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Length variation in the internal transcribed spacers of ribosomal DNA in *Picea abies* and related species

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Abstract The structure and variation of nuclear ribosomal DNA (rDNA) units of *Picea abies,* (L.) Karst. was studied by restriction mapping and Southern hybridization. Conspicuous length variation was found in the internal transcribed spacer (ITS) region of *P. abies*, although the length of this region is highly conserved both within and among most of the plant species. Two types of ITS variants (A and B), displaying a size difference of 0.5 kb in the ITS2 region, were present within individuals of *P. abies* from Sweden, Central Europe and Siberia. A preliminary survey of 14 additional Eurasian and North American species of *Picea* suggested that length variation in the ITS region is widespread in this genus. Alltogether three length variants (A, B and C) were identified. Within individuals of eight *Picea* species, two length variants were present within the genome (combinations of A and B variants in *P. glehnii*, *P. maximowiczii*, *P. omorika*, *P. polita* and *P. sitchensis* and variants B and C in *P. jezoensis*, *P. likiangensis* and *P. spinulosa*). Within individuals from five species, however only one rDNA variant was present in their genome (variant A in *P. aurantiaca, P. engelmannii*, *P. glauca*, *P. koraiensis* and *P. koyamai*; variant B in *P. bicolor*). The ITS length variation will be useful as a molecular marker in evolutionary studies of the *Picea* species complex, whose phylogeny is controversial. The presence of intraindividual variation in, and shared polymorphism of the, ITS length variants raises questions about the occurrence of interspecific hybridization during the evolutionary history of *Picea*.

Key words Evolution · rDNA
Internal transcribed spacers · *Picea*

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Introduction

The genus *Picea* includes 28–40 taxonomically unruly species, of which *P. abies* has the largest distribution, extending from Central Europe to the east coast of Asia (Wright 1955; Schmidt-Vogt 1977). *P. abies* is considered to be one of the most morphologically variable conifers and taxonomists disagree on whether to separate it into two or more subspecies, or even into separate species (Schmidt-Vogt 1977; Pravdin 1985). Allozyme studies have shown high levels of genetic variability in European *P. abies* populations and geographical patterns in the variation of certain allele frequencies (Lagercrantz and Ryman 1990). Intraspecific cpDNA polymorphism has also been observed in European poulations of *P. abies* (Sigurgeirsson 1992).

Ribosomal DNA (rDNA) variation has been widely used for studying the genetic structure in plant populations (reviewed in Schaal and Learn 1988) and to examine phylogenetic relationships between plant species (reviewed in Hamby and Zimmer 1991). In the present study, we have characterized the rDNA units of *P. abies* by restriction mapping and evaluated the use of variable rDNA regions as molecular markers by surveying geographically distant populations of *P. abies*, as well as individual trees from 14 other species in the genus of *Picea,* for the presence of observed polymorphisms.

rDNA forms large multigene families in conifer genomes, distributed at 6–8 nucleolus organizer loci (Brown et al. 1993; Karvonen et al. 1993). Each rDNA unit contains genes coding for 18*s*, 5.8*s* and 25*s* rRNAs, separated by two internal transcribed spacers (ITS1 and ITS2). Adjacent transcription units are separated by a nontranscribed intergenic spacer region (IGS), which typically contains subrepeating elements (Schaal and Learn 1988). Recent studies on conifer rDNA have suggested several deviations from the basic structure of the plant rDNA unit. Due to long intergenic spacer regions (IGS), the rDNA units of *Pinus* and *Picea* are two- or three-fold longer than in angiosperms, ranging in size from 18 kb to over 40 kb (Cullis et al. 1988; Bobola et al. 1992; Beech and Strobeck

1993; Karvonen et al. 1993). In addition, the ITS regions of *Picea mariana* (Bobola et al. 1992), *Pinus sylvestris* (Karvonen et al. 1993) and *Pinus pinea* (Maggini and Baldassini, personal communication) comprise about 3 kb, whereas this region is only 588–623 bp in angiosperm plants (Venkateswarlu and Nazar 1991). Sequencing studies have indicated that the length of the ITS region is conserved in a large number of angiosperm species (Venkateswarlu and Nazar 1991; D'Ovidio 1992; Ritland and Straus 1993), although sequence variation may exist within species and even within individuals (Baldwin 1992; Suh et al. 1993). In contrast, the present study reports length variation of the ITS region in *P. abies* and several other species of *Picea*, which should be of use in elucidating the taxonomic relationships in this genus.

