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Variation in *trn*-L/*trn*-V and *trn*-F/*trn*-T spacer regions of cpDNA in *Abies koreana* Wilson and *A. nephrolepis* Traut./Maxim.

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Abstract : The first evidence has been provided about the variation within *trn*L-*trn*V and *trn*F-*trn*T spacer regions of cpDNAs in Korean fir and Manchurian fir, revealed by PCR-RFLP analysis. Four cpDNA haplotypes have accordingly been recognized by being analyzed using the *trn*L-*trn*V/*Tru*1I primer-enzyme combination and 3 haplotypes using the *trn*F-*trn*T/*Tag*I combination, which exhibited inter and intraspecific variation. A total of 6 cpDNA haplotypes were recognized by pooling the PCR-RFLP variants observed in both combinations. Haplotypes 2 and 3 were common for both species investigated, whereas haplotypes 1, 4, and 5 were detected only in Korean fir and haplotype 6 was detected only in Manchurian fir. Although haplotypes 2 and 3 were common in both species, haplotype 2 was major haplotype for Korean fir and haplotype 3 was one of the 2 major haplotypes for Manchurian fir. Restricted occurrence of haplotype 4 in Mt. Halla and haplotype 5 in Mt. Jiri of the Korean fir may represent the existence of geographic isolation by the sea between them. Diagnostic potential of individual haplotypes in discriminating between the two species as well as between their populations is discussed.

Key words **:** trn*L*-trn*V spacer,* trn*F*-trn*T spacer, cpDNAs, Korean fir, Manchurian fir, PCR-RFLP analysis, haplotypes*

Introduction

Korean fir (*Abies koreana* Wilson) together with Manchurian fir (*A. nephrolepis*/Traut./Maxim.) and needle fir (*A. holophylla* Maxim.) are the only representatives of the genus *Abies* endemic to the Korean peninsula (Lee, 1996a; Lee, 1996b). The two former species are regarded to be systematically closely related, which belong to the section Elate according to the taxonomic account by Liu (1971) and/or to the section *Balsameae* according to the classification proposed by Farjon and Rushforth (1989). The *habitts* of Korean fir are confined to Korea peninsula, while those of Manchurian fir extend to the northern China and Siberia. The Mt. Halla on the Jeju island is the southernmost part of South Korea along with the Mt. Jiri in the southern part of the peninsula and the northernmost population of Mt. Deogyu. Those regions are the most prominent habitats of Korean fir known since the description of the species by Wilson in 1920 (Wilson, 1920). Conversely, Manchurian fir is more common in the northern part of the country with the populations at Mts. Seorak and Taebaek being best recognized. It is supposed that there exists also a transient zone between Mt. Deogyu and Mt. Gaya where both species occur commonly (Chang *et al.*, 1997).

The best reflection of both a striking similarity and differentiation between Korean fir and Manchurian fir is the Wilson's quotation in his respective paper: "…Dr. T. Nakai confused this species with *A. nephrolepis*, but when traveling together on Halla I pointed out differences and he readily concurred that the two were quite distinct species" (Wilson, 1920). However, in spite of this statement, no reliable diagnostic markers are still available for classification of these species. Probably the most distinct difference reported concerns the folia flavonoids by which Korean fir populations differ conspicuously from those of Manchurian fir (Chang *et al.*, 1997). Also, at the anatomical level a clinal variation was observed in positions of resin ducts. The clinal pat-

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tern has accordingly been distinct in northern populations of Korean fir containing prevailingly median resin ducts and in southern populations of Manchurian fir with marginally positioned resin ducts. Contrary to this anatomical trait, none of the morphological characters of cones and leaves proved to be of diagnostic value in this respect (Chang *et al.*, 1997). Recently, Kim (2003) reported that morphological characters, such as the shape of micro- and macrostrobili, crown shape, and surface structure of young shoots, may be useful for discriminating each species. The attempt has also been made to differentiate the respective species at the molecular level. Based on RAPD markers, the first data on genetic variation and phylogenetic relationships between Korean fir, Manchurian fir, and needle fir have been reported indicating close relationship but independent taxonomic status for Korean fir and Manchurian fir species (Kim *et al.*, 1996; Kim, 1998).

