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Allelic sequence polymorphisms in the intron region of the nuclear-encoded *GapC* gene preceded the speciation of three closely related *Abies* species (Pinaceae)

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Abstract The DNA sequences of *GapC* intron 8 were determined for three closely related *Abies* species, *Abies firma*, *Abies homolepis* and *Abies veitchii*, and ten alleles were identified. Although, in most cases, an allele was specifically detected in one species, some rare alleles were found in two species. The phylogenetic analysis of those alleles showed that they trace back to different ancestral sequences, and that a species possessed the different originated alleles. The polymorphism of the *GapC* gene observed in the three *Abies* species seemed to have preceded their evolutionary divergence.

Keywords *Abies* · ncDNA · *GapC* · Intraspecific polymorphism · Interspecific polymorphism

Introduction

Abies Mill. is a coniferous genus of the family Pinaceae. About 40 species are widely distributed in the Northern Hemisphere from temperate to subarctic zones. Recently, extensive molecular genetic studies of *Abies* species have been carried out, mainly with regard to the European species. In population genetic studies, allozymes have usually been used as a tool (Suyama et al. 1992, 1997; Konnert and Bergmann 1995; Vicario et al. 1995; Parducci et al. 1996; Siegismund et al. 1996; Davidson and El-Kassaby 1997; Nagasaka et al. 1997). With regard to organellar genomes, several studies at the DNA level have used RFLP (Tsumura et al. 1994), PCR-RFLP (Vicario et al. 1995; Ziegenhagen et al. 1995; Parducci and Szmidt 1999), the microsatellite of cpDNA (Vendramin and Ziegenhagen 1997; Ziegenhagen et al. 1998; Vendramin et al. 1999), and RFLP of mitochondrial DNA (Tsumura and Suyama 1998). With regard to the nuclear genome, a few studies were attempted using the RAPD technique (Vicario et al. 1995; Leibenguth and Shoghi 1998).

In some gymnosperms, RAPD markers (Aagaard et al. 1998; Tani et al. 1998, Thomas et al. 1999), STS markers (Tsumura and Tomaru 1999), rDNA ITS regions (Karvonen and Savolainen 1993; Quijada et al. 1998), and microsatellite markers (Thomas et al. 1999) of the nuclear genome have been used for population genetic studies.

We considered that the intron regions of nuclear genes would be particularly effective to develop suitable markers. In this report, DNA sequences of the intron region of the *GapC* gene (Cerff and Chambers 1979), which is the nuclear-encoded gene of cytosolic GAPDH (glyceraldehyde-3-phosphate dehydrogenase), were determined in three Japanese *Abies* species (*Abies firma* Sieb. et Zucc., *Abies homolepis* Sieb. et Zucc. and *Abies veitchii* Lindl.) and were used to investigate their intra- and inter-specific polymorphisms. Additionally, we explored the evolutionary relationships among alleles and discussed them in association with the speciation.

Materials and methods

Plant materials and DNA extraction

Needles from 13 individuals of *A. firma*, ten of *A. homolepis*, and ten of *A. veitchii* were collected for DNA analysis. Total genomic DNA was isolated from approximately 100 mg of needles using a modified protocol (Shiraishi and Watanabe 1995) of the CTAB method (Murray and Thompson 1980). Extracted total DNA was purified using Gene Clean III (BIO 101).

Primer design

Primers were designed based on the cDNA sequence of *Pinus sylvestris* (GenBank Acc. No. L07501). The sequence of *P. sylvestris GapC*1 was compared with the sequence of the *GapC*2 (GenBank Acc. No. X73151) and the *GapC*4 (GenBank Acc. No. X73152)

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Table 1 Nucleotide sequence of primers used for DNA sequence analysis of *GapC* gene intron 8

[a] Sequence indicated by lower case letters is the sequence of the -21M13/M13Rev primers