Materials and methods

DNA samples

The number and locations of *P. abies* DNA samples are presented in Table 1. The locations were selected on the basis of being distributed geographically as widely as possible. Two types of DNA samples were included. Single-plant samples were isolated from individual seedlings or trees. Composite DNA samples represent a mixture of individual seedlings or needles from individual trees collected in a single population. Single-plant samples from Siberian populations, representing either subspecies *P. abies* subsp. *obovata* (n=12) or presumed hybrids between European and Siberian subspecies (*P. abies* subsp. *fennica*, n=6) were also included (identified on the basis of morphology, Dr. K. Krutovskii, personal communication). The source of the European samples was the Institute of Forest Improvement, Sävar, Sweden. The Siberian samples were a kind gift of Dr. Krutovskii.

Table 1 List of *P. abies* DNA samples examined in the study

Region	Latitude (N)	Longitude (E)	Sample size[a]
Northern Sweden	67°18′	23°18′	1 (s)
	66°50′	22°23′	1 (s)
	65°27′	17°24′	1 (s)
	64°17′	14°55′	1 (s)
	64°17′	16°35′	1 (s)
	63°54′	18°02′	1 (s)
	62°10′	17°28′	1 (s)
	67°15′	23°03′	20 (c)
Southern Sweden	56°30′	16°04′	1 (s)
	58°57′	12°18′	10 (c)
	57°13′	14°35′	10 (c)
	57°13′	12°38′	10 (c)
	57°22′	18°41′	10 (c)
	56°38′	14°38′	10 (c)
	56°30′	16°04′	10 (c)
	56°02′	12°56′	19 (c)
Central Europe	49°35′	19°35′	1 (s)
	48°54′	13°26′	1 (s)
	49°35′	18°50′	20 (c)
Russia	55°31′	23°00′	1 (s)
	53°02′	27°36′	1 (s)
	56°08′	92°74′	12 (s)[b]
	58°01′	56°35′	6 (s)[c]

[a] Sample type in parentheses: (s) for single-plant samples and (c) for composite
[b] *P. abies* subsp. *obovata*
[c] *P. abies* subsp. *fennica*

Single-plant DNA samples from 14 other species of *Picea* were surveyed for the presence of ITS length variation. The source of the *P. aurantiaca*, *P. bicolor*, *P. engelmannii*, *P. glauca*, *P. glehnii*, *P. jezoensis*, *P. koyamai*, *P. maximowiczii*, *P. omorika* and *P. polita* samples was the Hørsholm Arboretum, Hørsholm, Denmark. Samples of *P. koraiensis*, *P. likiangensis* and *P. spinulosa* were obtained from the Bedgebury Pinetum, Bedgebury, Great Britain, and the *P. sitchensis* sample was from Yakutat, Alaska.

DNA isolation and Southern blots

Total DNA was isolated from needles of mature *P. abies* trees following Szmidt et al. (1986). This extraction method was applied to all samples except the Siberian population samples, from which total DNA was isolated from 2–3 week-old seedlings following the miniprep method of Doyle and Doyle (1990).

DNA samples (3 µg) were digested to completion with restriction enzymes (Boehringer) and fractionated on 0.8% agarose gels. DNA was transferred to a nylon membrane by vacuum blotting, hybridized at 65°C with rDNA probes labelled with digoxigenin (DIG labelling and detection kit, Boehringer), washed twice in 2×SSC, 0.1% SDS at room temperature followed by 2×15 min in 0.5×SC; 0.1% SDS at 65°C and then detected by immunological staining as described by Karvonen et al. (1993). The probes used for restriction mapping included pBG35, the complete rDNA repeat unit from flax (Goldsbrough and Cullis 1981), the 1.5-kb rDNA fragment from the 18*s* region of pBG35, and the 2.1-kb rDNA fragment from the 25*s* gene of pBG35. A homologous probe for restriction mapping was provided by PCR amplification and cloning of the ITS1 region of *P. abies* (clone pABI3, see below).

