Theor Appl Genet (2005) 110: 730–741
DOI 10.1007/s00122-004-1899-y

ORIGINAL PAPER

Višnja Besendorfer · Iva Krajačić-Sokol · Srećko Jelenić
Jasna Puizina · Jelena Mlinarec · Tonka Sviben
Dražena Papeš

Two classes of 5S rDNA unit arrays of the silver fir, *Abies alba* Mill.: structure, localization and evolution

Received: 25 July 2004 / Accepted: 1 December 2004 / Published online: 19 January 2005

Abstract The structure and organization of the 5S ribosomal DNA units of the silver fir, *Abies alba* Mill., as well as their position in the chromosome complement were investigated. PCR amplification of the gene and non-transcribed spacer region, sequence analysis and Southern hybridization, using a homologous probe, detected DNA sequences of approximately 550 bp and 700 bp. Sequence analysis of the spacers revealed that the difference in length between the sequences occurred in the middle spacer region as a result of the amplification of a 75-bp sequence of the short unit class, which is organized in four 54- to 68-bp tandem repeats in the long spacer unit. The 5S rDNA transcribed region is 120 bp long and shows high sequence similarity with other gymnosperm species. The comparative analysis of 5′ and 3′ flanking sequences of 5S rRNA genes of silver fir and other gymnosperms indicates that *A. alba* spacer units have the same rate of evolution and are more closely related to *Larix* and *Pseudotsuga* than to *Pinus* and *Picea*. Southern hybridization and fluorescence in situ hybridization of metaphase chromosomes of *A. alba* suggest that the short and long spacer units are organized as separate tandem arrays at two chromosomal loci on chromosomes V and XI.

Communicated by D.B. Neale

V. Besendorfer (✉) · I. Krajačić-Sokol
S. Jelenić · J. Mlinarec · T. Sviben · D. Papeš
Department of Molecular Biology,
Faculty of Science, University of Zagreb,
Rooseveltov trg 6, 10000 Zagreb, Croatia
E-mail: vbesend@zg.biol.pmf.hr
Tel.: +385-1-4826267
Fax: +385-1-4826260

J. Puizina
Department of Biology,
Faculty of Natural Sciences and Mathematics,
University of Split, Teslina 12, 21000 Split, Croatia

Present address: J. Puizina
Gregor Mendel Institute of Molecular Plant Biology,
Austrian Academy of Science, Rennweg 14, 1030 Vienna, Austria

Introduction

Silver fir (*Abies alba* Mill.) belongs to the genus *Abies*, which together with the genera *Picea*, *Keteleeria*, *Cathaya*, *Pseudotsuga* and *Tsuga*, is grouped in the subfamily Abietoideae. Although more than 70 fir species have been described, only between 39 and 55 species are currently recognized (Liu 1971; Rushforth 1987; Farjon 1990; Vidaković 1982). Of these, six species, including silver fir, grow in Europe. Silver fir is distributed in the mountainous regions of southern Europe, in both the east and west. This species, together with *Picea abies*, is a dominant component of forests in the mountain region of central and southern Croatia. The present geographical distribution of silver fir in Europe is the result of postglacial recolonization, which began about 11,000 years ago from warm refugia in southern Europe, where the species survived the last glaciations, and reached the limits of its present range 6,000 years BP (Vendramin et al. 1999). During the last decade, the genetic structure of silver fir populations has been more precisely studied in order to obtain the geographical pattern of genetic variation due to the genetic differentiation in the glacial refugia. The results obtained from chloroplast DNA and mitochondrial DNA (mtDNA) provide evidence that even a species with a very long lifespan and heavy pollen grains, such as *A. alba*, was able to establish highly efficient pollen-mediated gene flow between refugia. On the basis of the maternal inheritance of mtDNA, Liepelt et al. (2002) identified Croatia as an introgression zone between eastern and western *A. alba* lineages. Contact between these lineages was established approximately 7,000–7,500 years ago. In comparison with other gymnosperm species, little is known about the structure and organization of the *A. alba* nuclear genome and its genetic divergence.

The ribosomal 5S rRNA and 18S–5.8S–26S rRNA (18S rRNA) multigene families are the most widely used gene families in the determination of phylogenetic rela-

tionships between plant and animal species. In plants, as in most higher eukaryotes, 5S rRNA genes are organized in tandem repeats and the number of repeats varies from less than 1,000 to over 75,000 (Campell et al.1992; Sastri et al. 1992). 5S rDNA arrays are located at one or more chromosomal loci, which in most species are separated from 18S rDNA loci. 5S rRNA tandem units consist of the 5S rRNA gene and an intergenic, nontranscribed spacer (NTS). The 120-bp 5S rRNA gene sequence is highly conserved across species, while the NTS region exhibits variation in base composition and length (ranging from 100 bp to over 800 bp) from species to species. The high level of conservation of the 5S rRNA gene is associated with the precise function of 5S rRNA as a component of the large ribosomal subunit in all eukaryotic organisms. Some regions of the gene are more conserved than others, which are explained by the regulation of 5S rRNA transcription. In eukaryotic cells, the transcription of the 5S rRNA gene depends on the binding of transcription factor TFIIIA to DNA sequences Box A and Box C located within the gene (Szymanski et al. 1998). Alterations in the DNA sequence within either box as well as changes in the distance between boxes can prevent or reduce transcription (Hayes and Tullius 1992). Studies on the structure of 5S rRNA arrays indicate that the region 10–40 bp upstream of the gene also has a regulatory function (Scoles et al. 1988, Gottlob-McHugh et al. 1990).

