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Classifying *Abies* Species (Pinaceae) Based on the Sequence Variation of a Tandemly Repeated Array Found in the Chloroplast DNA *trn*L and *trn*F Intergenic Spacer

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Abstract

DNA sequences of the chloroplast spacer region between the *trn*L and *trn*F genes were determined in 18 species of *Abies* MILL. and another five species of Pinaceae (*Keteleeria davidiana*, *Tsuga sieboldii*, *Larix kaempferi*, *Pseudotsuga menziesii*, and *Picea bicolor*). A tandem repeat sequence composed of a 14-bp core sequence was found in all *Abies* species analyzed. This tandem repeat array was specific for genus *Abies*, since this array was not detected in the other five species of Pinaceae. Comparison of the tandem repeat region of *Abies* species revealed variation in the number of repeats and in the nucleotide sequences of the units among species. The number of repeats varied from two to five, and there were eight different unit types in nucleotide sequences. Eighteen *Abies*

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species were divided into nine groups by the tandem repeat type, which was defined by both the number of repeats and the unit type. This grouping was compared with two conventional classifications based on morphological traits. There were some differences between the results of this study and the conventional classifications.

Key words: Abies, tandem repeats, chloroplast DNA, spacer region, classification, Pinaceae.

Introduction

Pinaceae is one of the most prosperous families in gymnosperms, and about 200 species in 11 genera are distributed in the Northern Hemisphere. *Abies* MILL. is a representative genus of the subfamily Abietoideae, and is the second largest genus in Pinaceae following genus *Pinus*. About 40 species are distributed in a wide range from the semi-arctic to the Temperate Zone in the Northern Hemisphere. The differentiation of morphological traits among species in genus *Abies*, however, is extremely small compared to that of genus *Pinus*. This may be due to the more recent speciation of *Abies* species in comparison with genus *Pinus*; genus *Abies* occurred in the early Tertiary period (FLORIN, 1963), while genus *Pinus* occurred in the Jurassic period (MIROV, 1967). Many taxonomists have suggested different classification systems for genus *Abies*. This indicates the difficulty of classification of this genus.

A number of molecular phylogenetic studies by chloroplast DNA (cpDNA) analysis were reported in many coniferous species (STRAUSS and DOERKSEN, 1990; CHASE *et al.,* 1993; BRUNSFELD *et al.,* 1994; TSUMURA *et al.,* 1995). There were, however, few reports of molecular phylogenetic studies of genus *Abies* except for those that estimated interspecific relationship between a few species (KORMUTÁK *et al.,* 1993; VICARIO *et al.,* 1995; TSUMURA and SUYAMA, 1998). There is little molecular information specific to the phylogeny of genus *Abies* published to date.

Recently, DNA information has been widely used to estimate evolutional relationships. For plant species, cpDNA-encoding genes are used predominately. The conservative gene-coding regions are not as powerful in investigating closely related species. Intron regions and spacer regions are useful because they hold a much higher mutation rate (GIELLY and TABERLET, 1994; MANEN and NATALI, 1995).

In this study, we determined the DNA sequence of the cpDNA spacer region between the *trn*L and *trn*F genes in 18 *Abies* species, and we found a tandemly repeated array that was variable among these species. According to the variation in this tandem repeat region, we classified 18 *Abies* species and compared the results with two conventional systems proposed by LIU (1971) and FARJON (1989, 1990).

Materials and Methods

Eighteen species of genus *Abies* and five species of another five genera in Pinaceae were analyzed in this study, as shown in *table 1.* Total genomic DNA was isolated from ca. 100 mg of leaves using a modified protocol (SHIRAISHI and WATANABE, 1995) of the CTAB method (MURRAY and THOMPSON, 1980). The total DNA was purified with the GENECLEAN III KIT (BIO 101).

The cpDNA spacer region between the *trn*L and *trn*F genes was amplified from total genomic DNA as a template using the polymerase chain reaction (PCR). The primer pair used for the amplification was 5'-TGTAAAACGACGGCCAGTGGTTCAAGTCCCTCTATCCC-3' and 5'-CAGGAAACAGCTATGACCATTTGAACTGGTGACACGAG-3. They were designed based on the two primers (B49873 and A50272) of TABERLET *et al.* (1991).