Amplification and cloning of the P. abies ITS1

Polymerase chain amplification of the ITS1 region was performed as previously described for *P. rubens* by Bobola et al. (1992). The primers were 20-bp oligos located 237 bp upstream from the 3′ end of the 18*s* coding gene (primer DES18S2) and 30 bp from the 5′ end of the 5.8S gene (primer DES5.8S). A 50 µl reaction contained 1×*Taq* buffer, 1.5 mM of $MgCl_2$, 200 µM of each dNTP, each primer at 3 µM, 20 ng of *P. abies* DNA and 2.5 U of *Taq* DNA Polymerase (Promega), overlaid with 50 µl of mineral oil. The PCR cycles, 95°C for 5 min, 30×(93°C for 1 min, 53°C for 1 min 30s, 72°C for 2 min), were performed in a Hybaid OmniGene thermal cycler. Reaction mixtures were extracted with 200 µl of chloroform, and a 5-µl sample was analyzed in a 1% agarose gel to check the size of the PCR products. The PCR products were purified by phenol extraction and cloned into a pGEM-T vector (Promega). Plasmid DNA was isolated from several putative recombinant colonies. The inserts were excised by *NcoI*+*SalI* double digestion and analyzed in agarose gels. One of the clones (pABI3), which contained a 0.55-kb insert, was DIG-labeled and the ITS1 identity of the clone was confirmed by Southern hybridization. As the restriction maps of the rDNA repeats had been defined using flax rDNA probes, we were able to predict the fragment sizes to which the pABI3 probe should hybridize. pABI3 hybridized to the expected rDNA fragments, confirming its origin from the ITS1 region.

Results

Structure of the rDNA repeats in P. abies

Restriction mapping of the rDNA revealed two major repeat types within the *P. abies* genome. Restriction maps from the coding (transcribed) regions of the rDNA repeats

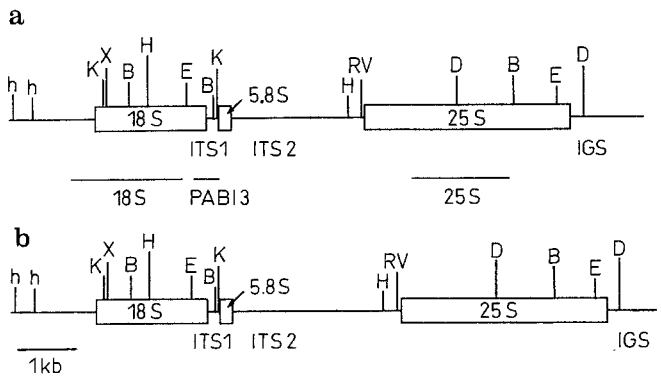

Fig. 1 Restriction maps of the two nuclear rDNA repeat types found in *P. abies*, containing **a** 2.5-kb and **b** 3.0-kb ITS2 regions. The position of the probes used for restriction mapping are shown below map **a**. In addition to these, pBG35, the whole flax rDNA repeat, was used as a probe. pBG35 hybridizes with only the rRNA coding regions of *P. abies* rDNA (not with the IGS). Restriction enzyme abbreviations: *B BamHI, D DraI, E EcoRI, H HindIII, K KpnI, RV EcoRV, X XbaI*. Polymorphic *Hin*dIII restriction sites are indicated by *lower-case letters*

Table 2 rDNA fragments detected with various probe-enzyme combinations within a Swedish *P. abies* individual. Only the most informative enzyme-probe combinations for localization of the site of repeat length variation are shown. Fragments giving weak hybridization signals are presented in parentheses

Enzymes	rDNA fragment sizes (kb)		
	18*s* probe	ITS1 probe	25*s* probe
*Xba*I+*Bam*HI	0.4, 1.6	1.6	5.6, 5.1
*Xba*I + *Eco*RV	5.0, 4.5	5.0, 4.5	>20
*Xba*I + *Dra*I	6.6, 6.1	6.6, 6.1	(6.6), (6.1), 2.2
*Xba*I + *Hin*dIII	0.7, 4.0, 3.5	4.0, 3.5	>20
*Hin*dIII	2.5, 2.2, 4.0, 3.5	4.0, 3.5	>20