Because the 5S rRNA genes are highly conserved, their sequence analysis is used for inferring phylogenetic relationships among deep branches of eukaryotes (Soltis et al. 1998). The spacer region is more informative for the study of phylogenetic relationships at the interspecific and intergeneric levels due to the faster rate of divergence in comparison to the highly conserved coding region. Differences in spacer regions appear as a result of duplication and/or deletion accumulated mostly in the middle spacer region (Scoles et al. 1988; Sastri et al. 1992).

Multiple copies of 5S rRNA gene families, as other multigene families, can undergo concerted evolution due to homogenizing forces that lead to the high level of identity of all gene copies within the species. Differences in the sequences are the result of normal levels of divergence between the orthologous genes in different species. Molecular processes that lead to concerted evolution are unequal crossing-over, gene conversion and replication slippage, but the rate at which the variant repeat types become homogenized depends upon other factors such as the number of repeats in an array, intensity of natural selection and effective population size (Smith 1976; Basten and Ohta 1992; Schlötterer and Tautz 1994).

In contrast to angiosperms, the structure and organization of the 5S rDNA locus has only been characterized in a few gymnosperm species in genera: *Pinus* (Van de Peer et al. 1990; Mashkova et al. 1990; Gorman et al. 1992; Moran et al. 1992; Liu et al. 2003b), *Larix* (Trontin et al. 1999), *Picea* (Brown and Carlson 1997) and *Pseudotsuga* (Amarashinge and Carlson 1998). In all the species investigated, the 5S rDNA repeats have a conserved 120-bp transcribed region and an NTS that varies not only in size (from 101 bp in *Picea glauca* to 880 bp in *Pseudotsuga menziesii*) but also in the number of different size classes. In Asian pines, the length of the NTS varies from 382 bp to 401 bp in *P. bungeana* and from 538 bp to 608 bp in four diploxylon pines (Liu et al. 2003b). Intragenomic and interspecific heterogeneity in five Asian pine species has been explained to be a result of the action of 'stronger' concerted evolution following the diversification of the two subgenera, *Pinus* and *Strobus*, and very 'weak' concerted evolution following the speciation of four diploxylon pines (Liu et al. 2003b). In *Pinus radiata*, the 5S rRNA genes occur in two size classes of about 525 bp and 850 bp in length (Gorman et al. 1992; Moran et al. 1992). Moran et al. (1992) have found that all 11 species in the subgenus *Strobus* have only a short 5S rDNA size class. The same study showed that New World species of the subgenus *Pinus* have both short and long size classes, whereas Old World species only have the longer size class. Two highly divergent size classes of 5S rDNA were identified in larch species (Trontin et al. 1999) and in *P. glauca* (Brown and Carslon 1997).

Chromosomal localization and physical mapping of 5S rDNA loci in most gymnosperm species investigated so far revealed a single 5S rDNA locus (Doudrick et al. 1995; Lubaretz et al. 1996; Brown and Carlson 1997; Amarasinge and Carlson 1998; Hizume 1999; Siljak-Yakovlev et al. 2002; Liu et al. 2003a). In some *Pinus* species, one to three loci were detected (Liu et al. 2003b), whereas in *P. radiata*, Gorman et al. (1992) found 5S rRNA genes spread on all chromosomes. There is no information on the chromosomal distribution of the two size classes of 5S rDNA in *Larix decidua* and *Larix kaempferi* (Trontin et al. 1999). One 5S rDNA locus detected in the chromosome complement of *L. decidua* by Lubaretz et al. (1996), however, suggests that both size classes may be present at the same locus. Sequence heterogeneity among repeats within individual arrays (loci) is probably the case in *P. glauca*, in which Brown and Carlson (1997) also found only one 5S rDNA locus. These results demonstrate that concerted evolution forces in those species were not strong enough to overcome the forces generating variation.

In this paper, the 5S rDNA unit structure and organization in *A. alba* are described in terms of amplification patterns, nucleotide sequence, composition of tandem arrays and position of 5S rRNA genes in the chromosome complement.

Materials and methods

Plant material and DNA isolation

The analyses of 5S rDNA were performed on two *A. alba* Mill. genotypes (DD1 and DD2) collected from the

Donja Dobra population in the Gorski Kotar region (central part of Croatia). Total DNA was extracted from young needles, using the cetyltrimethyl ammonium bromide method described by Doyle and Doyle (1987). For chromosome preparations, silver fir seedlings were provided by the Forestry Research Institute 'Jastrebarsko'.

