Chloroplast DNA Inversion Polymorphism in Populations of Abies and Tsuga

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Polymorphism for a 42-kb chloroplast DNA inversion was detected in five species of Abies and two species of Tsuga based on a sample of 1,281 individuals and both Southern hybridization and polymerase chain reaction (PCR) analyses. Two haplotypes were observed in all populations and species. The 42-kb inversion is associated with a short inverted repeat that includes trnS, psaM, and trnG. The frequencies of the two haplotypes within species were very similar among the five species of Abies. This polymorphism has been maintained within populations and species in both Abies and Tsuga, probably because the mutation rate of the inversion is high. Haplotype frequencies had no geographical tendencies for any species except Abies mariesii, in which haplotype frequencies varied clinically, possibly as a result of rapid dissemination after the most recent glacial period and random genetic drift.

Introduction

Although the chloroplast genome is generally conservative in its evolution, intraspecific variation in chloroplast DNA (cpDNA) has been observed and studied in many species (reviewed by Soltis, Soltis, and Milligan 1992). The nature of intraspecific cpDNA polymorphism is typically limited to restriction site changes and insertion/deletion mutations. In a few cases, intraspecific variation has been ascribed to an inversion (Palmer, Jorgensen, and Thompson 1985; Brunsfeld, Soltis, and Soltis 1992; Hong, Hipkins, and Strauss 1993), but such intraspecific variation in the chloroplast genome is thought to be rare.

The cpDNA in conifers has several distinct features relative to that of angiosperms, including lack of a large inverted repeat (Lidholm et al. 1988; Strauss et al. 1988; Rauhueson and Jansen 1992; Tsumura et al. 1993), paternal inheritance (Neale, Wheeler, and Allard 1986; Neale, Marshall, and Sederoff 1989; Mogensen 1996), relatively high levels of intraspecific variation (Wagner et al. 1987; Hong, Hipkins, and Strauss 1993; Dong and Wagner 1994; Tsumura et al. 1994), and a different pattern of RNA editing (Wakasugi et al. 1996). The presence of an inverted repeat may influence the frequency of inversion events. For example, the legumes are one of only a few angiosperm groups that lack the large inverted repeat, and some legumes have a high frequency of inversion events. Palmer et al. (1987) proposed that lack of the inverted repeat structure in the legume chloroplast DNA was implicated in its relative instability. The rearranged cpDNAs typical of Pinaceae and several legumes alternatively may be due to the presence of numerous dispersed repeated sequences (Milligan, Hampton, and Palmer 1989).

Intraspecific cpDNA variation has been studied in many species of Pinaceae. Such studies have investigated levels of cpDNA diversity and the geographic and taxonomic tendencies of cpDNA variation (Wagner et al. 1987; Hong, Hipkins, and Strauss 1993; Dong and Wagner 1994), although the cause of the variation has not always been reported. The chloroplast genome of conifers contains several short repeats (Tsai and Strauss 1989; Tsudzuki et al. 1992), and recombination events might occur through these repeats (Strauss et al. 1988). For example, Hipkins et al. (1995) documented the fact that a mutational hot spot in the cpDNA of Douglas fir (Pseudotsuga menziesii) is caused by variability in the number of direct repeats derived from a partially duplicated tRNA gene. They indicated that this polymorphism was most likely generated by partial trnY gene duplication and that the sequences were then expanded by slipped-strand mispairing and unequal crossing over.

We previously reported evidence of intraspecific cpDNA variation in Abies mariesii (Tsumura et al. 1994). In the current study, we surveyed cpDNA variation in 47 populations of five species of Abies. To determine whether the same cpDNA polymorphism is maintained in other genera, we surveyed for the polymorphism in one population of each of two Tsuga species, T. diversifolia and T. sieboldii. According to a molecular phylogeny of conifers (Tsumura et al. 1995), Keteleeria is more closely related to Abies than Tsuga is, but in Japan, we could collect population samples of only Tsuga because there are no natural populations of Keteleeria. We also studied the cause of the variation using gene mapping by PCR and sequence analysis of cpDNA. The mechanism responsible for this cpDNA variation and possible reasons for the maintenance of the polymorphism in Abies and Tsuga are discussed.

Materials and Methods

Plant Materials

Fresh needles were collected from 1,281 trees from 47 natural populations of 5 Abies species widely dispersed across the natural distributions of the species in Japan (fig. 1). Fourteen and six specimens of T. diversifolia and T. sieboldii, respectively, were also sampled.