Table 1. – List of species used in this study.

species	source*1
Abies alba	Arboretum 1
A. amabilis	Arboretum 2
A. balsamea	Arboretum 1
A. bracteata	Arboretum 2
A. concolor	Arboretum 1
A. densa	Natural forest 1
A. firma	Arboretum 3
A. grandis	Arboretum 1
A. holophylla	Arboretum 1
A. homolepis	Arboretum 1
A. koreana	Arboretum 4
A. lasiocarpa	Arboretum 1
A. mariesii	Natural forest 2
A. nephrolepis	Arboretum 1
A. nordmanniana	Arboretum 1
A. sachalinensis	Arboretum 1
A. sibirica	Arboretum 1
A. veitchii	Natural forest 3
Keteleeria davidiana	Arboretum 5
Tsuga sieboldii	Arboretum 6
Larix kaempferi	Arboretum 7
Picea bicolor	Arboretum 8
Pseudotsuga menziesii	Arboretum 9

*1) Arboretum 1: Arboretum of University Forest in Hokkaido, University of Tokyo, Japan; Arboretum 2: Arboretum of the University of British Colombia, Canada; Arboretum 3: Arboretum of Tohoku Breeding Station, National Forest Breeding Center, Japan; Arboretum 4: Arboretum of University Forest, Seoul University, Korea; Arboretum 5: Arboretum of Kyushu University, Japan; Arboretum 6: Arboretum of Forestry and Forest Products Research Institute (FFPRI), Japan; Arboretum 7: Arboretum of Faculty of Agriculture, Shinsyu University, Japan; Arboretum 8: Tama Forest Science Garden, FFPRI, Japan; Arboretum 9: Arboretum of University Forest, Kyoto University, Japan; Natural forest 1: Natural forest, Butane; Natural forest 2: Natural forest in Hatimantai, Akita Prefecture, Japan; Natural forest 3: Natural forest in Akaishi Mountains, Gifu Prefecture, Japan.

Each 20-μL reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 200 μM each dNTP, 0.5 μM each Primer, 0.5 unit *AmpliTaq* DNA polymerase Stoffel Fragment, and 2 ng template DNA. DNA amplification was performed at 94 °C for 60 s, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, followed by 60 s at 72 °C with the GeneAmp PCR System 9600 (Perkin-Elmer). The PCR products were electrophoresed on a 1.5% agarose gel, and the fractions of DNA were excised from the gel under long wave UV light. DNAs were recovered from the gel particles and purified using a QIAEX Gelextraction Kit II (QIAGEN). The sequence reactions were performed with an Auto Sequencer Core Kit (TOYOBO) using the purified DNA as templates. The sequences were determined from both strands (SHIRAISHI and WATANABE, 1995). The DNA sequences were initially aligned using the program CLUSTAL V (HIGGINS et al., 1992), and we then manually checked the sequences.

Results and Discussion

DNA sequence in the trnL and trnF spacer region

The DNA sequence of the cpDNA spacer region between the *trn*L and *trn*F genes was determined for 18 *Abies* species. This

region varied in length from 403bp to 455bp among species; sequence variations were found at 13 sites. In these 13 variable sites, 10 sites were variation by point mutations, two sites were variation by 4- or 10- base insertion/deletion, and one site was tandem repeat variation. In this spacer region, 10 DNA sequence variations were detected among 18 *Abies* species (*Table 2*).

Table 2. – Tandem repeat type (TRT) for 18 *Abies* species and comparison to LIU's (1971) and FARJON's (1990) systematic classification.