Polymerase chain reaction amplification of 5S rDNA, cloning and sequencing

Polymerase chain reaction (PCR) amplification was carried out in a 25-µl reaction containing 5–10 ng silver fir DNA, 0.4 µM of primers P3 and P4 or primers P2 and P5, 200 µM dNTPs, 10 mM Tris-HCL (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$ and 2 U *Taq* DNA Polymerase (Fermentas). Two primers, P3 (5′-GAGTTCTGATGGGATC CGGTG-3′) and P4 (5′-CGCTTGGGCTAGAGCAG-TAC-3′), used by Brown and Carlson (1997) for 5S rDNA amplification in *P. glauca*, were chosen for amplification of the entire spacer region and partial amplification of the 5S rRNA gene (Fig. 1a). Primer P2 (5′-GGGTGCAA-CACTAGGACTTC-3′), described by Brown and Carlson (1997), and primer P5 (5′-ACGGTGCGTAGAT CCCCTCGG-3′), designed according to the conserved part of the nontranscribed spacer of the short spacer class of *A. alba* 5S rDNA, were used for the amplification of the entire 5S rRNA gene (Fig.1a). After an initial denaturing step at 94°C for 3 min, 20 amplification cycles were performed on a Gene Amp PCR System 2400 (Applied Biosystems) thermal cycler, each consisting of denaturation at 94°C for 1 min, annealing at 58°C for 10 s and primer extension at 72°C for 30 s. Amplification was ended by a final extension step at 72°C for 10 min. Amplified fragments were separated on 1% (w/v) agarose gels. For cloning purposes, the amplified fragments were gel-purified using a DNA Extraction Kit (Fermentas) and cloned into the pCR 2.1 plasmid (TA Cloning Kit, Invitrogen) according to the manufacturer's instructions. Chemically competent *Escherichia coli* TOP10 cells (Invitrogen) were transformed, and plasmid extractions from recombinant clones were performed using a Qiagen Miniprep Kit. Dideoxy chain-terminating sequencing reactions were carried out by VBC-Genomics Bioscience Research (Vienna, Austria).

Southern hybridization

Genomic DNA (1–2 µg) was digested overnight at 37°C with 10–20 U/µg of the restriction enzyme *Sca*I (Fermentas), separated by electrophoresis on a horizontal agarose gel (1% w/v) and transferred to a nylon membrane (Amersham Biosciences) by capillary blot. The probe, a 694-bp fragment corresponding to the longer spacer class of *A. alba*, was cut out of clone IKS702, using the enzyme *Eco*RI (Fermentas), gel-purified using a QIAEX II purification kit (Qiagen) and radioactively labelled using [α−^{32}P]dATP and a Klenow fragment (Fermentas). The labelled probe was

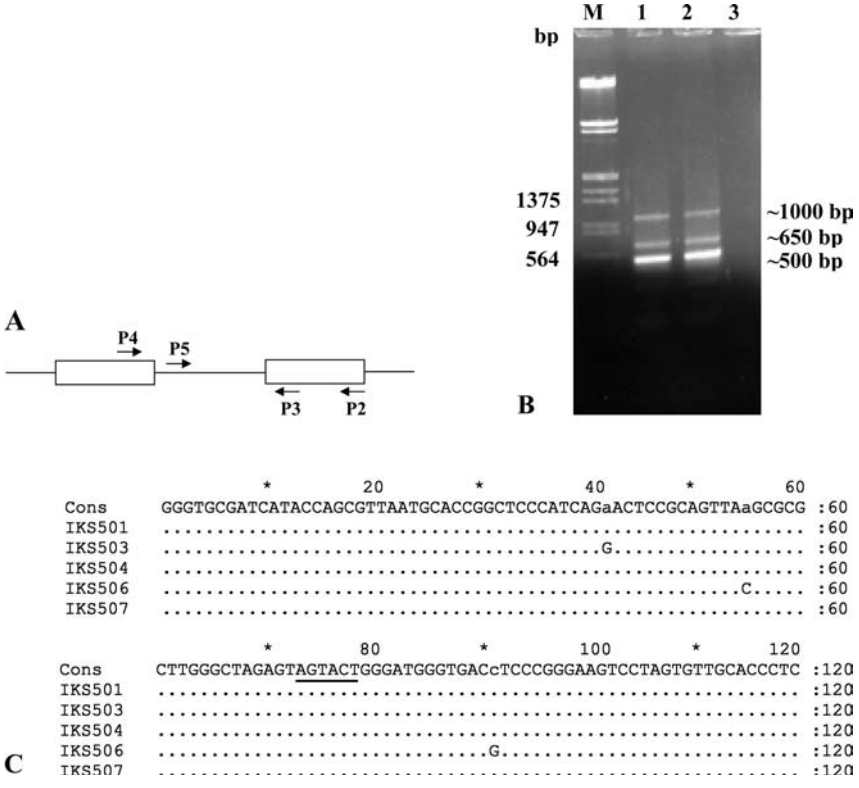

Fig. 1 Position of primer pairs, polymerase chain reaction (PCR) amplification pattern and sequence comparison of *Abies alba* 5S rRNA gene clones. **a** Schematic drawing shows position and orientation of the two primer pairs, P3/P4 and P2/P5, used for the amplification of *A. alba* 5S rDNA repeat units. The 5S rRNA genes are shown as *boxes* and the nontrascribed spacer (NTS) as a *line*. **b** Products of PCR amplification of the 5S rRNA gene together with the middle and upstream spacer region with the P2/P5 primer pair separated by agarose gel electrophoresis. *Lane M* λDNA *Hin*dIII/*Eco*RV marker, *lanes 1* and *2 A. alba*, *lane 3* negative control (no DNA template). **c** Sequence comparison of five *A. alba* 5S rRNA gene clones. The recognition sequence of the *Sca*I restriction enzyme used in Southern hybridization analysis is *underlined*. *Periods* represent identical residues

purified using a nucleotide removal kit (Qiagen). Hybridization was carried out at 65°C overnight in 0.25 M phosphate buffer, 7% (w/v) SDS and 0.1% (w/v) BSA. The blot was washed twice at 65°C for 10 min in 2× SSC, 0.1% SDS and once for 20 min in 1× SSC, 0.1% SDS. Blots were autoradiographed and recorded using Molecular Imager FX and Quantity One software (Bio-Rad).