In order to contribute to the ongoing discussion about genetic relationship between these species, a comparative study on their chloroplast DNA (cpDNA) has been undertaken using a PCR-RFLP analysis.

Material and Methods

1. Needle samples

Needle samples of the Korean fir were collected from Mt. Deogyu (27 individuals), Mt. Jiri (12 individuals), and Mt. Halla in Jeju island (46 individuals). Needle samples of the Manchurian fir were collected from 41 individual trees in Mt. Taebaek (Figure 1). Sampled needles were kept in a refrigerator until DNA extraction.

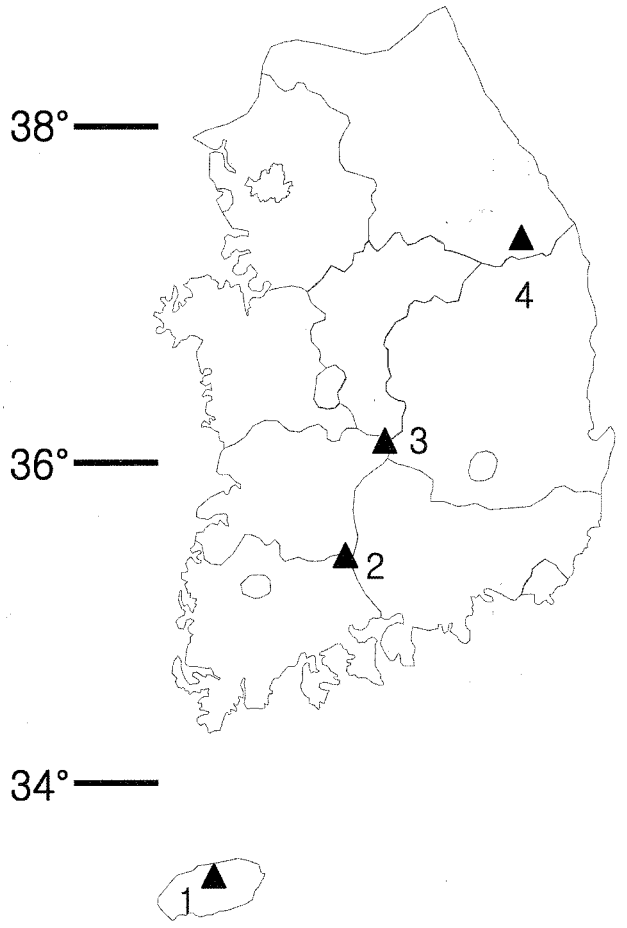

Figure 1. Localities of investigated species and their location on a map of South Korea. Population names refer to Table 1.

2. DNA isolation, PCR amplification, and restriction enzyme digestion

Genomic DNA was isolated from needles of individual trees using NucleoSpin Plant kit (Mackerey-Nagel GmbH & Co. KG, Germany). The primer pairs of trnL-trnV and *trn*F-*trn*T were used for PCR. The former includes the intergenic spacer between the *trn*L (UAG) and *trn*V (GAC) genes, whereas the latter between the *trn*F (GAA) and *trn*T (UGU) genes (Tsumura *et al.*, 1994). The primer sequences of *trn*L-*trn*V are 5'-CTGCTTCCTAAGAGCAGCGT-3' and 5'-TTGACATGGTGGAAGTCATCA3'. They were designed on the basis of the complete cpDNA sequence of *Pinus thunbergii* (Wakasugi *et al.*, 1994). The primer sequences of *trn*F-*trn*T are 5'-ATTTGAACTGGTG ACACGAG-3' and 5'-CATTACAAATGCGATGCTCT-3' (Taberlet *et al.*, 1991).