TRT	Seq. Type[a]	Species	Liu (1971)	Farjon (1990)	
			Section	Section	Subsection
ABOO	1	*A. bracteata*	*Bracteata*	*Bracteata*	
ABO	2	*A. concolor*	*Grandes*	*Grandis*	
	2	*A. grandis*	*Grandes*	*Grandis*	
ABD	3	*A. mariesii*	*Homolepides*	*Amabilis*	
ABF	4	*A. balsamea*	*Balsamea*	*Balsamea*	*Laterales*
ABBBF	5	*A. lasiocarpa*	*Balsamea*	*Balsamea*	*Laterales*
AO	6	*A. koreana*	*Elate*	*Balsamea*	*Medianae*
	6	*A. nephrolepis*	*Elate*	*Balsamea*	*Medianae*
	6	*A. sachalinensis*	*Elate*	*Balsamea*	*Medianae*
	6	*A. veitchii*	*Elate*	*Balsamea*	*Medianae*
	6	*A. densa*[b]	*Pindrau*	*Pseudopicea*	*Delavayianae*
	7	*A. sibirica*	*Pichta*	*Balsamea*	*Laterales*
	7	*A. firma*	*Momi*	*Momi*	*Firmae*
	7	*A. homolepis*	*Homolepides*	*Momi*	*Homolepides*
AG	8	*A. holophylla*	*Homolepides*	*Momi*	*Holophyllae*
BE	9	*A. amabilis*	*Grandes*	*Amabilis*	
CB	10	*A. alba*	*Abies*	*Abies*	
	10	*A. nordmanniana*	*Abies*	*Abies*	

[a]) Chloroplast haplotype defined by entire sequence of *trn*L-*trn*F spacer region.
[b]) *A. densa* was included with *A. spectabilis* by LIU (1971).

Interspecific polymorphisms of *Abies* cpDNA have been observed using restriction fragment length polymorphism (RFLP) and PCR-RFLP analyses (KORMUTÁK *et al.,* 1993; ZIEGENHAGEN and FLADUNG, 1997; PARDUCCI and SZMIDT, 1999). Intraspecific variations have also been recognized in several species (ZIEGENHAGEN and FLADUNG, 1997; PARDUCCI and SZMIDT, 1999). PARDUCCI and SZMIDT reported that most of the cpDNA variations observed in their work were attributed to length mutations. In our study, variations among *Abies* species were investigated at the sequence level. Of 13 mutation sites detected in the *trn*L-*trn*F region, only three were due to length mutations, and the remaining sites were caused by point mutations. This might indicate that cpDNA possesses point mutations more frequently than length mutations in *Abies.*

Partial sequences of the cpDNA spacer region between the *trn*L and *trn*F genes in *Abies grandis*, *Keteleeria davidiana*, *Tsuga sieboldii*, *Larix kaempferi*, *Pseudotsuga menziesii*, and *Picea bicolor* are shown in *figure 1.* An imperfect tandem repeat region composed of a 14 nucleotide core sequence (unit) was found between nucleotide positions 87 and 128 of the sequence only in *A. grandis.* The other five species of the five genera in Pinaceae characterized had a sequence homologous with this core sequence; they were not, however, tandemly repeated. All of the 18 *Abies* species investigated possessed a tandem repeat sequence in this region.

Tandem repeat variation among Abies species

A close comparison of the tandem repeat regions among *Abies* species showed that the number of repeats ranged from 2 to 5. There were also nucleotide sequence variations among the units (*Fig. 2*). Eight different types of units were identified according to their nucleotide sequences. Unit A was the most

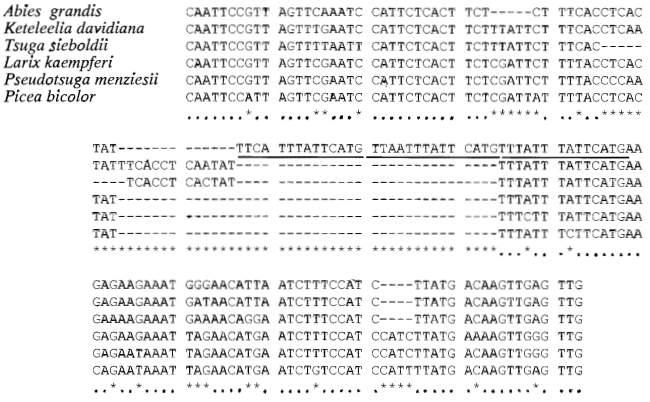

Fig. 1. – Partial sequences of the cpDNA spacer region between the *trn*L and *trn*F genes in *Abies grandis*, *Keteleeria davidiana*, *Tsuga sieboldii*, *Larix kaempferi*, *Pseudotsuga menziesii*, and *Picea bicolor*. Underlining indicates a tandem repeat region.