Chromosome preparation and 5S rDNA in situ hybridization

Root tips were obtained from seedlings germinated on moist filter paper at 25°C in a thermostat germinator after 1 month of stratification at 4°C. Following pretreatment with 0.05% (w/v) colchicine (Sigma) for 16–20 h at room temperature, roots were fixed in 3:1 (v/v) ethanol-acetic acid at 4°C for 24–48 h and stored in 70% (v/v) ethanol. Root tips were rinsed with 0.01 M citrate buffer and digested with a mixture of 2.5% (w/v) cellulase (Calbiochem) and 2.5% (w/v) pectolyase (Sigma) in 0.01 M citrate buffer for 1–2 h at 37°C or in an enzyme solution containing 1% (w/v) pectolyase Y23 (Seishin Corporation), 4% (w/v) cellulase R10 (Onozuka) and 4% (w/v) hemicellulase (Sigma) in 0.05 M citrate buffer at 37°C for 1–1.5 h. Root tips were macerated and squashed in 45% (v/v) acetic acid.

The position and number of 5S rDNA sites were determined by fluorescence in situ hybridization (FISH), following Liu et al. (2003a), with slight modifications. Clone IKS702 (Table 1), which contained a 694-bp 5S rDNA fragment of *A. alba*, was used as a probe and labelled with digoxigenin-11-dUTP (DIG-dUTP) (Roche) in a PCR according to Schwarzacher and Heslop-Harrison (2000). Prior to hybridization, the chromosome preparations were treated with Pepsin (10 μg/ml, Sigma) or Proteinase K (100 μg/ml, Sigma) and fixed with 4% paraformaldehyde. Fifty microlitres of hybridization mixture containing the DNA probe at a final concentration of 40–60 ng/ml in 50% (v/v) formamide, 10% (w/v) dextran sulphate, 0.1% (w/v) sodium-dodecyl sulphate, 250 μg/ml salmon sperm and 2× SSC buffer was added to each slide and denaturated at 85°C for 15 min. After overnight hybridization at 37°C, slides were washed stringently at 42°C for 10 min in 40% formamide in 0.1× SSC, followed by subsequent washing in 2× SSC and 4× SSC/Tween 20. After incubation with anti-DIG-FITC (Roche), slides were washed three times for 8 min in 4× SSC/Tween 20 at 40°C. Slides were counterstained with DAPI (2 μg/ml) and mounted in antifade solution (AF1 citifluor). For the simultaneous localization of both 5S and 18S rDNA, an additional probe was used. A 2.4-kb *Hin*dIII fragment (entire 18S rDNA) from *Cucurbita pepo* cloned into pUC19 (Toress-Ruiz and Hemleben 1994) was labelled with Cy3-dCTP (Amersham Biosciences) by nick translation according to the manufacturer's protocol. Preparations were examined under an Olympus BX51 microscope with an appropriate filter set. Hybridization signals were imaged by the highly sensitive digital camera (Olympus DP70), and images were merged using Adobe Photoshop, version 6.0.

Sequence alignment and phenetic analysis

Silver fir 5S rDNA sequences were subjected to a similarity search against the nonredundant nucleotide sequence database, using the National Centre for Biotechnology Information BLAST network service. Sequence alignments were performed by using the Clustal X program and edited by hand. For phenetic analysis, multiple sequence alignments were done using the BioEdit program. Phenetic analyses were carried out on the alignment data, using PAUP, version 4.0 (Swofford 1998).

Results

PCR amplification and sequence analysis of the 5S rRNA gene

Primers P2 and P5 were used for PCR amplification of the 5S rRNA gene. Three fragments of approximately 500, 650 and 1,000 bp, consisting of the entire 5S rRNA gene as well as upstream and middle spacer regions of the NTS were obtained (Fig. 1b). Two fragments of 500 bp and 650 bp in length were expected according to

Table 1 Accession number, length of the entire 5S rDNA and nontranscribed spacer (NTS), G + C content and pairwise comparison of the short (IKS560, IKS561, IKS565) and long (IKS700, IKS702) unit clones sequenced in *Abies alba*

Accession no.	Length[a] (bp)	G + C content[a] (%)	Clone	Similarity score[a] (%)				
				IKS560	IKS561	IKS565	IKS700	IKS702
AY830934	557/437	59.3/59.5	IKS560	100				
AY830935	552/431	58.7/58.9	IKS561	91/89	100			
AY830936	548/428	58.6//58.5	IKS565	90/88	98/97	100		
AY830937	705/585	56.3/56.1	IKS700	63/57	63/56	62/55	100	
AY830938	706/586	56.5/56.3	IKS702	63/57	63/56	62/55	100/100	100