The reaction mixture for PCR contained 5~10 ng of template DNA, 75 mM Tris-HCl pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.1 M of each primers and 0.6 unit of *Taq* polymerase (ABgene, UK), in a total volume of 10 μl. The PCR was run in PTC-200 Peltier Thermal

Table 1. Chloroplast DNA haplotypes observed in each combination of primer/restriction enzyme.

Population	Haplotypes							Total
	*trn*L-*trn*V/*Tru*11				*trn*F-*trn*T/*Taq* I			
	I-1	I-2	I-3	I-4	II-1	II-2	II-3	
1. Mt. Halla (Korean fir)	41	2	2		44		1	45
2. Mt. Jiri (Korean fir)	11			1	12			12
3. Mt. Deogyu (Korean fir)	24	3			27			27
4. Mt. Taebaek (Manchurian fir)	18	23			38	3		41

Cycler (MJ Research, USA). DNA amplification was performed at 94°C for 30 sec, followed by 34 cycles at 94°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min. The final strand elongation at 72°C was allowed for additional 2 min. To confirm successful amplification and to determine the size of the amplified fragment, 2 μl of the PCR products was analyzed by electrophoresis using 1% agarose gel containing ethidium bromide (125 ng per 250 ml of gel mixture) prepared with 1x TBE buffer. After electrophoresis, the DNA fragments were visualized and photographed over UV trans-illuminator.

The PCR products of *trn*L-*trn*V were digested with the restriction enzyme of *Tru*1I, while those of *trn*F-*trn*T with *Taq*I. Of the total PCR amplification mixture, 6 μl of the PCR product was digested by 5 unit of restriction enzyme following manufacturer's instruction (MBI Fermentas, UK; Promega, USA). The generated cpDNA restriction fragments were fractionated electrophoretically in 2% agarose gel with ethidium bromide (125 ng per 250 ml of gel mixture) prepared with 1x TBE buffer. Electrophoresis was run at 6V/cm for 3 hours and DNA fragments were visualized and photographed over UV trans-illuminator. The size of DNA fragments was estimated by comparison with a DNA size standard (GeneRuller™ 100bp DNA Ladder Plus, MBI Fermentas, UK).

3. Statistical analysis

The observed variants of the PCR-RFLP were recorded as the presence (1) versus the absence (0) of the restriction site on the homologous fragment. To estimate genetic diversity within populations, 2 estimates of nucleotide diversity (Lynch and Crease, 1990) and haplotype diversity (Nei, 1987) within populations were calculated using the programs of HAPLO (Lynch and Crease, 1990) and Arleguin 2.0 (Schneider *et al.*, 2000), respectively. Level of genetic differentiation among populations was estimated by 3 different statistics on the bases of individual haplotypes (Φ_{ST}; Excoffier *et al.*, 1992) and nucleotide variation (N_{ST}; Lynch and Crease, 1990), respectively. AMOVA for estimating Φ_{ST} was performed at 2 and 3 hierarchical levels on the basis of genetic distance calculated by Euclidean metric of Excoffier *et al.* (1992) using Arleguin 2.0 program (Schneider *et al.*, 2000). Genetic relationships among populations were reconstructed by UPGMA (phylip v3.5c; Felsenstein, 1993) on the basis of pairwise Nei's genetic distance (Nei, 1972) between populations computed by RAPDDIST v1.0 (Black, 1996).

Results

For preliminary experiments, a total of 58 primer-enzyme combinations, employing 11 primer pairs (*trn*S-*psb*C, *rbc*L_1-*rbc*L_2, *rpo*C1, *rpl*2, *psb*B, *psb*D, *trn*K, *trn*C-*trn*D, *trn*F-*trn*T, *trn*Q-*trn*G, and *trn*L-*trn*V) and 21 restriction enzymes, were tested to detect the restriction site variants which could be used for classification of Korean fir and Manchurian fir using PCR-RFLP, respectively. Of these, only 2 combinations of *trn*L-*trn*V/*Tru*1I and *trn*F-*trn*T/*Taq*I revealed intraspecific restriction site variation which has enabled to discriminate between populations studied and to some degree also between the respective species.