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Abies sachalinensis is found on Hokkaido Island (the northernmost part of Japan) between 10 and 1,650 m above sea level. Abies mariesii is found in the subalpine regions from central to northern Honshu Island, where it occurs primarily between 1,000 and 1,800 m (northern Honshu) and between 1,800 and 2,900 m (central Honshu). Abies veitchii is largely restricted to central Honshu at an elevation of 1,200–2,800 m, except for a few isolated populations, including the Mitsunomi population of the Kii Peninsula and the Ishizuchi population on Shikoku Island. The Ishizuchi population of A. veitchii is isolated from the main distribution of the species (fig. 1) and is treated as A. veitchii var. sikokiana (Liu 1971). The distribution of Abies homolepis ranges from Fukushima Prefecture, south through central Honshu and Shikoku, to Kyushu Island. Its range is generally between 1,000 and 1,800 m above sea level. The Mitsumine population of Abies firma is considered a hybrid population between A. firma and A. homolepis, named A. umbellata (Mayr 1890; Liu 1971). This hybrid population is distributed only in a narrow area of central Japan. Tsuga sieboldii grows in hills and mountains at elevations between 500 and 1,500 m from central Honshu Island to Kyushu Island and is usually associated with A. firma and some other conifers. Tsuga diversifolia occurs in the mountains at elevations between 700 and 2,000 m from central to northern Honshu and is common in mixed coniferous forests with A. homolepis, A. veitchii, A. mariesii, and other conifers (Farjon 1990). Fresh needles from the specimens of the 47 Abies populations and the 2 Tsuga populations were stored at −30°C until DNA extraction.

DNA Extraction and Analysis

Total DNA was extracted from needles of each species by a modification of the method of Tsumura et al. (1995). For preliminary screening, DNA from six individuals of each of the five Abies species was digested by 18 restriction endonucleases, BamHI, BglII, DraI, EcoRI, EcoRV, HaeIII, HindIII, HinfI, HhaI, KpnI, PstI, PvuII, StyI, Sall, Smal, TaqI, XbaI, and XhoI. DNA fragments were separated electrophoretically at 15 V for 20 h in 0.7% agarose gels in TAE buffer (40 mM Tris-HCl, 20 mM sodium acetate, 2 mM EDTA [pH 8.0]). After electrophoresis, the DNA fragments were transferred to
a nylon membrane (Hybond-N+, Amersham Co. Ltd.) following the protocol of Amersham. Heterologous probes were used to detect variation in the cpDNA of the five species using the procedure of Tsumura et al. (1994). Clones pCS3, pCS5, pCS7, pCS8, pCS9a, pCS9b, pCS10, pCS11, and pCS12 of Cryptomeria japonica (sugi) cpDNA (Tsumura et al. 1993) and clones pTBA3, pT66, pT88, pT20, pT25, pTB7, and pTB10 of tobacco cpDNA (Shinozaki et al. 1986) were used as probes.

Based on complete cpDNA sequence data from Pinus thunbergii (Wakasugi et al. 1994), we designed 100 specific PCR primers for amplification of an entire P. thunbergii chloroplast genome in order to construct a chloroplast gene map of Abies species and to identify regions of intraspecific variation in the Abies chloroplast genome. PCR primers were carefully designed using the OLGIGO program, version 4.0 (National Bioscience), and then synthesized. Using DNA samples of A. mariesii, Pinus parviflora, Picea koyamae, Larix kaempferi, and P. thunbergii as templates, 105 primer combinations were tested to see if they yielded PCR products. DNA materials were those used in a previous study (Tsumura et al. 1995). After checking PCR amplification, we undertook a structural analysis of the Abies cpDNA and constructed a cpDNA gene map for the genus. In this analysis, we investigated the inversion, insertion, and deletion of cpDNA in the five Abies species. Pinus thunbergii cpDNA was also used to confirm the sizes of PCR products.

After detecting regions of intraspecific cpDNA variation in Abies, we designed the following three primers to quantify two major variants by PCR: (A) 5′-CGTTTACCGAGGGTTCGAATC-3′, (B) 5′-CCAAAGTTCATCTCATCTC-3′, and (C) 5′-CTTCTGTCTTCTACCATGAC-3′. These sequences are located in the psbI, trnR, and clpP genes, respectively. PCR amplification of the cpDNA regions was performed using the procedure of Tsumura et al. (1995).