common and appeared in 15 out of the 18 species investigated. The second most common were units O and B, which appeared in 11 and 9 species, respectively. The nucleotide sequence of unit O perfectly matches the homologous sequence of *Keteleeria davidiana* and *Larix kaempferi.* Both units A and B differed from unit O in the third nucleotide of the core sequence. Units D, F, and G were detected in only one or two species, and these types were also different from unit O in one nucleotide. Unit O is the only one that can form units D, F, and G by changing one nucleotide, assuming that the possibility of a reverse mutation is ignored. Units C and E were detected in one species, with these units being formed by one mutation from units B and D, respectively. The phylogenetical relationships among the units as inferred are shown in *figure 2.* Unit O appears to be the ancestral type, with a one-nucleotide change in unit O causing the formation of units A, B, D, F, and G. A further one-nucleotide mutation occurring in units B and D would form units C and E, respectively.

Nine tandem repeat types (TRTs) characterized by the arrangement of eight types of core units were detected in 18 *Abies* species (*Fig. 3* and *Table 2*). The evolutionary relationships of these TRTs were inferred. TRT: ABBBF can be formed by unit B in TRT: ABF being duplicated twice. TRT: ABOO may appear to be a duplicate of unit O in TRT: ABO. TRT: ABD and ABF can be derived from one point mutation of unit O in TRT: ABO. Since unit E appears to be formed by a one-nucleotide change from unit D, it is reasonable to conclude that TRT: BE is derived from TRT: ABD; that is, a nucleotide substitution in unit D and a deletion of unit A have occurred in TRT: ABD. TRT: CB may be formed by a duplication of unit B, followed by a nucleotide change in the first unit. TRT: AG is likely derived from TRT: AO, as unit G can not be derived from any of the other units except for unit O without reverse mutation.

Classification of Abies species according to the TRTs

Taxonomists have proposed a variety of different classifications for *Abies*, with LIU (1971) and FARJON (1989, 1990) being the most recent. Their classifications are, however, different in many points, which may be indicative of the difficulty of classifying *Abies.*

The grouping based on the TRTs, and the conventional systems proposed by LIU and FARJON are shown in *table 2*. The two conventional systems agree with each other in some selections. Two European species, *A. alba* and *A. nordmanniana*, are classified into sect. *Abies.* These two species possessed TRT: CB, differentiating them from the other 16 species. Two North American species, *A. concolor* and *A. grandis*, are classified