[a]Values for entire clone sequence/values for NTS region

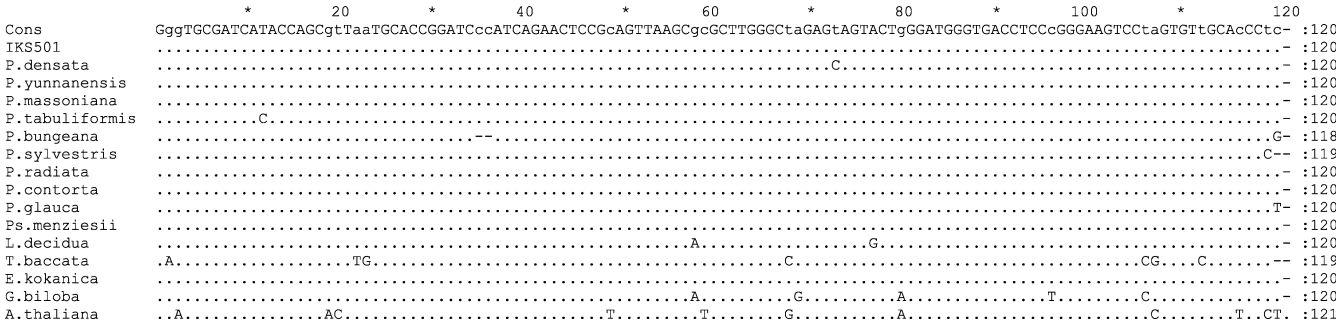

Fig. 2 Sequence comparison of the *A. alba* 5S rRNA gene clones with the 14 gymnosperm 5S rRNA genes published to date. The clones with the highest match scores in the BLAST search and those from the 5S ribosomal RNA database (Szymanski et al. 1998; http://rose.man.poznan.pl/5Sdata/5SRNA.html) were compared with the silver fir clone IKS501. Only 21 variable nucleotide positions with 17 substitutions were found. *Periods* represent identical residues to those of the *A. alba* clone IKS501 120-bp sequence, and *dashes* indicate alignment gaps

the position of the P5 primer in the short and long spacer classes. The fragment of approximately 1,000 bp probably occurred as a consequence of unspecific primer annealing. A 500-bp fragment was cloned into the pCR 2.1 cloning vector, and five clones were chosen for further analysis. All clones were obtained from silver fir genotype DD2.

The alignment of 5S rRNA gene sequences of five clones (IKS501, IKS503, IKS504, IKS506 and IKS507) obtained by amplification with primers P2 and P5, as well as five clones (IKS560, IKS561, IKS565, IKS700 and IKS702) obtained by amplification with primers P3 and P4, confirmed a conserved 120-bp coding region, beginning with GGG and ending with CTC (Fig. 1c). This is in agreement with the previously identified 5S rRNA genes in other gymnosperm species (Van de Peer et al. 1990; Gorman et al. 1992; Moran et al. 1992; Brown and Carlson 1997; Liu et al. 2003b). The sequence similarity among clones was 98–100%. Three clones (IKS501, IKS504 and IKS507) were identical, while in clones IKS503 and IKS506, one and two substitutions were observed, respectively (Fig. 1c). The sequence of the silver fir 5S rRNA gene (clone IKS501) was compared with 14 previously sequenced gymnosperm 5S rRNA genes (Fig. 2). The clones with the highest match scores in the BLAST search and those from the 5S ribosomal RNA database (Szymanski et al. 1998; http://rose.man.poznan.pl/5Sdata/5SRNA.html) were compared with the silver fir clone. In order to compare the sequence similarity of the 5S rRNA genes between gymnosperm and angiosperm species, *Arabidopsis thaliana* was included. The sequence comparison revealed high sequence identity among gymnosperm species (from 93% identity with *Taxus baccata* to 100% identity with some *Pinus* species, *Picea glauca*, *Pseudotsuga menziesii* and *Ephedra kokanika*) with only 21 variable positions, of which 17 were substitutions (Fig. 2). Most substitutions were transitions (88%). A high similarity score (86–91%) was also recorded between gymnosperm species and *A. thaliana*.

PCR amplification and sequence analysis of 5S rDNA NTS

In order to amplify the intergenic NTS, the primer pair P3 and P4 was used. PCR amplification produced two fragment classes, a prominent one of approximately 550 bp and a faint one of approximately 700 bp (Fig. 4c). The 550- and 700-bp fragments consisted of an entire repeating unit, with the exception of 12 bp between the 5′ ends of P3 and P4 primers, as was expected according to the results of Brown and Carlson (1997) obtained in *P. glauca* with the same primer pair. Three clones containing the short spacer class (IKS560, IKS561 and IKS565) and two clones with the long spacer class (IKS700 and IKS702) were sequenced. Clone IKS560 originated from the DD1 genotype, whereas the remaining four clones originated from the DD2 genotype.

The accession number of the clones (AY830934–AY830938); the entire sequence length derived from the 5S rRNA gene sequence of the five clones IKS500–IKS507 and the NTS clones IKS560, IKS561, IKS565, IKS700 and IKS702: the length of NTS; G + C content; and pairwise similarity scores are shown in Table 1. After correction for the 5S rRNA gene sequence, the length of the NTS sequences was measured to be 427 bp in clone IKS565, 431 bp in clone IKS561 and 437 bp in clone IKS560, while in clones IKS700 and IKS702, the NTS length was 585 bp and 586 bp, respectively. The difference in G + C content between the two spacer classes was low, varying from 56.1% in clone IKS700 to 59.5% in IKS560, and the values are similar to those found in other gymnosperm species (Liu et al. 2003b; Trontin et al. 1999).