To confirm the sequence of the intraspecific cpDNA variation in five species of Abies and T. diversifolia, we conducted sequence analysis of two regions: region I (psbI-trnS-psaM-trnG) and region II (clpP-ΨtmG-psaM-trnS). DNA samples from our previous study (Tsumura et al. 1995) were used except for A. firma, which was collected in the arboretum of the Forestry and Forest Products Research Institute in Japan. We used the following primers for PCR amplification of these regions: (a) 5′-TTGGAAGCTGCTAGT3′ and (b) 5′-TCGGGACTCTCAGCA-3′ for region I, and (c) 5′-CTTCTGTCTTCTACCATGAC-3′ and (d) 5′-TTTATACCCACAGGTAC-3′ for region II. PCR products were purified by MicroSpin S-400 HR Columns (Pharmacia Co. Ltd.) to prepare the template DNA for sequencing. The following five primers were used for sequencing: (e) 5′-TGTTTACCGAGTATGGTAT-3′, (f) 5′-GTCACTAGCCATCATCCTCTC-3′, (g) 5′-AACGAATCCCATCTTACCAC-3′, (h) 5′-CCGTCAGGAGTTGATAC-3′, and (i) 5′-GCGGTATAGTTAGTGTA-3′. Sequencing was done using a dye terminator cycle sequencing kit (Perkin-Elmer) on an ABI 377 automated sequencer.

Statistical Analysis

Phenotypic cpDNA variation in each population was determined by analysis of Southern hybridization and PCR, and haplotypes were subsequently determined. The frequencies of haplotypes in each population were then estimated, considering the chloroplast genome as one locus with the haplotypes as alleles. Birky, Maruyama, and Fuerst (1983) and Birky, Fuerst, and Maruyama (1989) developed the theory for estimation of organelle gene diversity based on Nei's (1973, 1978) theory. In this study, haplotype diversity (H) was calculated in each population using the haplotype frequencies using Nei's method. Haplotype diversity statistics were also calculated (Nei 1973, 1987) using the haplotype frequencies to understand the partitioning of the diversity in Abies and then to consider the evolutionary mechanisms affecting cpDNA variation, such as genetic drift and gene flow. These statistics partition genetic diversity into subdivisions within and among populations. Total gene diversity (HT) consists of the genetic variation within (HS) and among (DST) populations: HT = HS + DST. GST, the coefficient of genetic differentiation among populations, was then calculated to determine how gene diversity was partitioned, using the equation GST = DST/HT. Using the FSTAT program (Goudet 1995), θ (Cockerham 1969), equivalent to Wright's (1965) FST, was also estimated, together with its standard deviation, by the method of Weir (1990), and the statistical significance of θ was evaluated by the permutation test (Weir 1996).

Results

Intraspecific Variation of Abies cpDNA Detected by Southern Hybridization

Sixty combinations of restriction endonucleases and probes were used in Southern hybridization for preliminary screening of polymorphism using 30 individuals (six individuals per species) from five species of Abies. Intraspecific cpDNA variation was detected with 11 combinations: HindIII + pCS10, HindIII + pCS7, BgIII + pCS7 (Tsumura et al. 1994), DraI + pCS11, KpnI + pCS7, KpnI + pCS11, PvuII + pCS7, PvuII + pCS11, Smal + pCS11, XhoI + pCS7, and XhoI + pCS11. Two types of variants were found when we probed with the pCS7 and pCS10, which occupy neighboring positions on a physical map of cpDNA in C. japonica. Furthermore, using the pCS11 probe in combination with any one of five restriction enzymes, we detected two variants whose patterns agreed with those found using the pCS7 and pCS10 probes. With the other combinations, we detected some interspecific variation (data not shown) with less frequent forms, most of which were confined to single species such as A. mariesii or A. veitchii.

We investigated all samples of Abies (1,281 specimens taken from 47 populations of 5 Abies species) using only two combinations of probe and endonuclease,
F I G . 2.—Intraspecific variation of chloroplast DNA in Japanese Abies species: the Southern hybridization pattern using the combination of pCS7 and HindIII and the PCR pattern using three primers to detect intraspecific variation (see Materials and Methods). The “M” indicates the λ/HindIII DNA marker, and the numbers 1–14 indicate specimens of the Odaigahara population of Abies homolepis in both (a) and (b). The arrows A and B indicate the two haplotypes.

pCS7 + HindIII (fig. 2) and pCS10 + HindIII. We expected results similar to those of these two combinations if we used the remaining nine probe-enzyme combinations, because pCS7 and pCS10 were adjacent on a physical map of cpDNA in C. japonica. The patterns from these two combinations were in complete agreement with each other.