The amplified products with approximate length of 2,200 bp and 1,477 bp were generated by PCR using the primers for *trn*L-*trn*V and *trn*F-*trn*T, respectively. No variation in size of PCR products was observed in the analyzed individual trees of both species. Following restriction enzyme digestion of the *trn*L-*trn*V PCR products with *Tru*1I in the gel. Contrary to haplotypes I-1 and I-2, the haplotypes I-3 and I-4 were detected only in the individuals of Korean fir. The profile of cut DNA

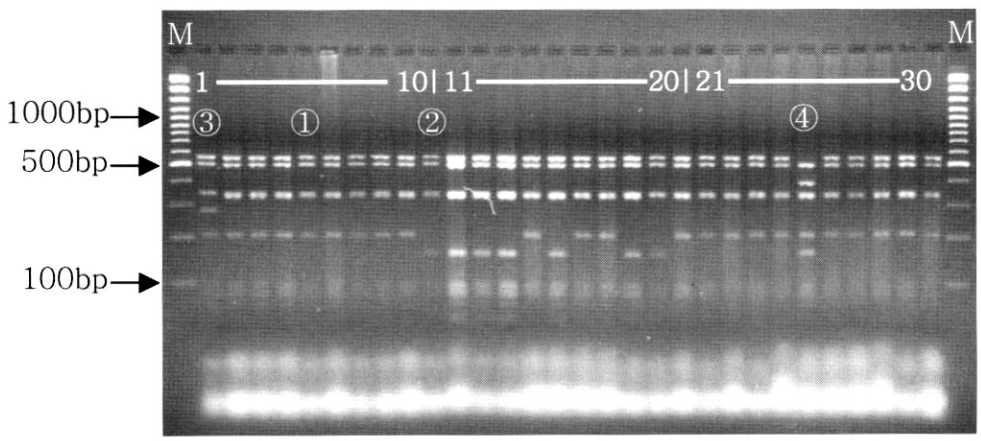

Figure 2. Examples of restriction fragment patterns of individual trees in Mt. Halla (1~10), Mt. Taebaek (11~20) and Mt. Jiri (21~30) populations generated by the combination of *trn*L-*trn*V/*Tru*1I. Encircled figures denote 4 restriction fragment profiles recognized among sampled trees.

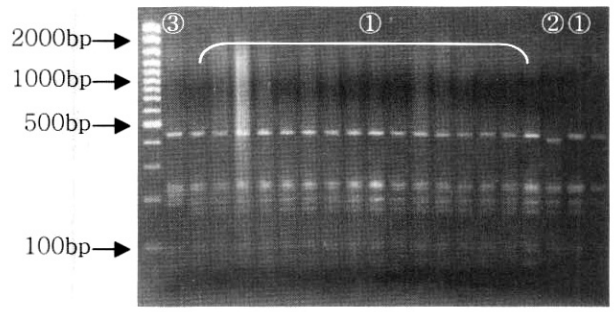

Figure 3. Three haplotypes observed from the combination of *trn*F-*trn*T/*Taq*I.

fragments of the haplotype I-3 resembled that of the haplotype I-1 except for one additional fragment of 280 bp instead of the 50 bp fragment. For the haplotype I-4, compared to other haplotypes, there were 2 additional fragments of 380 bp and 170 bp instead of a 550 bp fragment that was present commonly in other haplotypes (Figure 2).

Digestion of the *trn*F-*trn*T PCR product with *Taq*I revealed 2 restriction site changes that yielded 3 different haplotypes (Figure 3). The haplotype II-1 was common for both species and consisted of 8 DNA fragments of the size 420, 220, 210, 190, 180, 100, 90, and 70 bp, respectively. The haplotype II-2 was distinguished from the haplotype II-1 by possessing a 410 bp fragment instead of a 420 bp fragment, which might be resulted from indel mutation on the same fragment. This haplotype was detected only in Manchurian fir population. Conversely, the haplotype II-3 was found only in Korean fir in Mt. Halla. It differed from haplotype II-1 by possessing abundant fragment of 200 bp instead of a 180 bp fragment.