Within each size class, alignment was straightforward and pairwise comparison resulted in high similarity scores. However, lower sequence similarity, 62% and 63% in the entire 5S rDNA sequences and 55–57% in the NTS, between short and long unit classes was recorded. The number of variable sites was found to be higher between short unit clones obtained from different silver fir genotypes (88 and 89%). Between clones

IKS561 and IKS565, obtained from the DD2 genotype, only five substitutions were present, while in clone IKS560, obtained from the DD1 genotype, 41 substitutions with a trasition/trasversion ratio of 19/22 were observed in comparison to DD2 genotype clones IKS561 and IKS565 (data not shown). In addition, some short deletions and duplications were also present. In two short unit class clones, IKS561 and IKS565, as well as in both long unit class clones, IKS700 and IKS702 obtained from the same silver fir genotype, the 4-bp alignment gap was observed at position −204 (−1) bp upstream of the 5′ end of rRNA gene (Fig. 3b). This gap probably occurred as a consequence of CGCA sequence duplication in clone IKS560. In clone IKS565, another 4-bp alignment gap appeared at the end of GA-rich repeat motifs (Fig. 3b). Such repeating motifs could be involved in slippage and looping out of the parental strand during replication (Scoles et al. 1988) and, therefore, the 4-bp gap in this clone could be considered as a deletion.

The alignment of the two unit classes showed that deletions/duplications and base pair changes appeared more frequently in the middle spacer, closer to the upstream region of the 5S rRNA gene (Fig. 3a, b). Small 3-bp and 5- to 6-bp gaps were present upstream at positions −175 (−1) bp and −123 (−1) bp in the GA-rich regions of the short unit class and could be accounted for as deletions. Three of the largest gaps in the long unit class were observed downstream at position 15 (−1) (11-bp gap) and upstream at positions −37 (−1) (8-bp gap) and −79 (−1) (10-bp gap) (Fig. 3a, b) and could also be accounted for as deletions. These deletions probably did not disturb the function of regions involved in the transcriptional regulation of the 5S rRNA genes. In the upstream region, the gaps appeared after the AT- and GA-rich putative regulatory elements, sequences which were highly conserved in all clones. The same situation was observed downstream of the 5S rRNA gene, where a T-rich element involved in termination of replication was preserved.

Diagrammatic representation of the two unit classes (Fig. 4a) shows that the length difference occurred as a consequence of the four tandemly arranged repeated elements (A, B, C and D) located in the middle region of the long unit class. Sequence comparison showed a 59–73% similarity of these elements, with the 75-bp sequence located in the middle spacer region of the short unit class (Fig. 4a, b). When compared with the 75-bp sequence of the short unit class, elements A and D had the highest similarity score (67% and 73%, respectively). Generally, the sequence comparison demonstrated that these elements have accumulated mutations such as deletions and base pair changes, but a 25-bp long sequence upstream of the 3′ end seems to be more preserved than other parts of the repeated sequences (Fig. 4b, underlined).

Encouraged by results obtained by Trontin et al. (1999) in larch species, we compared the silver fir short unit class with the short unit class of radiata pine. Surprisingly, we found 100 bp of a mostly TC-rich middle spacer sequence at position 135, downstream of the radiata pine 5S rRNA gene, 66% similar to the sequence at position 105/106, downstream of the 5S rRNA gene in the short unit class of silver fir (Fig. 5). This 100-bp silver fir–radiata pine common sequence was interrupted by a 20-bp insertion in silver fir clones. Moreover, a 75-bp sequence of the silver fir short unit class that was amplified in the long class clones is part of a 100-bp sequence of radiata pine shown in Fig. 5 (underlined). Immediately downstream of this region, GA-rich sequences were found as a characteristic of silver fir short and long unit classes (Fig. 3b).

The organization of 5S rDNA units

In order to confirm two distinct classes of 5S rDNA in genomic DNA of silver fir, we performed Southern hybridization, using a 694-bp fragment from the longer unit class of clone IKS702 as a probe. Different amounts of genomic DNA was digested with the *Sca*I restriction enzyme, for which there is a single recognition site within the 5S rDNA gene region in *A. alba*. Whereas partial digestion with *Sca*I produced a ladder-like pattern of hybridization fragments (data not shown) characteristic of DNA sequences organized in tandem arrays, complete digestion resulted in two strongly labelled bands of approximately 550 bp and 700 bp (Fig. 4d, lane 1). Additional weak bands of approximately 1,100 bp and 1,400 bp were obtained when larger amounts of DNA were digested (Fig. 4d, arrows in lanes 2 and 3). These bands correspond to dimers of the respective repeat units, and the double-ladder pattern suggests that the two 5S rDNA units are organized tandemly at separate loci in the genome. The difference in signal strength between the two unit arrays suggests that the short spacer units are more abundant than the long ones in the silver fir genome.

