gunma Museum of Natural History, http://www.gmnh.pref.gunma.jp/storageo/YB00001702/pageYB00001702.html). Therefore, although the phylogeography of tree species has been discussed mainly in the context of recolonization patterns following the last glacial maximum (LGM), detected cpDNA haplotypes and their distribution patterns in *B. maximowicziana* might have occurred and formed over a long time, and may have pre-dated the LGM. In support of this, recent studies in Europe suggest that longer temporal scales and episodes, such as repeated ice ages in the Quaternary or plate tectonic events in the Tertiary, should also be considered when discussing factors affecting modern phylogeographical structure (e.g., Magri et al. 2006, 2007; Ingvarsson 2008; Lascoux et al. 2008).

Gene transfer between northern and southern haplotypic groups

In previous nuclear SSRs analysis of *B. maximowicziana* (Tsuda and Ide 2005), two northern and southern clusters (assumed to be demes) were detected using Bayesian clustering STRUCTURE analysis developed by Pritchard et al. (2000). In the cited study, the populations in Hokkaido and the northern Tohoku region (H1–5 and T1–T6) belong to the northern cluster and the populations in the Kanto region and Central Honshu (K1–3 and C1–6) to the southern cluster. The STRUCTURE analysis indicated that both clusters accounted for ca. half of the ancestry of some populations located in the geographically intermediate zone (the southern Tohoku region between northern Tohoku and the northern central Honshu region) such as the Naruko (T7), Zao (T8) and Katsurao (T9) populations. These results show that geographically intermediate populations are also genetically intermediate according to the bi-parentally inherited nuclear DNA analysis. However, the maternal cpDNA variation of these populations was clearly structured, and their cpDNA haplotypes clearly belong to the southern group (with haplotype D predominating). These findings may reflect differences in gene flow dynamics between populations originating from northern and southern lineages, especially via pollen. In particular, these patterns may be influenced by past population colonization and admixing. For example, if the southern populations traveled rapidly northwards to the southern Tohoku region in lower densities than the northern population established there, the few southern immigrants could suffer from pollen limitation and might often receive pollen originating from northern populations. Since these areas are considered to represent the contact zone between northern and southern groups, such asymmetrical nuclear transfer and exchange via pollen will have occurred repeatedly and been complicated by hybridization between the groups and further backcrossing and/or hybridization between hybrids and purely southern or purely northern individuals. In contrast, clear separation between northern and southern cp genome in these areas suggests that transfer and exchange of the nuclear genome from populations in the northern Tohoku region (which have northern haplotypes) via seed to populations in the southern Tohoku region (which have southern haplotypes) has been less frequent than pollen flow.

Although the G'_{ST}-based pollen/seed migration ratio, *r*, of *B. maximowicziana* (387.5) we obtained is lower than the reported value for *Carpinus betulus* (849.8, Grivet and Petit 2003; Coart et al. 2005), it appears to be substantially higher than that of several other tree species, for example *B. pendula* (287.2, Palmé et al. 2003a; Rusanen et al. 2003), *Fagus sylvatica* (60.6, Demesure et al. 1996; Comps et al. 2001), *Fraxinus excelsior* (54.1, Heuertz et al. 2004a, b) and *Quercus suber* (44.6, Toumi and Lumaret 1998; Belahbib et al. 2001) (all G'_{ST}-based *r* values calculated by Y.T.). Thus, the potential pollen flow to potential seed flow ratio of *B. maximowicziana* seems to be within the higher part of the range for tree species. Therefore, the high value of the pollen/seed migration ratio of *B. maximowicziana* supports the hypothesis that pollen flow dominates in gene transfer events between the northern and southern groups.

Haplotype sharing among species

Ancestral polymorphism and introgression/hybridization are considered to be the main factors associated with the sharing of cpDNA haplotypes among related species. These two factors can be distinguished by estimating common ancestral haplotypes and their geographical distributions among species (Palme et al. 2004). Ancestral haplotypes are common among species (Watterson and Guess 1977); therefore, if the shared haplotypes are rare among species and are peripheral in the haplotype network, ancestral polymorphism is unlikely to provide an adequate explanation for the sharing (Palme et al. 2004). In addition, if the species shared haplotypes only as a result of ancestral polymorphism, their geographical distribution patterns should be independent of each other (Palme et al. 2004). Therefore, if hybridization and introgression had occurred frequently, both common and rare haplotypes should theoretically be shared, and show similar geographical patterns among species, as observed among *B. pendula*, *B. pubescens* and *B. nana* in central Europe by Palme et al. (2004). In contrast, common haplotypes, especially haplotype A were shared among the Japanese birches, and the

geographical patterns of the shared haplotypes seem to be more species-specific than those of the European species, although limited numbers of populations were examined here. Therefore, AMOVA did show significant genetic variation among Japanese species in this study, whereas it did not detect significant genetic variation among species of European birches (Palme et al. 2004; Maliouchenko et al. 2007), suggesting that the species-component of genetic variation was stronger than the geographic component for Japanese birch species; European species exhibit the opposite pattern (Palme et al. 2004). These results suggest that current sympatric introgression/hybridization among species is very rare although ancestral polymorphism cannot be excluded as a possible factor explaining the cpDNA haplotype sharing detected in this study. Such rare hybridization is supported by our field observation that phenotypic hybrids between Japanese birches occur very rarely in natural populations (authors' personal observations). However, further extensive sampling of Japanese birches, analysis of DNA sequences, physiological, palynological and palaeogeographical data are needed not only to elucidate the factors and processes that have shaped their haplotype sharing (e.g., ancestral polymorphism, ancient introgression and/or recent local hybridization–introgression) but also to draw clear conclusions about the population history of *B. maximowicziana*, including asymmetrical gene flow in the contact zone.

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