were generated for a Swedish *P. abies* individual using seven restriction endonucleases and four probes (Fig. 1). The highly conserved *Xba*I site at the 5′ end of the 18*s* gene was used as a starting point for mapping. Single digestions with either *Xba*I or *Eco*RV generated a single, over 20-kb, rDNA fragment, suggesting single recognition sites of these enzymes to be present in the *P. abies* rDNA repeat. Based on these digestions, the rDNA repeat size in *P. abies* is in the range observed in other *Picea* species (Bobola et al. 1992; Beech and Strobeck 1993). The length of the ITS1 region was determined from the PCR amplification of the region between the 3′ end of the 18*s* gene and the 5′ end of the 5.8*s* gene. The PCR products were ligated to a pGEM-T vector. In 13 out of the 15 confirmed ITS1 clones, the size of the insert was about 550 bp and in two clones about 650 bp, of which 237 bp are from the 18*s* gene and 30 bp from the 5.8*s* gene. Therefore, PCR analysis indicated that the ITS1 region of *P. abies* rDNA is about 300 or 400 bp, and shows length variation.

In addition to the length variation observed in the PCR analysis of the ITS1 region, length variation of *P. abies* rDNA was also observed in the RFLP analysis. The presence of two types of rDNA repeats within the *P. abies* genome was supported by several single and double digestions, which generated double bands with a consistent 0.5-kb size difference. These double bands appeared in the following double digestions, *Xba*I+*Dra*I, *Xba*I+*Eco*RV, *Xba*I+*Bam*HI, *Xba*I+*Eco*RI, *Xba*I+*Hin*dIII, *Eco*RI+*Dra*I, *Bam*HI+*Dra*I, as well as in *Bam*HI, *Eco*RI and *Hin*dIII single digestions. The site of the 0.5-kb repeat length variation was localized to the ITS2 region by using separately the 18*s*, 25*s* and ITS1 probes. For example, bands of 4.5 and 5.0 kb in the *Xba*I+*Eco*RV double-digested *P. abies* DNA hybridized with the 18*s* and the ITS1 probes, but not with the 25*s* probe (Table 2), suggesting that they are located in the region spanning the 18*s* gene and the transcribed spacers. In *Bam*HI and *Xba*I+*Bam*HI digestions (Table 2), a 1.6-kb rDNA fragment hybridized with the 18*s* and ITS1 probes, and the double bands (5.1 and 5.6 kb) hybridized with only the 25*s* probe. Therefore, the 0.5-kb indel is located between the *Bam*HI site in the ITS1 and the *Bam*HI site in the 25*s* gene (Fig. 1. Table 2). In this way, using small probes and all enzyme combinations, it was possible to locate the 0.5-kb size difference to the ITS2 region; between the 5.8*s* gene and the *Hin*dIII and *Eco*RV restriction sites of ITS2 (Fig. 1).

Survey of P. abies populations

Single-plant and composite DNA samples of *P. abies* from Central European, Swedish and Russian populations (see Table 1) were surveyed for the presence of ITS2 rDNA variants. As exemplified in Fig. 2, all *P. abies* DNA samples exhibited the presence of the two rDNA length variants when digested with *Xba*I+*Dra*I and hybridized with pBG35. The 6.1- and 6.6-kb bands, extending from the 5′ end of the 18*s* gene to the middle of the 25*s* gene, were present in each DNA sample. Only slight variation in relative proportions (copy numbers) of the rDNA variants could be detected between individuals, as judged from the intensity of hybridization signals. The two rDNA variants were also present in the 12 studied *P. abies* subsp. *obovata* individuals, as well as in the six studied *P. abies* subsp. *fennica* individuals. However, as judged by hybridization intensity (example in Fig. 2b), the relative proportion of the shorter rDNA variant was higher than that of the longer variant both in *P. abies* subsp. *obovata* and *P. abies* subsp. *fennica*.