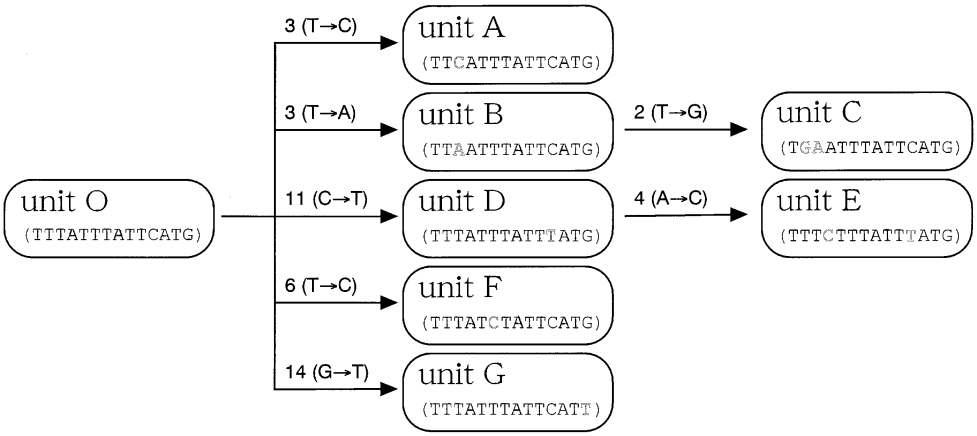

Fig. 2. – Sequences of repeat units and their relationship estimated from nucleotide substitution. The letters above the arrows show the changed nucleotide at the site in parentheses.

```
ABO   ... TTCATTTATTCATG TTAATTTATTCATG TTTATTTATTCATG ...
ABOO  ... TTCATTTATTCATG TTAATTTATTCATG TTTATTTATTCATG
                                        TTTATTTATTCATG ...
ABD   ... TTCATTTATTCATG TTAATTTATTCATG TTTATTTATTTATG ...
BE    ... -------------- TTAATTTATTCATG TTTCTTTATTTATG ...
ABF   ... TTCATTTATTCATG TTAATTTATTCATG TTTATCTATTCATG ...
ABBBF ... TTCATTTATTCATG TTAATTTATTCATG
                        TTAATTTATTCATG
                        TTAATTTATTCATG TTTATCTATTCATG ...
CB    ... -------------- TGAATTTATTCATG
                        TTAATTTATTCATG -------------- ...
AO    ... TTCATTTATTCATG -------------- TTTATTTATTCATG ...
AG    ... TTCATTTATTCATG -------------- TTTATTTATTCATT ...
```

Fig. 3. – Sequences of 9 tandem repeat types among *Abies* species.

into sect. *Grandes* (sect. *Grandis*), and their TRT (ABO) also differentiated them from other species. The TRT groupings for these four species, therefore, agree with the conventional classifications.

A. bracteata differ in many morphological traits from other *Abies* species, and have been classified into a monotypic section. *A. bracteata* is classified into subgenus *Pseudotorreya*, sect. *Bracteata* by LIU, and sect. *Bracteata* by FARJON. Both of LIU and FARJON treated this species as a monotypic group, and considered that there is no related species. The TRT of this species was ABOO, which was not seen in any other species. This TRT was, however, similar to TRT: ABO of *A. concolor* and *A. grandis* of sect. *Grandes* (sect. *Grandis*), only differing in the duplicated unit O. The classification of *A. bracteata* should be reconsidered with the phylogenetic relationships based on more DNA information.

Classification of the remaining 13 species differs at many points between LIU and FARJON. FARJON has classified the Japanese species, *A. mariesii*, into sect. *Amabilis* with a North American species, *A. amabilis*. On the other hand, LIU has classified *A. mariesii* into sect. *Homolepides*, which includes *A. homolepis* (distributed in Japan) and *A. holophylla* (distributed in the Korean Peninsula), and *A. amabilis* into sect. *Grandes*, which include the North American species, *A. grandis*, and so on. Also in this study, the TRTs of *A. mariesii* and *A. amabilis* were different from each other. However, as mentioned above, the TRTs of these two species are relatively closely related in the evolutional relationships. This could indicate that the TRT data supports the classification proposed by FARJON.

FARJON has classified North American species *A. balsamea* and *A. lasiocarpa* into sect. *Balsamea* with some Asian species such as *A. koreana*, *A. nephrolepis*, *A. sachalinensis*, *A. veitchii*, and *A. sibirica*. LIU, however, has differentiated the two North American species from the Asian species. The results of this study have shown all these Asian species to have TRT: AO, but *A. balsamea* and *A. lasiocarpa* have TRT: ABF and ABBBF, respectively. Although the TRTs of *A. balsamea* and *A. lasiocarpa* are different, they can be considered to be closely related because the only difference appears to be due to the duplication of unit B. This suggests that the TRTs of the two North American species are of the same origin, but that these two species are of different origin from the Asian species. Consequently, the classification by LIU, in which *A. balsamea* and *A. lasiocarpa* are differentiated from the Asian species, seems to be suited to the TRT grouping. There are some questions that still remain in LIU's classifications, as he divided the Asian species into three sections. These divisions should be reconsidered with more molecular information. Even though FARJON has classified *A. balsamea* and *A. lasiocarpa* into the same section as the Asian species, he has differentiated them into subsections. FARJON has divided sect. *Balsamea* into two subsections, subsect. *Laterales* (*A. balsamea* and *A. lasiocarpa*) and *Medianae* (*A. koreana*, *A. nephrolepis*, *A. sachalinensis* and *A. veitchii*). This seems to agree with the TRT grouping. *A. sibirica* (TRT: AO), however, was classified into subsect. *Laterales*, which includes *A. balsamea*. Some questions still remain in these classifications.