Chromosomal localization of the 5S rDNA sites

The chromosome complement of silver fir consists of 24 metacentric and submetacentric chromosomes. The corresponding haploid chromosome complements are shown in Fig. 6a and b. The chromosomal location of 5S rDNA was determined by FISH with digoxigenin-labelled probe, clone IKS702, and detected with anti-DIG-FITC. The green fluorescent signals were found on two chromosome pairs (Fig. 6b). One 5S rDNA locus was located on the long arm, close to the secondary constriction, of metacentric chromosome pair V, and the second had an intercalary position on submetacentric chromosome pair XI (Fig. 6b). The double FISH with both 5S and 18S rDNA probes confirmed that the 5S rDNA locus on chromosome pair V is positioned close to the 18S rDNA locus

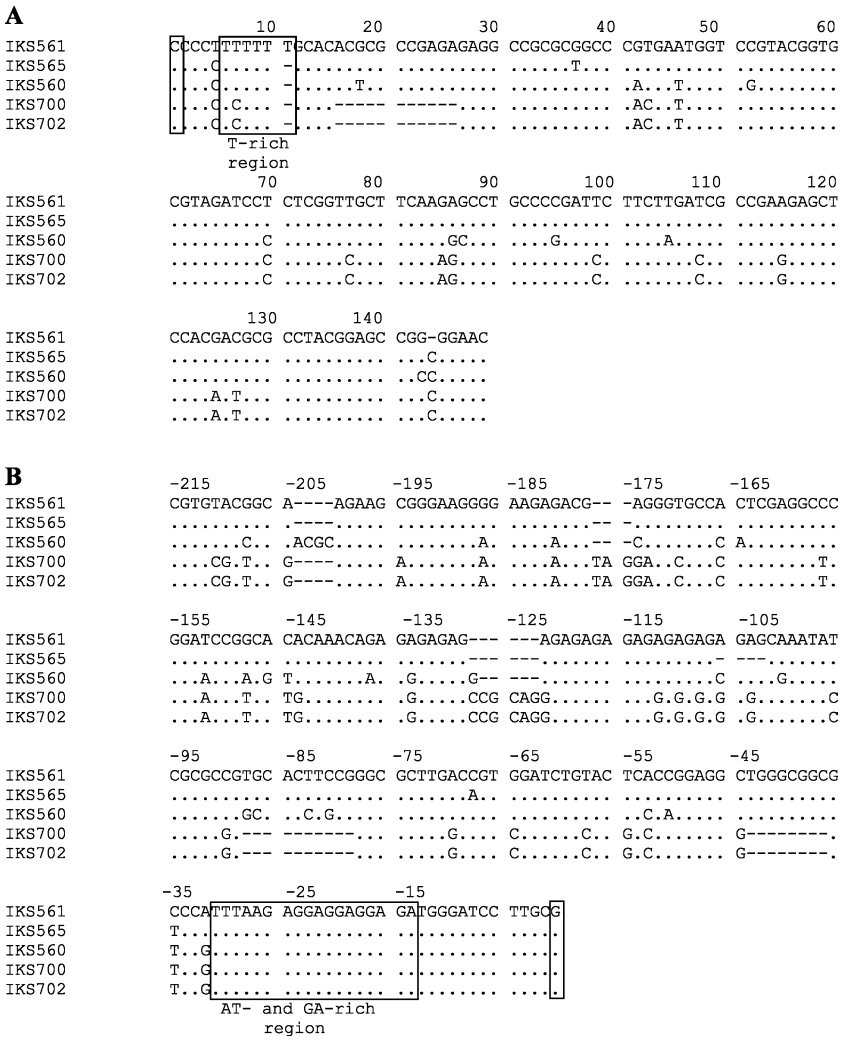

Fig. 3 Comparison of the **a** downstream (149-bp) and **b** upstream (215-bp) spacer regions of silver fir short (IKS560, IKS561 and IKS565) and long (IKS700 and IKS702) unit classes. The nucleotides are numbered relative to the 5S rRNA gene region. *Periods* represent residues identical to those of the IKS561 short unit class clone sequence. The putative regulatory elements involved in the regulation of gene expression (AT- and GC-rich region upstream of the 5′ end of the 5S rRNA gene) and the termination of transcription (T-rich region downstream of the 3′ end of the 5S rRNA gene) as well as the 5′ and 3′ ends of the 5S rRNA gene are *boxed*. Note the higher variability of the upstream region visible as the number of deletions/insertions and substitutions increases in comparison to the downstream region. The largest deletions appeared after and before regulatory elements

(Fig. 6e). This facilitates distinguishing between the two nonhomologous 5S rDNA loci in interphase nuclei (Fig. 6d). We also noticed that 5S rDNA nonhomologous loci are usually found at separate locations in interphase cells, which may suggest a lack of interlocus exchange (Fig. 6d). In most of the analysed prophase and metaphase chromosomes (Figs. 6c and e), differences in FITC signal strength within and among loci were observed and could indicate an unequal content of 5S rDNA repeating units.