Variations in the structure of the rDNA units in different species of Picea

Single-plant DNA samples of 14 species of *Picea* (Table 3) were double-digested with *Xba*I and *Dra*I restriction enzymes and hybridized with pBG35. Four kinds of hybridization patterns emerged (Fig. 3 a). In *P. glehnii*, *P. maximowiczii*, *P. omorika*, *P. polita* and *P. sitchensis*, the rDNA hybridization pattern was similar to that of *P. abies*, suggesting the presence of the two ITS length variants (A and

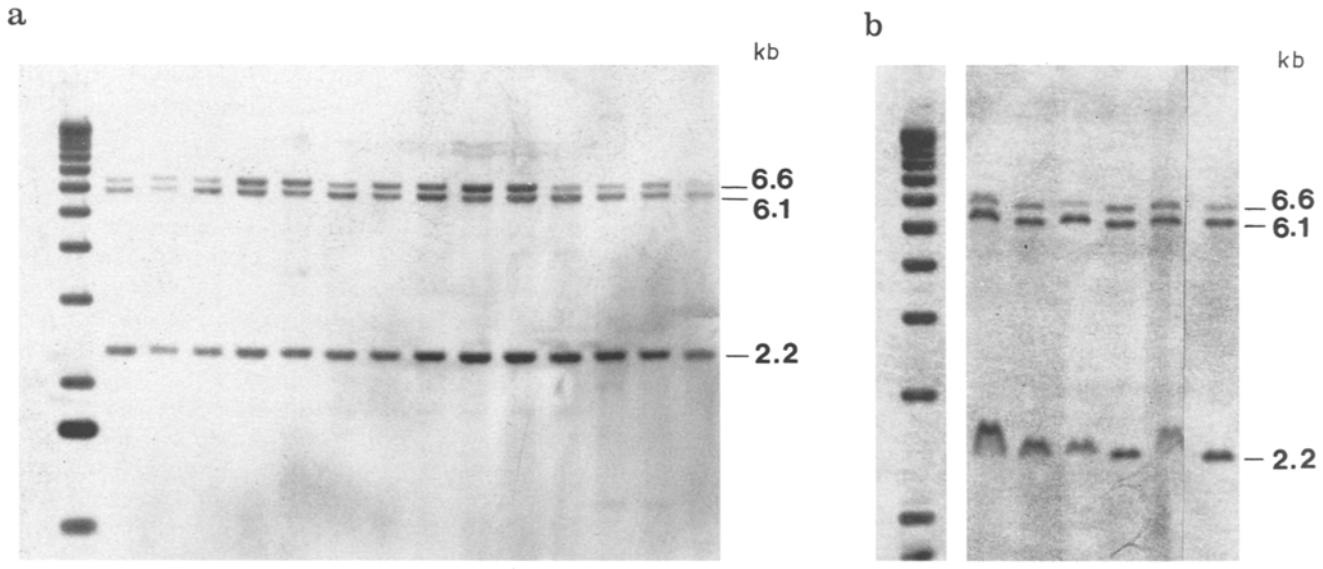

Fig. 2 rDNA hybridization patterns of *P. abies* individuals from **a** European and **b** Siberian populations. DNA samples were double digested with *Xba*I and *Dra*I and hybridized with digoxigenin-labelled pBG35. The 6.1-kb rDNA fragment is from the A variant and the 6.6-kb rDNA fragment from variant B (Fig. 1). They span the region between the *Xba*I site in the 5′end of the 18*s* gene and the *Dra*I site in the middle of the 25*s* gene. The 2.2-kb rDNA fragment is similar in A and B variants. It extends from the *Dra*I site in the middle of the 25*s* gene to the *Dra*I site to the 3′ end of the 25*s* gene

Table 3 rDNA repeat types[a] detected within single individuals of different *Picea* species

Species	Distribution	rDNA repeat types
P. abies (L.) Karst	Europe, Asia	A, B
P. aurantiaca Mast.[b]	Central China	A
P. bicolor (Maxim.) Mayr	Central Japan	B
P. engelmannii (Parry) Engel.	Western North America	A
P. glauca (Moench) Voss	North America	A
P. glehnii (Fr. Schmidt) Mast.	Japan, Sachalin, Kuriles	A, B
P. jezoensis (Sieb. et Zucc.) Carr.	Japan, East Siberia, Korea	B, C
P. koraiensis Nakai[c]	Korea/Manchuria	A
P. koyamai Shirasawa	Central Japan	A
P. likiangensis (Franch.) Pritz	Central China	B, C
P. maximowiczii Regel et Mast.	Central Japan	A, B.
P. omorika (Pancic) Purkyne	Serbia	A, B
P. polita (Sieb. et Zucc.) Carr.	Japan	A, B
P. sitchensis (Bong.) Carr.	Western North America	A, B
P. spinulosa (Griff.) Henry	Himalayas	B, C