By pooling restriction fragment profiles generated by both primer/restriction enzyme combinations, a total of 6 individual cpDNA haplotypes were recognized from 125 individuals (Table 2). As to the frequency distribution of individual haplotypes, there were several haplotypes observed only in a single population, such as haplotype 1 and 4 in Mt. Halla of Korean fir, haplotype 5 in Mt. Jiri of Korean fir, and haplotype 6 in Mt. Taebaek of Manchurian fir, respectively. The prevalence of haplotype 2 in both populations of Korean fir resulted in a relatively low haplotype diversity index for Mt. Halla population (0.21) and for Jiri population (0.167), respectively (Table 3). The haplotype 2 was also observed in Manchurian fir at high frequency. Haplotype 3 was also observed in both species with much higher frequency in Manchurian fir. Although level of genetic diversity was not equivalent between 2 different estimates of nucleotide diversity (Lynch and Crease, 1990) and haplotype diversity (Nei, 1987) on account of the different nature of raw data (i.e., nucleotide variants vs. haplotype variants), overall trends of comparative level of genetic diversity was consistent (Table 3). Population of Mt. Taebaek of Manchurian fir, known to be progenitor species of the Korean fir, showed the highest genetic diversity that was about 3 times of those for the populations of Korean fir (Table 3). Of the 3 populations of Korean fir, population of Mt. Deogyu showed higher level of genetic diversity.

To test distribution pattern of genetic diversity among populations and species, 3 statistics were estimated on the bases of individual haplotypes (Φ_{ST}; Excoffier *et al.*, 1992), nucleotide variation (N_{ST}; Lynch and Crease,

Table 2. Individual cpDNA haplotypes determined by pooling restriction fragment profiles observed in 2 combinations of primer/restriction enzyme.

Population	Haplotypes						Total
	1	2	3	4	5	6	
Mt. Halla (Korean fir)	1	40	2	2			45
Mt. Jiri (Korean fir)		11			1		12
Mt. Deogyu (Korean fir)		24	3				27
Mt. Taebaek (Manchurian fir)		18	20			3	41

Table 3. Genetic diversity within population.

Population	Indivi duals	[a]He (SE)	[b]$\hat{H}$ (SE)
1. Mt. Halla (Korean fir)	45	0.016 (±0.013)	0.21 (±0.08)
2. Mt. Jiri (Korean fir)	12	0.009 (±0.012)	0.167 (±0.13)
3. Mt. Deogyu (Korean fir)	27	0.014 (±0.009)	0.205 (±0.09)
4. Mt. Taebaek (Manchurian fir)	41	0.037 (±0.028)	0.578 (±0.04)
Average		0.019	0.290

a: estimates of nucleotide diversity within populations (Lynch and Crease, 1990)
b: estimates of haplotype diversity within populations (Nei, 1987)

Table 4. Estimates of distribution pattern of genetic diversity.

	[a]Haplotype Variation	[b]Nucleotide Variation
Among populations	0.298	0.308
Within populations	0.602	0.692
Between populations of Korean fir	0.012	0.035
Within populations	0.988	0.965

a: variance component (Φ_{ST}; Excoffier *et al.*, 1992)
b: fraction of nucleotide variation (N_{ST}; Lynch and Crease, 1990)

Table 5. Results of AMOVA with individual haplotypes. Groups represent 2 species.