Primer	Nucleotide sequence (5′ to 3′)[a]
gC8U	tgtaaaacgacggccagtGGTTGATGGACCGTCAAACAAG
gC8L	caggaaacagctatgaccCAACTTTCCATTGAGGGCTGG
FO-515	tgtaaaacgacggccagtTACCTGTGCAAAGATCAGTAAGGC
FO-516	caggaaacagctatgaccGGGAGAATGTGCTAAAACTGCC

genes of *Zea mays*, for characterising the intron positions. A pair of primers (gC8U and gC8L in Table 1) was designed at the position corresponding to exons 8 and 9 of *Z. mays*. PCR with these primers could amplify a partial genomic DNA sequence of the *GapC* gene; that is, the region corresponding to intron 8 of *Z. mays*. The sequences of Perkin-Elmer-21M13 primer (TGTAAAACGACGGCCAGT) and M13Rev primer (CAGGAAACAGCTATGACC) were tagged on the 5′-end of the primers.

In *Abies* species, the *GapC* intron 8 was about 1 kbp in length. Therefore, the entire sequence could not be determined in one sequencing process. A new pair of primers (FO-515 and FO-516, Table 1) was designed from the sequence information determined by the first process, followed by determination of the rest of the sequence.

PCR amplification

PCR was performed using the GeneAmp PCR System 9600 (Perkin-Elmer) with the following reaction. Each 20-µl reaction contained 1× *ExTaq* buffer, 3 mM of $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, 0.5 units of *ExTaq* DNA polymerase (Takara), and 200 ng of template DNA. A "Touchdown" temperature profile (Don et al. 1991) was adopted to inhibit a non-specific amplification. DNA amplification was carried out by pre-denaturing at 94°C for 1 min, followed by 30 cycles of denaturing at 94°C for 30 s, annealing for 30 s, and extending at 72°C for 90 s. In the initial ten cycles, the annealing temperature was progressively lowered from 60°C to 52°C by 0.8°C every cycle. After reaching the final annealing temperature of 52°C, reactions were subjected to an additional 20 cycles of amplification. At completion of the last cycle, a final extension at 72°C was carried out for 1 min.

Sequence analysis

PCR products were separated by electrophoresis in 1.5% agarose gel and the target fractions were excised from the gel. DNAs were recovered from the gel particles and were purified using QIAEX II Gel Extraction (QUIAGEN). The sequence reaction was carried out by a Thermo Sequenase Pre-mixed Cycle Sequencing Kit (Amersham Life Science) using the purified DNA as a template. Primers used for the sequencing reaction were Perkin-Elmer-21M13 and M13Rev primers. The sequences were determined using a HITACHI DNA sequencer 5500.

Data analysis

The sequences were aligned manually. Since much insertion/deletion (indel) information was included in the sequences of the alleles, two types of data sets, which excluded or included indel information, were prepared. For excluding indel information, gap positions were scored as missing data. For including indels, each indel was scored and entered as a separate presence/absence character, while still treating gap positions as missing data. Parsimony analyses were performed by PHYLIP var. 3.572 (Felsenstein 1996). To evaluate the robustness of the internal branches of the parsimony trees, 100 bootstrap replications were executed.

Results

DNA sequence of the *GapC* intron in three *Abies* species

PCR amplification of the entire region of *GapC* intron 8, including a 3′ partial exon-8 and a 5′ partial exon-9 region, was carried out for each individual of *A. firma*, *A. homolepis*, and *A. veitchii*, and their sequences were determined (Fig. 1). Comparison of the sequences with the *P. sylvestris* cDNA sequence revealed extremely high homology (94%) in the exon region, and the PCR product amplified with primers gC8U and gC8L was confirmed to be a partial *GapC* gene (Fig. 1A). The intron was inserted at the same position as the *Z. mays GapC*2 and *GapC*4 genes.