[a] The rDNA repeat types are presented in Fig. 3 b
[b] *P. asperata* var. *aurantiaca* (Mast.) Boom
[c] *P. abies* var. *koraiensis* or *P. koyamai* var. *koraiensis*

B, Fig. 1) within the genome. In contrast, five species (*P. aurantiaca*, *P. glauca*, *P. engelmannii*, *P. koraiensis* and *P. koyamai*) displayed only the shorter ITS length variant (A), whereas *P. bicolor* showed the presence of only the B-type variant. In *P. jezoensis*, *P. likiangensis* and *P. spinulosa* there were two rDNA length variants within their genome: variant B and a new variant type (C). Schematic restriction maps of the rDNA length variants (A–C) observed in *Picea* species, as detected by the *Xba*I+*Dra*I digestion, are presented in Fig. 3 b. Table 3 lists the rDNA repeat types encountered in the species studied, together with the species distribution. Note that the *Picea* species found in Japan, many of which are endemic and have an extremely narrow geographical distribution, show all the rDNA repeat-type combinations (only A, only B, A+B, B+C) encountered in this study.

Discussion

The present study demonstrates that length variation in the transcribed spacers within and between species of *Picea* is common. Although there are numerous observations of IGS length variation within plant species as well as within individual plants (reviewed in Schaal and Learn 1988), there are few reported cases of ITS length variation. Within and between angiosperm species the length of the transcribed rDNA region varies little (Venkateswarlu and Nazar 1991; D'Ovidio 1992; Ritland and Straus 1993), although nucleotide divergence in ITS1 and ITS2 regions among species can be considerable (Baldwin 1992; Suh et al. 1993). In the few conifer species studied, the ITS region is longer and shows more length variation than in angiosperm species. In *Picea mariana* and *P. rubens*, multiple-size ITS1 fragments were consistently produced in amplification reactions (Bobola et al. 1992). In *P. sylvestris*, a low-copy number variant carrying a 0.4-kb deletion in the ITS1 region was identified (Karvonen and Savolainen 1993).

In the present study, we observed two ITS length variants present at high-copy numbers within individuals of *P. abies* (combination of A and B variants) and within individuals of eight additional *Picea* species (variant combinations A+B or B+C). The ITS variants A and B were

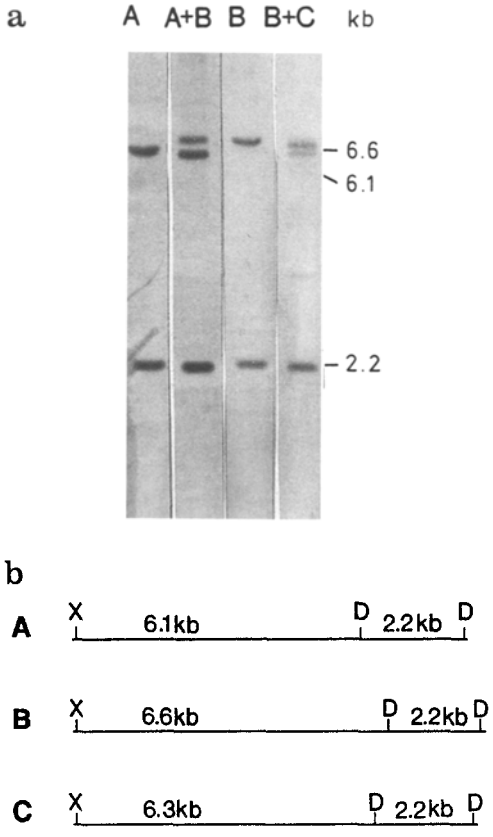

Fig. 3 a, b Length variation in the coding region of rDNA repeats between and within species of *Picea*. **a** The four observed rDNA hybridization patterns of *Picea* species, double-digested with *Xba*I and *Dra*I and hybridized with pBG35. The letters *A, B and C* refer to the rDNA length variants producing the different hybridization patterns. **b** Schematic restriction maps of the rDNA length variants

present in all surveyed individuals as well as in composite samples of *P. abies* from geographically distant locations, including Siberian samples of *P. abies* subsp. *obovata* and *fennica*. There was no indication of geographic differentiation in the distribution of ITS length variants, as all samples contained both variants. However, the proportion or copy numbers of the ITS variants within individuals from Siberian populations seemed to differ slightly from European samples. The shorter ITS variant showed more intensive hybridization signals than the longer variant, indicating a higher copy number within trees. In no individual tree of *P. abies*, either from Europe or Siberia, were the variants present as a single, fully homogenized, rDNA repeat type.