Source of variance	d.f	Component Variance
Among populations	3	29.8%
Within populations	121	60.2%
Among groups	1	41.1%
Among populations within groups	2	-0.4%
Within populations	121	59.3%

$\Phi_{ST} = 0.407 : \Phi_{SC} = -0.007 : \Phi_{CT} = 0.411$
Φ_{ST}: genetic differentiation among populations
Φ_{SC}: genetic differentiation among populations within species
Φ_{CT}: genetic differentiation between 2 species

1990), and restriction site mutations (θ; Weir, 1990), respectively. All the 3 estimates revealed high level of genetic differentiation among populations (Table 4). When AMOVA was performed with 4 populations without considering species classification, 29.8% of the observed haplotype variation was allocated to genetic differentiation among populations (Table 5). However, AMOVA at the 3 hierarchical levels with species grouping resulted in much higher level of genetic differentiation between species (Φ_{ST} = 0.407) and negative value among populations within species (Table 5). However, all 3 estimates of genetic differentiation between populations of Korean fir revealed that majority of genetic diversity might be allocated to individuals within populations (Table 4). Dendrogram reconstructed by UPGMA revealed that 3 populations of Korean fir were genetically closely related (Figure 4). Mean genetic distance between

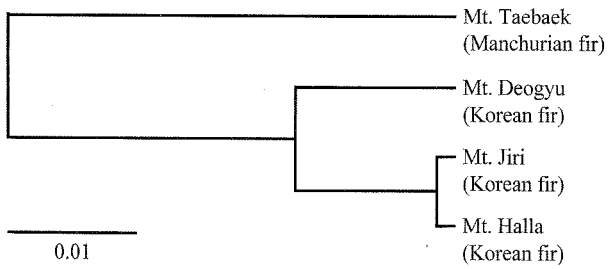

Figure 4. Dendrogram reconstructed by UPGMA based on Nei's average number of differences between populations (Nei, 1972).

3 populations of Korean fir and Manchurian fir was 0.07 and that among 3 populations of Korean fir was 0.026. This observation suggests that 3 populations of Korean fir are genetically rarely differentiated and that majority of genetic differentiation is allocated to genetic difference between Korean fir and Manchurian fir.

Discussion

The differentiation of morphological traits among Abies species is extremely small compared to species of the genus *Pinus* (Isoda *et al.*, 2000). This is especially true for Korean fir and Manchurian fir that have been known to be difficult to distinguish on the basis of the 4 needle characters, 4 seed characters, and 7 cone characters (Chang *et al.*, 1997). It follows from the most recent taxonomic accounts by Liu (1971) and Farjon and Rushforth (1989) that there are some additional pairs of species or their groups in the genus *Abies* whose taxonomic status is unclear. This prompted significantly the systematic studies of the genus at the DNA level. According to Isoda and Shiraishi (1999), DNA markers could provide more objective information on genetic differentiation than morphological characteristics. In case of Korean fir and Manchurian fir, phylogenetic relationship reconstructed by neighbour-joining method on the basis of 189 RAPD markers revealed that the two species might be diverged from a common hypothetical ancestor (Kim, 1998). The comparative study on genetic relationships among populations in South Korea, reconstructed by both UPGMA and neighbour-joining tree method, showed two distinct groups of 8 Korean fir and 5 Manchurian fir populations, where the two species were proposed to be treated as different taxa (Kim, 1998).

At the cpDNA level, no difference was observed between Korean fir and Manchurian fir in their *trn*L-*trn*F spacer region. On the basis of tandem repeat sequences, both species belonged to the AO group involving also 5 additional species of *Abies* native to Japan (Isoda *et al.*, 2000). Present data on restriction site polymorphism observed within the *trn*L-*trn*V and *trn*F-*trn*T spacer regions are the first evidence of genetic differentiation between Korean fir and Manchurian fir at the cpDNA level. In spite of the conservative nature of the gene coding regions of chloroplast genome, the cpDNA molecule in conifers harbors also a lot of variation in its intron, a spacer region (Isoda, 2000). On account of much higher mutation rate for introns than coding regions, they are regarded to be useful for investigating closely related species (Gielly and Taber, 1994).