The length of the intron was about 830 bp for *A. firma* and about 970 bp for the remaining two species. Intron 8 of *A. firma* possessed two large deletion sites. One was a 28-bp deletion from the 70th to the 97th nucleotide position and the other was a 146-bp deletion from the 619th to the 764th nucleotide position of *A. homolepis* and *A. veitchii* (Fig. 1B). The sequence of the 146-bp indel site was highly homologous with the adjacent sequence. It was necessarily inferred that the large length difference of intron 8 between *A. firma* and the other two species was caused by the duplication of the 146-bp sequence (indicated with '=>' in Fig. 1B).

DNA sequence polymorphism within and among species

In order to investigate intra- and inter-specific sequence variations, PCR amplification using primers FO-515 and FO-516 was carried out for many individuals of each species. The intron region of the 307th to the 778th nucleotides could be amplified by this PCR (Fig. 1). DNA sequences could be determined for 23 PCR products amplified in 13 *A. firma*, 11 in 10 *A. homolepis*, and 10 in 10 *A. veitchii*.

The DNA sequencing for these 44 PCR products found ten different putative alleles (Fig. 2). Of these ten alleles, four (g8a, g8b, g8f, and g8g) were detected in *A. firma*, two (g8d and g8e) in *A. homolepis*, one (g8c) in both *A. firma* and *A. homolepis*, and three (g8h, g8i, and g8j) in *A. veitchii* (Table 2). Comparison of each allele revealed polymorphisms with an approximately 140-bp indel at one site, a point mutation at 19 sites, and short indels (1 bp to 6 bp) at seven sites (Fig. 2).

Because g8a and g8b possessed no duplication site, they were about 140-bp shorter than the other alleles. These two alleles were found only in *A. firma*, and most