The presence of two ITS variants within individuals of *P. abies* was unexpected given the occurrence of concerted evolution in multigene families. As a result of homogenizing mechanisms associated with concerted evolution, rDNA repeats (most notably the transcribed regions involving the rRNA genes and transcribed spacers), are usually very similar within individuals and species, although differences may accumulate between species (reviewed by Hillis and Dixon 1991). Due to low levels of intraspecific variation compared to interspecific variation, rDNA is well suited for use as a phylogenetic marker. Shared polymorphisms between species may be expected only in cases of explosive radiation and/or interspecific hybridization, when the speciation rate exceeds the homogenization rate of the gene copies within a multigene family (Hillis and Davis 1988).

The presence of two distinct ITS types at high-copy numbers within individuals of *Picea* indicates that the homogenization of the transcribed rDNA regions has not been complete. Except for *P. abies*, our sampling scheme was not designed to evaluate the amount of rDNA diversity within species. However, the distribution of ITS variants among species (represented by single individuals) gives a somewhat puzzling picture, as several species show the combination of two ITS variants while in other species only one variant could be detected within the genome. The observed pattern of variation can be explained by two alternative hypotheses. Either variation in the length of the ITS region is an ancestral polymorphism characteristic of this genus, or else it is an indication of past hybridization between ancestral species (e.g. the combination of A and B results from hybridization between ancestral species having variants A and B as fully homogenized gene families). Biparental inheritance and a additivity of parental rDNA types (IGS variants) has been documented in several hybrid plant taxa (Doyle et al. 1985; Doyle and Doyle 1988; Doyle et al. 1990). However, it has not been clear whether more than one type of rDNA will be maintained in hybrids over time due to the homogenizing effects of concerted evolution and possible backcrossing with parental species (Rieseberg and Brunsfeld 1992). In the case of *Picea*, the distribution of a high number of rDNA repeats in numerous loci (Brown et al. 1993) may contribute to the slowness of homogenization in two ways. First, restoration of parental rDNA genotypes in the backcross situation at six or seven loci (the observed rDNA locus number in *P. glauca*, Brown et al. 1993) is less probable ($1/2^7$) than in angiosperm species which generally have one or two loci ($1/2^1$). Second, the homogenization mechanism may redistribute the ITS variants among loci, reducing the within-array homogeneity and the probability of getting "pure" parental rDNA genotypes in the subsequent generations after the initial hybridization. In the long run, homogenization events should result in homogeneous ITS types across all loci. The time required for complete homogenization may be long, as exemplified by the persistence of two ITS sequence variants in several species of the evolutionarily old plant taxon Winteraceae (Suh et al. 1993).

Among species of *Picea*, low levels of morphological (Wright 1955), cytogenetical (Khoshoo 1961) and chloroplast DNA divergence (Sigurgeirsson and Szmidt 1993) have been observed, and removal of geographic isolation barriers has often resulted in the production of hybrids (Wright 1955). Several morphologically distinct species have exhibited high interfertility in artificial crossing experiments (Wright 1955; Mikkola 1969). In natural populations, crossability and interspecific gene flow have been demonstrated to occur between *P. glauca* and *P. sitchensis* (Szmidt et al. 1988; Sigurgeirsson et al. 1991), as well as

between *P. sitchensis* and the *P. glauca/P. engelmannii* species complex (Sutton et al. 1991), using cpDNA polymorphisms as molecular markers.

The most complete picture of the phylogenetic relationships among species can be obtained by employing both cytoplasmic and nuclear markers. Although the evolutionary origin of the ITS length variation within and among species of *Picea* remains speculative at this stage, this rDNA polymorphism provides new useful markers for clarifying the phylogeny of, and the evolution in *Picea*. Studies are currently in progress towards sequencing the ITS length variants of *Picea*.

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