Detection of Intraspecific Polymorphism of cpDNA in Abies by PCR

We screened for intraspecific polymorphisms in Abies cpDNA using 100 primers that, taken together, can amplify a whole chloroplast genome of species of Pinaceae. The 105 combinations of primer pairs were used to survey the variable region of cpDNA. We found two large cpDNA inversions among species of Pinaceae and many minor variations, such as deletions, insertions, and small inversions, within species (data not shown). One of the large inversions was located between ORF25-trnT and trnE-trnG in species of Pinaceae. Another inversion point may lie in the region between atpA and atpE previously reported by Strauss et al. (1988), but the exact inversion point was found to be between trnS-psaM-trnG and P'trnG-psaM-trnS. This inversion exists among genera of Pinaceae (Strauss et al. 1988), but it also occurs within species of Abies. The intraspecific cpDNA variation in Abies was caused by a large inversion of 42 kb associated with a short inverted repeat of trnS-psaM-trnG. We investigated all samples using three PCR primers, A, B, and C, to compare the results of Southern hybridization and to confirm the presence of the 42-kb inversion. Two cpDNA haplotypes were detected in each population, producing exactly the same results obtained from Southern hybridization with the combinations pCS7 + HindIII and pCS10 + HindIII (fig. 2).

Sequence of the Short Inverted Repeat Associated with the 42-kb Inversion in Abies and Tsuga

An Abies cpDNA gene map was also constructed by PCR analysis (fig. 3). The genome size was 120 kb, very similar to the sizes of other cpDNAs in Pinaceae (Strauss et al. 1988). The inversion points for the 42-kb inversion were trnS-psaM-trnG and P'trnG-psaM-trnS, and they have a short inverted repeat structure. The lengths of the inverted repeats were 1,180 bp for A. firma and A. homolepis, 1,186 bp for A. veitchii and A. sachalimensis, 1,235 bp for A. mariesii, and 1,164 bp for T. diversifolia. Sequence similarity of the inverted repeat regions among the five Abies species exceeded 99%, but between Abies and Tsuga it was 66%. These six species from two genera maintain the complete inverted repeat structure. Their accession numbers in the DNA Data Bank of Japan (DDBJ) are AB020785–AB020796.

Frequencies of cpDNA Haplotypes in Populations and Species

The frequencies of cpDNA haplotypes found in each population appear in table 1. We named the two
haplotypes A and B, respectively (fig. 2). At the species level, frequencies of the two haplotypes were mostly equal, but A. homolepis exhibited a somewhat lower frequency of haplotype A. We tested for deviation from a ratio of 1:1 in each population and species. There were no significant deviations from a 1:1 ratio at the population, species, and genus levels according to a chi-square test, except in two populations of A. mariesii and a species of A. homolepis. We also investigated frequencies of the two cpDNA haplotypes in one population each of T. diversifolia and T. sieboldii, for which the frequencies of haplotype A were 50% and 29%,
spectively. The frequency of haplotype A in *T. sieboldii* was not significantly lower than 50% according to a chi-square test. No geographic trends in these frequencies were found for any species except *A. mariesii*, which showed a geographical cline with latitude across its distribution (Tsumura et al. 1994).

We also estimated levels of cpDNA genetic diversity (table 2). In five species of *Abies*, *H_S* values ranged from 0.369 to 0.487, while *H_T* values were approximately 0.500. *G_ST* (θ) values ranged from 0.023 (−0.006) in *A. sachalinensis* to 0.225 (0.121) in *A. homolepis*, and, for the populations of the five *Abies* species overall, *G_ST* = 0.017 (0.024).

### Discussion

**Cause of Intraspecific cpDNA Variation in Abies**

Polymorphism for a 42-kb inversion was detected within and between species. The regions flanking the inversion were repeated, and the length of the inverted repeated region ranged from 1,164 to 1,235 bp in different species. The sequence similarity of the repeated regions among the five *Abies* species was 99.0%–100.0%, and that between *Abies* and *Tsuga* was 66.1%–66.7%. The repeats occur at the same genomic location in both genera and all seven species.