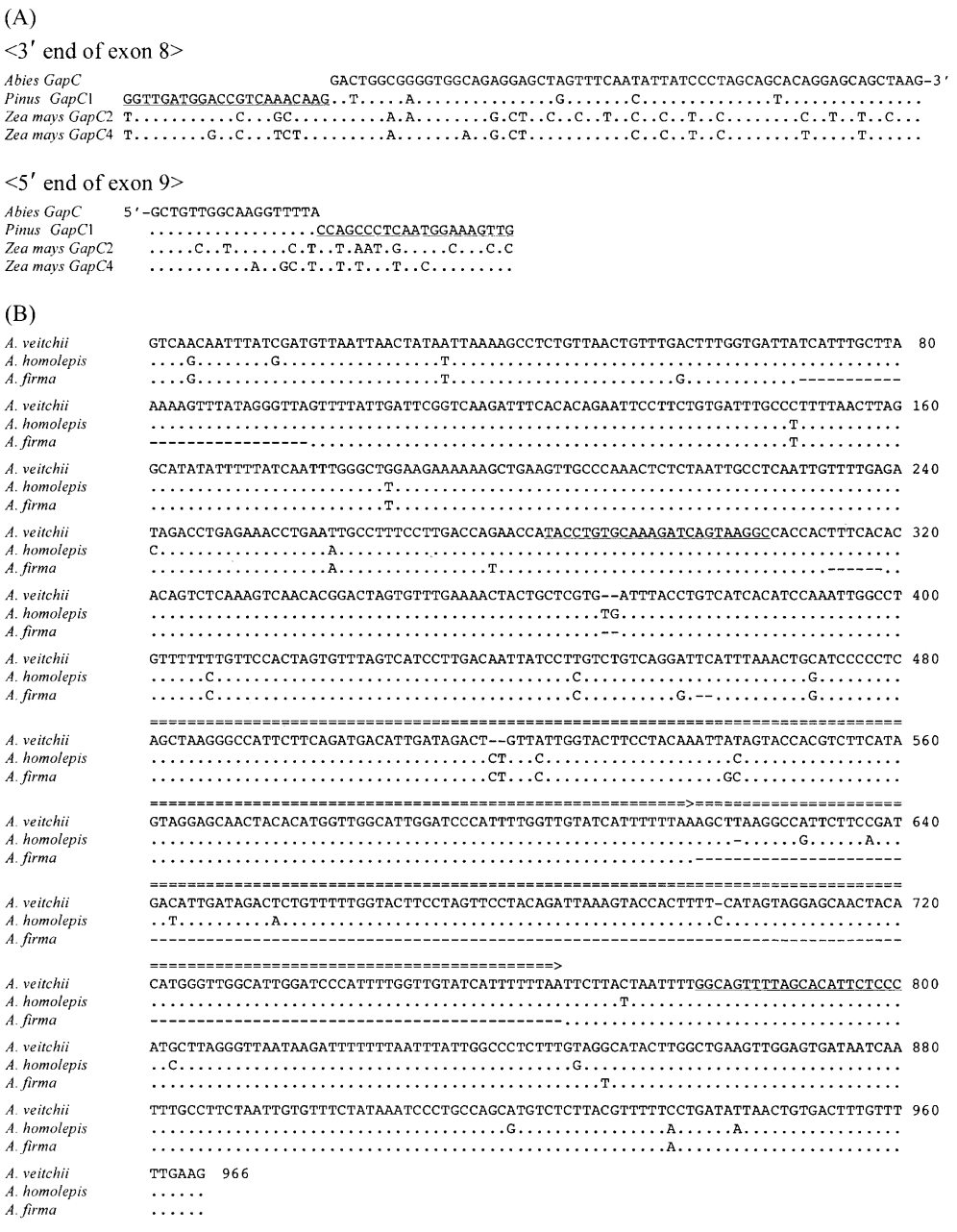

Fig. 1 (**A**) alignment of the 3′-end sequence of exon8 and the 5′-end sequence of exon9 of *Pinus sylvestris GapC*1 cDNA, *Zea mays GapC*2 genomic DNA, *Zea mays GapC*4 genomic DNA, and *Abies firma GapC* genomic DNA determined in this study, and (**B**) alignment of the *GapC* intron region among three *Abies* species. '-' indicates gaps, '.' indicates identical base as in the uppermost sequence. An *asterisk* indicates an intron insertion site. An *underline* indicates a primer annealing site. '=>' indicates a tandem repeat motif

of the individuals of *A. firma* possessed one of the alleles. Although the details are not shown in this report, PCR analysis for 68 individuals of *A. firma* showed that 59 produced only a PCR fragment with the same length as g8a and g8b (approximately 380 bp). PCR products with different lengths were obtained only in nine individuals whose sequences were determined. Of these nine individuals, eight possessed the approximately 830-bp fragment (the same length as g8a and g8b) and the approximately 970-bp fragment (the same as g8f and g8g). The exceptional individual possessed g8f. On the other hand, g8a and g8b were detected in neither *A. homolepis* nor *A. veitchii*. PCR analysis of 192 individuals of *A. homolepis* and 188 of *A. veitchii* revealed that none of them produced the approximately 380-bp PCR products.

In allele g8c, although the approximately 140-bp duplication could not be found in the same manner as in g8a and g8b, only 31 bp of the 3′ end of the 140-bp motif was duplicated. This may imply that this region is susceptible to duplication. Apart from the 31-bp duplication, there was no variation between g8c and g8a. This allele was found only in one individual of *A. firma* and *A. homolepis*, respectively. In the PCR analysis of the 448 individuals described above, no PCR product with the same length (approximately 410 bp) as g8c was detected, except in the two sequenced individuals.