As discussed in the prior section, TRT: AG is likely derived from TRT: AO. The eight TRT: AO species and one TRT: AG species (*Table 2*), show many differences from the classifications of LIU and FARJON. Since the TRTs do not offer enough information to clarify the taxonomical relationships among these 9 species, more DNA information is needed in this taxon.

Conclusion

Systematic analysis at the DNA level is quite important, especially for a taxon that is difficult to classify based on morphological information such as in the genus *Abies*. In this study, we detected variation in the *Abies* species in relation to the tandem repeated array at the chloroplast spacer region between the *trn*L and *trn*F genes. Even though additional DNA data would be needed to clarify the taxonomic relationship of 9 *Abies* species, the DNA results are compatible with FARJON's classification of *A. amabilis* and *A. mariesii* into sect. *Amabilis*, and with LIU's separation of the North American species of *A. balsamea* and *A. lasiocarpa* from the Asia species, *A. koreana*, *A. nephrolepis*, *A. sachalinensis*, *A. veitchii* and *A. sibirica*. A comparison between the tandem repeat variation and the

conventional classification systems by LIU (1971) and FARJON (1989, 1990) suggests the necessity of reconstructing the currently accepted systematics. An accumulation of more DNA information in addition to our results will clarify the systematics and phylogenetic relationships of genus *Abies*.

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Determination of the Selfing Rate in a Hinoki (*Chamaecyparis obtusa*) Seed Orchard by Using a Chloroplast PCR-SSCP Marker

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Abstract

The selfing rate in a Hinoki (*Chamaecyparis obtusa*) seed orchard, representing the most common coniferous species in Japan, was determined by using a highly specific PCR-SSCP (Polymerase Chain Reaction – Single-Strand Conformation Polymorphism) marker which recognizes the spacer region between the genes *trn*D and *trn*Y of the chloroplast DNA. One-thousand and three open-pollinated offsprings were analyzed from plus tree clone YKZ5. This clone has a single point mutation in comparison with 32 wild type clones which are also present in the orchard. Among the offsprings tested, the mutant chloroplast haplotype was detected in 23 samples. Based on the paternal inheritance of the chloroplast DNA marker, the mean selfing rate of clone YKZ5 was determined to 2.3% in good agreement with the theoretical value expected. Data demonstrate that the newly-developed PCR-SSCP marker derived from chloroplast DNA provides a powerful tool for accurate and effective analyzing of gene flow within a Hinoki seed orchard.

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Key words: PCR-SSCP, chloroplast DNA; selfing rate, seed orchard, *Chamaecyparis obtusa*.

Introduction

Hinoki (*Chamaecyparis obtusa*) is one of the most common conifers in Japan. The species is widely used in reforestation with exception of the island Hokkaido, representing the most northern Japanese island. Superior genotypos (plus trees) have been selected and grown in single orchards. The seeds harvested from such orchards yield now 40% of the plant stock used in Japan (National Forest Tree Breeding Center, 1997).

Researchers recently characterized the genetic information of Hinoki trees by using isoenzyme markers. Based on these markers, the genetic variation in both natural and artificial forests (SHIRAISHI *et al.,* 1986; SEIDO *et al.,* 1987, UCHIDA *et al.*, 1991), the genetic variability of plus trees (UCHIDA *et al.*, 1993), and the breeding structure of natural populations have been reported (SEIDO, 1990). Seed orchards planted by plus trees are designed and managed in order to produce a large amount of seeds for reforestation. If cross-fertilization, however, is limited in seed orchards, and if self-fertilization occurs at a high level,