The chloroplast genomes of most land plants contain a large inverted repeat (IR), usually ranging from 20 kb to 30 kb in size (Palmer and Delwiche 1998). All chloroplast genomes with this large IR exist as two equimolar populations of molecules differing only in the relative orientations of their single-copy sequences (Bohnert and Loffelhardt 1982; Palmer 1983; Palmer, Jorgensen, and Thompson 1985; Palmer et al. 1987). Several interesting recombination properties are associated with the large IRs of plastid genomes: (1) "flip-flop" recombination maintains a 1:1 mixture of the two different genomic configurations, that is, inversion isomers differing only in the relative orientations of their single-copy regions; (2) copy-correctional recombination maintains absolute identity between the two repeats within a genome; and (3) a potential inhibition of recombination is responsible for evolutionary inversions (Palmer and Delwiche 1998). Another type of recombination is related to shifts in the sizes of the IR; these shifts are thought to occur by a gene conversion–like process involving short tracts of DNA (Goulding et al. 1996).

The cpDNAs of *Abies* and *Tsuga*, like those of other conifers, do not contain a large inverted repeat (Lidholm et al. 1988; Strauss et al. 1988; Tsumura et al. 1993; Wakasugi et al. 1994), and their Southern hybridization patterns clearly showed two different haplotypes. It is likely, therefore, that the intraspecific variation we found was caused by the same mechanism of flip-flop recombination as that leading to variation in cpDNA that does contain large inverted repeats. The mutation is considered the same phenomenon qualitatively but differs quantitatively. This intramolecular recombination appeared at lower frequencies than in genomes with a large inverted repeat. The largest repeat sequences consist of 10%–35% of a whole chloroplast genome in plants (Palmer and Delwiche 1998), but in *Abies* and *Tsuga*, they make up only about 1% of the cpDNA. The recombination rate is much lower than that of flip-flop recombination with a large IR due to the difference in physical structure.

In the chloroplast genomes of most flowering plants, the gene order can be derived from an ancestral genome by one to three inversions (Howe 1985; Hira.tsuka et al. 1989; Doyle et al. 1992). Conifers, six tribes of Fabaceae (Palmer et al. 1987, Lavin, Doyle, and Palmer 1990), *Erodium* and *Sarcocaulon* (Geraniaceae) (Downie and Palmer 1992), and *Conopholis* (Orobanchaceae) (Downie and Palmer 1992) have lost one copy of the large inverted repeat from the chloroplast genome independently. Thus, the rearrangement in these groups may have been accelerated by the absence of the large inverted repeat commonly found in other types of cpDNA, a structure thought to give an element of stability to the chloroplast genome (Palmer and Thompson 1982; Strauss et al. 1988). However, not all cpDNAs with only one IR region have undergone rearrangement. The rearranged cpDNAs typical of Pinaceae and several

### Table 2

<table>
<thead>
<tr>
<th>Species</th>
<th>Populations</th>
<th><em>H_S</em></th>
<th><em>H_T</em></th>
<th><em>G_ST</em></th>
<th>θ (SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. firma</em></td>
<td>7</td>
<td>0.422</td>
<td>0.500</td>
<td>0.158</td>
<td>0.014 (0.029)</td>
</tr>
<tr>
<td><em>A. homolepis</em></td>
<td>8</td>
<td>0.369</td>
<td>0.476</td>
<td>0.225</td>
<td>0.121* (0.064)</td>
</tr>
<tr>
<td><em>A. veitchii</em></td>
<td>13</td>
<td>0.476</td>
<td>0.498</td>
<td>0.044</td>
<td>−0.005 (0.013)</td>
</tr>
<tr>
<td><em>A. mariesii</em></td>
<td>13</td>
<td>0.428</td>
<td>0.500</td>
<td>0.144</td>
<td>0.117* (0.054)</td>
</tr>
<tr>
<td><em>A. sachalinensis</em></td>
<td>5</td>
<td>0.487</td>
<td>0.499</td>
<td>0.023</td>
<td>−0.006 (0.012)</td>
</tr>
<tr>
<td><em>A. umbellata</em></td>
<td>1</td>
<td>0.444</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Genus</td>
<td>47</td>
<td>0.486</td>
<td>0.495</td>
<td>0.017</td>
<td>0.058* (0.024)</td>
</tr>
<tr>
<td><em>T. diversifolia</em></td>
<td>1</td>
<td>0.408</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><em>T. sieboldii</em></td>
<td>1</td>
<td>0.500</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Note:—Haplotype diversity statistics (Nei 1973, 1987): *H_S* is the average diversity within populations; *H_T* is the diversity in the total population; and *G_ST* is the relative extent of differentiation among populations. θ is Wright's fixation index (Wright 1965; Weir 1990).