Alleles g8d, g8e, g8f, g8g, g8h, g8i and g8j possessed the approximately 140-bp duplication, and were distinguished by 1–6-bp short indels at five sites and by point mutations at 18 sites. The sequence lengths of these al-

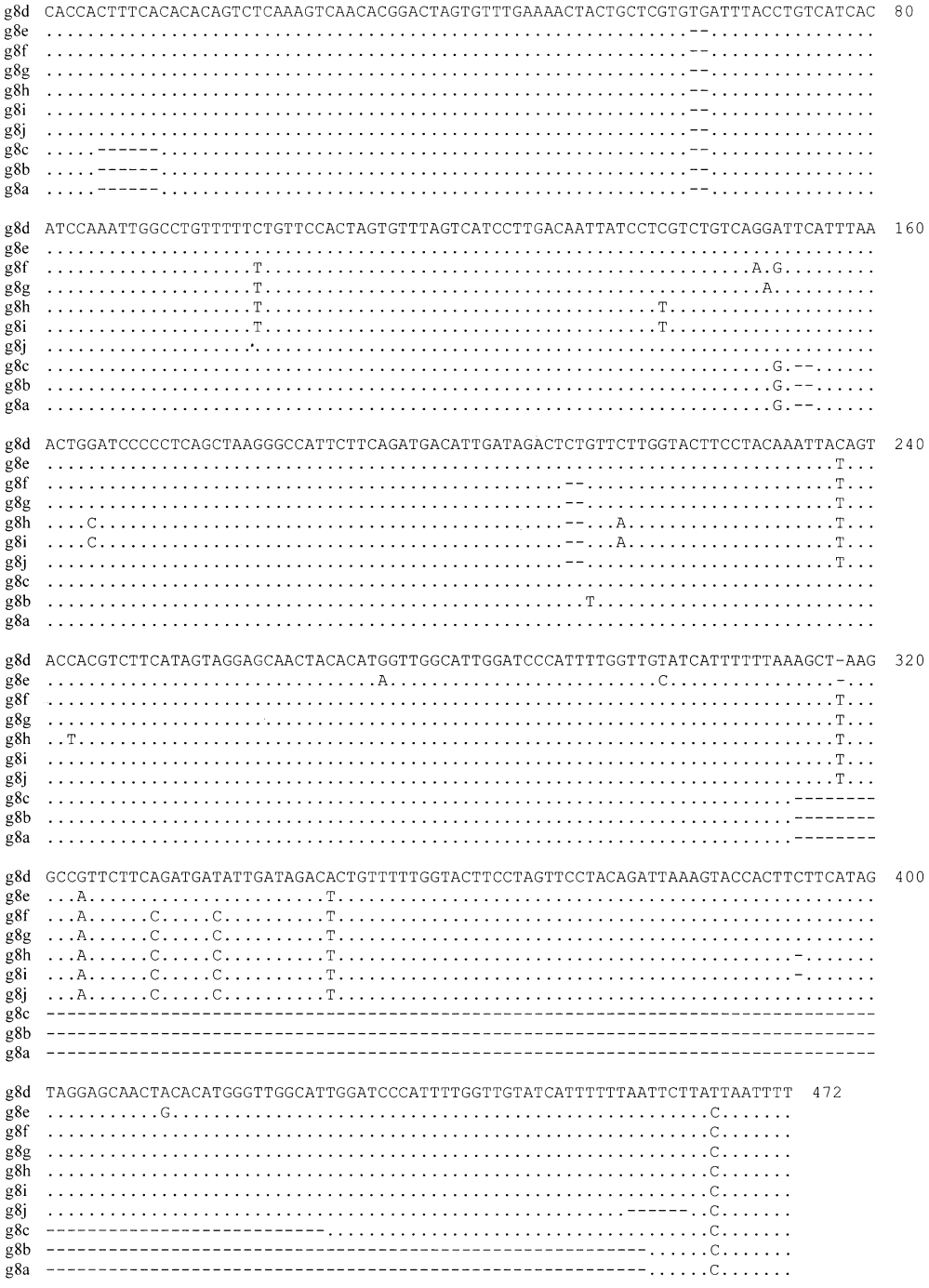

Fig. 2 Alignment of a partial DNA sequence of the *GapC* intron region for ten alleles in three *Abies* species. *Dashes* indicate gaps, '.' indicates a base identical to the uppermost sequence

leles were 471, 469, 468, 468, 467, 467 and 462 bp, respectively. Of the seven alleles, g8d and g8e were detected in *A. homolepis*, g8f and g8g in *A. firma*, and g8h, g8i and g8j in *A. veitchii*.