Of the 4 haplotypes detected within the *trn*L-*trn*V region and the 3 haplotypes recognized within the *trn*F-

*trn*T region of Korean fir and Manchurian fir, the *trn*L-*trn*V haplotypes 3 and 4, and *trn*F-*trn*T haplotypes 2 and 3 were turned out to be species specific ones. However, it should not be excluded that such specificity might be resulted from the small sample size. In other words, if more number of samples were analyzed, those tentatively species unique haplotypes could be observed in both species. Therefore, additional analysis should be necessary either to validate or to deny the potential of these haplotypes as diagnostic markers for the species classification.

At the intra-specific level, the differential occurrence of the *trn*L-*trn*V haplotypes 3 and 4, and *trn*F-*trn*T haplotype 3 in the 3 Korean fir populations suggests, in a preliminary way, differentiation among them. A small sample size of Mt. Jiri population is a big drawback that does not allow us to draw a far-reaching conclusion about its genetic structure. However, it is worth of noting the postulation of the most differentiation of Mt. Jiri population from other populations of the species (Chang *et al.*, 1997). The absence of *trn*L-*trn*V haplotypes 2 and 3, and *trn*F-*trn*T haplotypes 3 together with the presence of *trn*L-*trn*V haplotype 4 within such a small number of sampled trees corroborates the unique status of the Mt. Jiri population.

A unique feature of conifer cpDNA is its paternal inheritance. For *Abies*, it was demonstrated by Ziegenhagen *et al.*, (1995). Isoda *et al.*, (2000) have efficiently used the principle of paternal transmission of cpDNA in conifers for identification of natural hybrids between *A. veitchii* and *A. homolepis*. It is important to point out that any of the haplotypes described above was found to be of hybrid nature. This excludes the possibility of involvement of the intermediate forms into the sampled groups, which are supposed to exist in the areas with common occurrence of both respective species (Chang *et al.*, 1997).

Owing to conservative nature of chloroplast genome, cpDNA markers were considered initially as good indicators of genetic divergence of distant taxa but insensitive for detection of differentiation at the intra-specific level (Palmer, 1987). Restriction site analysis of cpDNA has accordingly been looked upon as one of the most popular techniques in plant systematic studies, such as phylogenetic reconstruction below the family level, preferentially at the genera and species levels (Liston, 1992; Soltis *et al.*, 1992). However, Wagner *et al.*, (1987) demonstrated that certain regions of cpDNAs in *Pinus contorta* and *P. banksiana* appear as "hot spots" showing also intra-specific variation. In *Abies*, the first report illustrating unequivocally the presence of intra-specific variation at the cpDNA level was that by Tsumura *et al.*, (1994). The authors proved the existence of two types of variants in each of the *p*CS10/*Hind* III, *p*CS7/*Hind* III, and *p*CS7/*Bgl* II probe-enzyme combinations showing a gradual cline along the latitude and altitude in 7 natural populations of *A. mariesii* in Japan. The variation within the *trn*S-*psb*C spacer region of *A. alba* has also been described by Ziegenhagen and Fladung (1995). The two haplotypes revealed by the *Hae*III digestion were found to be common not only among *A. alba* but also among *A. nordmanniana* individuals (Ziegenhagen and Fladung, 1997). The two cpDNA phenotypes recognized by *Eco*RI within the *atpA* gene of *A. sachalinensis* have enabled to differentiate the southern populations of the species in Japan (Hayashi *et al.*, 2000). Presented results of the observed variants in Korean fir and Manchurian fir are additional in the series of contributions unraveling the variability of *Abies* cpDNA. Though preliminary, their potentials for population study of both species seem to be high. To confirm it more conclusively, further experiments are necessary involving a more number of populations from both species distributed on the entire Korean peninsula. Undeniable is the contribution of presented data in validating the opinions of Parducci and Szmidt (1999) regarding the presence of multiple variable regions in *Abies* cpDNA. They are supposed to be scattered throughout the whole genome. The cpDNA in the genus *Abies* has accordingly been suggested to be more polymorphic than that in other conifers. As to variation of the *trn*L-*trn*V and *trn*F-*trn*T spacers in other species of *Abies*, the question remains open.

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