* Test of the significance of θ by permuting haplotypes or genotypes within the total population with 1,000 permutations. The null hypothesis is that θ is not greater than 0.

* P < 0.001.
legumes may be due to the presence of numerous dispersed repeated sequences that permit recombinating and rearrangement, rather than the loss of one copy of the IR (Milligan, Hampton, and Palmer 1989, reviewed in Solits and Solits 1998). Large numbers of inversions have also been reported in some species (Quigley and Weil 1985; Palmer et al. 1987; Knox, Downie, and Palmer 1993; Hoot and Palmer 1994; Cosner et al. 1997). The endpoints of most inversions lie adjacent to at least one tRNA gene, suggesting that tRNA genes might be involved in rearrangement of cpDNA (Howe 1985; Sugihara 1989). The 42-kb inversion found in Abies and Tsuga is also associated with a short inverted repeat that includes the tRNA genes trnS and trnG. This inversion may occur at the same location as that found as a distinguishing feature between lobolly pine and Douglas fir chloroplast genomes (Strauss et al. 1988), and if so, the predisposition to undergo the inversion is considered ancient because the polymorphism exists in different genera of Pinaceae (Strauss et al. 1988). This kind of inversion is thought to occur as an evolutionary event during speciation (Jansen and Palmer 1987; Palmer et al. 1987; Doyle et al. 1992), but the inversion that caused the intraspecific variation in Abies and Tsuga is unusual in that the variation has been maintained within populations and species.

We did not detect any patterns in the Southern hybridization analysis that suggested heteroplasmy. In the PCR analysis, however, we always observed a pattern consistent with heteroplasmy when PCR cycles were increased to 40 from 30, but the intensity of one of the two fragments was always very low compared with the other. We identified the major cpDNA genome type in each individual, and the major cpDNA types for all samples identified by PCR coincided with the results of Southern hybridization analysis. These results may be explained by the presence of only one type of cpDNA in some individuals or the preferential transmission of the major haplotype through pollen to the next generation after drift in meiotic cells. Heteroplasmy of cpDNA in Abies and Tsuga probably exists, based on the results from PCR. Thus, we need to clarify the quantity of the two haplotypes in a single tree and a single pollen grain using a quantitative PCR method.

High Rate of Inversion

All of the studied populations have two haplotypes that differed by a 42-kb inversion, and the haplotype frequencies in each population and species were generally similar. The average cpDNA diversity (H) across the five Abies species was 0.436, and $G_{ST}$ among species was 0.017. These five species have generally similar haplotype frequencies and are not differentiated from each other. From these results, we hypothesized that the rate of inversion was high and/or the rate of gene flow was high, especially given that the mode of inheritance of cpDNA in conifers is paternal (Ziegenhagen et al. 1995) and the breeding system is wind-pollinated and outcrossing.