In some individuals of *A. homolepis* and *A. veitchii*, the direct sequencing of PCR products failed because these individuals were heterozygous of for two different alleles, which differed in length because of the short indels. However, the sequences of both sides of the gaps could be determined even in these individuals, and the allele sequences of such individuals could be estimated based on the incomplete sequences. On the basis of these estimated sequences, it was presumed that some individuals of *A. homolepis* possessed allele g8g, and some individuals of *A. veitchii* possessed allele g8e (Table 2).

Phylogenetic relationship of alleles

Evolutionary relationships among the ten alleles detected in the three *Abies* species were estimated by constructing their parsimony trees. As there were many in-

Table 2 The number of alleles detected in each species. Homozygotes were counted as two. Numbers whose sequences we could not determine completely are in parentheses

Species	Putative allele									
	g8a	g8b	g8c	g8d	g8e	g8f	g8g	g8h	g8i	g8j
A. firma	13	1	1	–	–	6	3	–	–	–
A. homolepis	–	–	1	16 (10)	3 (8)	–	– (7)	–	–	–
A. veitchii	–	–	–	–	– (1)	–	–	11	7 (1)	2

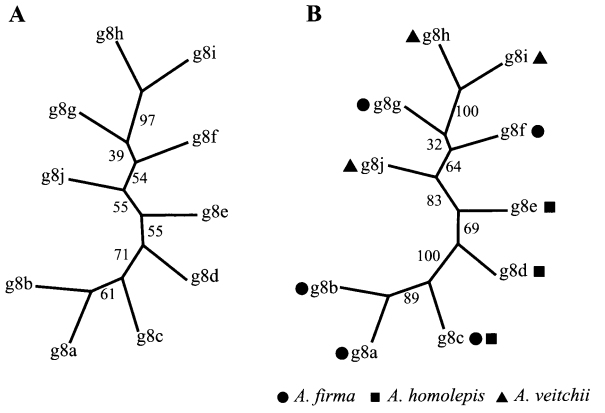

Fig. 3 (**A**) phylogenetic tree of ten *CapC* alleles inferred from the DNA sequence of the intron region using the parsimony method without indel information, and (**B**) with indels. *Numbers* along the nodes denote the bootstrap value of 100 replicates

dels in the sequences of these alleles, parsimony analyses were performed on the two data sets that excluded and included the indel information. The majority rule consensus tree of 100 bootstrap replications is shown in Fig. 3. The bootstrap of a consensus tree inferred from a data set including the indel information (Fig. 3A) showed lower values than the other tree (Fig. 3B). Two of the most-parsimonious trees showed an identical topology. This suggested that most of the indels observed in *GapC* intron 8 were acquired within the evolutionary lineage.

Three alleles, g8a, g8b and g8c, showed quite-high sequence homologies, and formed a single clade in the consensus trees. An allele most-closely related to these alleles was g8d, followed by g8e. The alleles g8f, g8g, g8h and g8i were closely related to each other, and were suggested to have been derived from a common ancestral sequence. *A. firma* possessed the alleles g8a, g8b, g8c, g8f and g8g, and they were inferred to be from multiple ancestral sequences. Correspondingly, the alleles g8h, g8i and g8j detected in *A. veitchii* may have had different origins. The alleles g8c, g8d and g8e found in *A. homolepis* were likely to have been derived from the same ancestral sequence. However, this species has a greater possibility to possess the g8g allele which originated from another ancestor, as described before.

Discussion

Evolutionary interpretation of the *GapC* sequence polymorphisms

The number of alleles found in each species was five in *A. firma*, three in *A. homolepis*, and three in *A. veitchii*. Except for g8c, found in both *A. firma* and *A. homolepis*, the remaining nine alleles were detected respectively in only one species. Considering also the incomplete sequences mentioned above, however, some of the nine alleles were assumed not to have become species-specific because they were found in more than one species (Table 2).