Birky, Maruyama, and Fuerst (1983) provided an equation for the equilibrium value of $K_e$ given that $K_e$ is small and that $K_e >\mu$: $K_e = 2N_{eo}\mu/(2N_{eo}\mu + 1)$, where $K_e$ is the probability that the two sampled genes from different individuals within a population will be different alleles, and $K_e$ is the probability that the two sampled genes from a single adult cell will be different alleles. This equation can be rearranged to express $\mu$ in terms of $K_e$ and $N_{eo}$: $\mu \sim 1/(1/K_e - 1)(2N_{eo})$ (Rand and Harrison 1992). Rand and Harrison (1992) estimated $\mu = (1-2) \times 10^{-4}$ from their cricket data. However, this equation does not include the migration rate. An equation giving the equilibrium expectation of $G_{ST}$ in an organellar genome, which includes migration and mutation rates, has also been derived: $G_{ST} \approx 1/[1 + 2N_{eo}L/(L - 1)](m_e + \mu)$, where $N_{eo}$ is the total population size, $L$ is the number of subpopulations, $m_e$ is the migration rate, and $\mu$ is the mutation rate (Birky, Fuerst, and Maruyama 1989). Birky, Fuerst, and Maruyama (1989) also documented that the effective number of organelle genes is approximately equal to the number of females and the effective migration rates of organelle genes are lower than the actual migration rates when the organelles show maternal inheritance. Assuming a large number of subpopulations, $L/(L - 1)$ is nearly 1; thus, $G_{ST} \approx 1/[1 + 2N_{eo}(m_e + \mu)]$. When the migration rates of pollen and seed are $m_p$ and $m_s$, respectively, we can assign the migration rates for chloroplast ($m_p$) and nuclear ($m_s$) genomes in conifers as $m_e = m_p$ and $m_n = (m_s + m_p)/2$, respectively. In the case where $m_p \gg m_s$, $m_s = m_p/2$. If the species is monoecious, such as Abies and Tsuga, we can set $N_{eo} = N_{ec} = N_{eo}$. Govindaraju and Wagner (1988) reported that cpDNA varied within individual trees from the P. banksiana—P. contorta sympatric region according to Southern hybridization analysis, but that study gave no direct evidence of somatic mutation. In our study, we have some evidence of heteroplasmy by PCR analysis but the quantity of it could not determined from our results. If heteroplasmy existed in the cpDNA of Abies, the value of $K_e$ would be expected to be very small based on the results of Southern hybridization analysis, and in this case, we can use the equation $G_{ST} = 1/(1 + 2N_{eo}(m_e + \mu))$ (Birky, Fuerst, and Maruyama 1989). Therefore, if we use allozyme data to estimate $N_{eo}$ and $m_e$, we can estimate the mutation rate of the cpDNA variation. Using the above assumptions, we estimated the inversion rate and compared it with mutation rates for nuclear genes. Yahara et al. (1991) estimated $N_e$ from the heterozygosity ($H$) and mutation rate ($\mu$) as $N_e = H/4(1 - H)\mu$, where $\mu$ was an estimate of the allozyme mutation rate. When we substituted different mutation rates ($1.3 \times 10^{-7}$ [Kimura 1983], $1.81 \times 10^{-6}$ [Mukai and Cockerham 1977], and $10^{-5}$ [Schoven and Brown 1991]) in the calculations, the derived $N_e$ value was also lower, depending on the mutation rate. We estimated $N_e$ to be roughly on the order of $10^{-3}$–$10^3$ depending on these mutation rates from allozyme data of A. mariesii and A. sachalinensis, respectively (Nagasaki, Wang, and Tanaka 1997; Suyama, Tsumura, and Ohba 1997). $G_{ST}$ of cpDNA in Abies was found to be 0.017. In this case, we estimated $(m_e + \mu)$
sequences, we found the plast genome, with some repeats (4). These repeat sequences are dispersed in the chloroplast genome; fig. 4). Hong, Hipkins, and Strauss (1993) observed only two individuals of Pinus muricata with intraspecific variation that could be caused by an inversion. This is one piece of evidence showing that this kind of mutation may occur in Pinus cpDNA, but the rate is likely to be very low compared with that in Abies. There is no repeat sequence around the inversion points (trnG-trnS and trnS-ΨtrnG) in Abies; therefore, we infer that inversion events might easily occur in Abies and Tsuga. If other species of conifers have cpDNA structures similar to the inversion observed in Abies, their chloroplast genomes might well show tendencies similar to those observed in Abies and Tsuga and may illuminate key characteristics of the origin and maintenance of cpDNA variation in conifers.

Genetic Drift and Mutation Rate

Only A. mariesii shows a geographical trend in haplotype frequencies. Sequence data of a cpDNA spacer region and rbcL in five Abies species showed that A. mariesii was distinct from the other four species (Suyama et al. 1996; Tsumura and Suyama 1998). However, at the species level, A. mariesii has the same level of cpDNA variation as the other four Abies species. After the last glacial period, this species was thought to have experienced a rapid northward expansion, especially in the Tohoku district (Sugita 1990). Strong genetic drift in the species at that time could explain this geographical cline (Suyama, Tsumura, and Ohba 1997). Because genetic drift during the period of rapid expansion could have exceeded the mutation rate, these populations may still show evidence of the putative strong genetic drift.

Three populations along major mountains in the Tohoku district, Hakkoda, Hachimantai, and Kurikoma, have a
high frequency of haplotype B, but southern populations have high frequencies of haplotype A. The results suggest that individuals with haplotype B were largely responsible for the northward expansion of A. mariesii after the last glacial period and that haplotype A has since arisen in these populations due to the high mutation rate and/or migration.

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LITERATURE CITED


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