In order to clarify whether this phenomenon was caused by interspecific gene flow or by these alleles originally being shared between species, the phylogeny of the alleles was inferred from the parsimony trees (Fig. 3). Three alleles of *A. firma* (g8a, g8b and g8c) were in the same clade of the tree, and were demonstrated to have been derived from the same ancestral sequence. However, the remaining two alleles of *A. firma* were located in a different clade. This could indicate that the alleles of this species were derived from at least two ancestral sequences. Correspondingly, in *A. veitchii*, the alleles were suggested to have multiple origins. In *A. homolepis*, the alleles' multiple origins were not indicated from the complete sequence data determined from the sequencing of both strands. The incomplete sequence data, however, suggested that this species also had allele g8g, which could have been derived from a different ancestral sequence from the other alleles of this species. According to these results, it is appropriate to conclude that alleles found in each species had not diverged from a species-specific ancestral sequence after their speciation, but had already existed in the ancestral species and then evolved or remained in each species to the present day. The origin of the sequence polymorphisms found in the *GapC* gene intron 8 can be considered to predate the speciation of the three *Abies* species.

Applying the molecular marker to population analysis

With regard to the *Abies* species, many studies using isozymes have revealed genetic features such as the population genetic structure of *Abies alba* (Konnert and Bergmann 1995; Parducci et al. 1996), *Abies mariesii* (Suyama et al. 1992, 1997) and *Abies sachalinensis* (Nagasaka et al. 1997), the mating system of *Abies procera* (Siegismund et al. 1996), the genetic relationships

between *A. alba* and an endemic species, *Abies nebrodensis* (Vicario et al. 1995), and so on. Though isozymes constitute an adequate tool for population genetic studies, they nonetheless may have weaknesses with regard to the limit of the number of markers and the small amount of information they provide. Recently, progress in molecular genetics has made it possible to analyze a large number of samples and to obtain abundant genetic information at the DNA level. However, it may be difficult to apply the microsatellite marker developed for a particular species even in the study of closely related species, as this marker is highly species-specific (Echt et al. 1999).

In this study, we investigated the sequence polymorphism of intron 8 of the *GapC* gene, and discussed its utility for population genetic analysis on a natural interspecific hybrid. Of the three *Abies* species used in this study, *A. firma* and *A. homolepis* have been recognized to hybridize naturally (Liu 1971; Takasugi 1971). Takasugi (1971) also pointed out the occurrence of a large-scale introgression between these two species, and suggested that most of the variations reported previously were the result of either hybridization or introgression. Furthermore, he advocated that most of the *A. firma* naturally distributed at present had been genetically influenced by *A. homolepis*. If large-scale introgression has been occurring between them, a major allele of one species can be expected to also appear frequently in the other species. In our study, however, the major alleles in *A. firma* were not detected in *A. homolepis*, and those in *A. homolepis* were not found in *A. firma*, and only two rare alleles (g8c and g8g) existed in both species. No evidence for such a large-scale introgression between the two species as suggested by Takasugi (1971) could be obtained from the genetic information of the *GapC* locus. Investigation of a wider range of natural forests using DNA markers is necessary to elucidate the real condition of introgression between the two species.

To apply intron sequence polymorphisms such as the *GapC* intron to population genetics effectively, we need to analyze more DNA regions. Apart from the *GapC* gene, the DNA sequence of the nuclear-encoded *Adh* gene intron region (Denda et al. 1995), the *CAD* gene intron region (Schubert et al. 1998), and the *cdc*2 gene intron region and its pseudogene (Kvarnheden et al. 1995), have been reported. These may be utilized to develop nuclear DNA markers as a powerful tool for population genetics. Many reports have also described cDNA sequences. It is possible to develop a novel nuclear DNA marker by designing primers based on cDNA sequence information and by determining the genomic DNA sequence (Harry et al. 1998).

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