Sequence comparison with other gymnosperm species 5S rDNA spacers

The short and long unit clone sequences of *A. alba* were compared with seven gymnosperm species of the subfamilies Abietoideae (*Picea glauca* and *Pseudotsuga menziesii*), Laricoideae (*Larix decidua*) and Pinoideae (*Pinus massoniana*, *P. densata*, *P. tabuliformis* and *P. yunnanensis*). Due to the high diversity of the spacer regions among the selected species, we found that only 5′ and 3′ flanking sequences of the 5S rRNA gene could be used for phylogenetic analysis. We therefore compared 78-bp sequences of the upstream and downstream spacer regions that were unambiguously aligned without gaps, except for those characteristics of the silver fir long spacer class. Parsimony analysis as implemented in PAUP, version 4.0 (Swofford 1998), was used to infer phylogenies based on nucleotide substitutions in aligned sequences. Parsimony analysis generated an unrooted tree with a tree length of 88, a constancy index of 0.80 and a retention index of 0.85. The phenetic tree was obtained on the basis of 37 parsimony informative characters and as expected, showed that both the short and long spacers of *A. alba* fall into one group (Fig. 7). The phenetic tree also showed the clustering of investigated spacer sequences. *P. menziesii* and *L. decidua* form one cluster, and *Pinus* species form another more distant one, while silver fir clones were grouped separately but closely to *Larix* and *Pseudotsuga*. The position of *P. glauca* in the phenetic tree showed that this species is closer to the *Pinus* group than to *A. alba*.

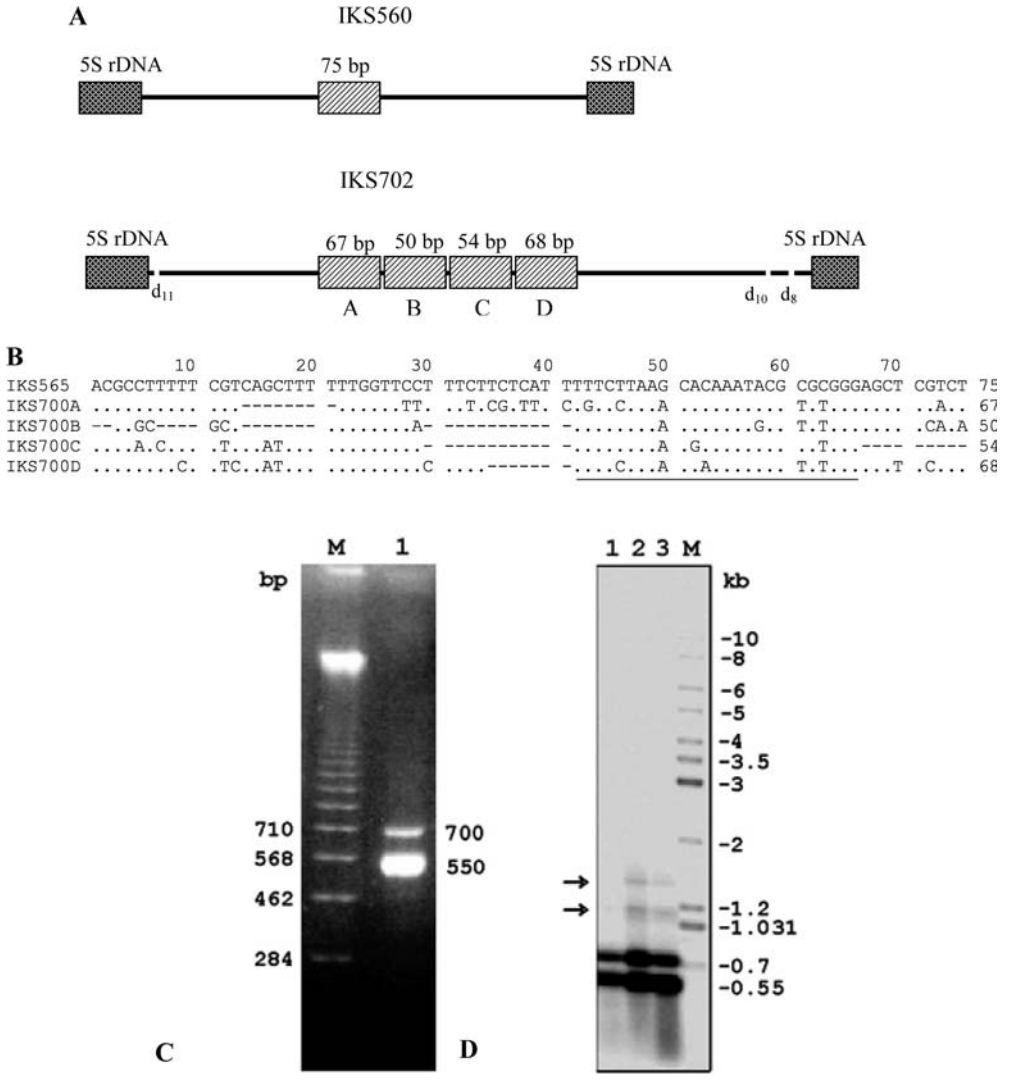

Fig. 4 Diagrammatic representation of the structure of *A. alba* 5S rDNA short and long unit classes, their PCR amplification and Southern hybridization pattern. **a** Diagrammatic representation shows the largest deletions (d_{11}, d_{10} and d_8) within the long unit class as interruptions, and the 75-bp element in the middle spacer region of the short unit class and tandemly repeated elements (A, B, C and D) in the middle spacer region of the long unit class that occurred as duplications of the 75-bp element of the short unit class (*labelled boxes*). **b** Sequence comparison of the tandemly duplicated elements identified in the long unit class showed differences between elements, which occurred as a consequence of deletions and base pair changes. The most conserved region in all sequences is *underlined*. **c** PCR-amplified short (550 bp) and long (700 bp) unit classes (entire NTS and part of the 5S rRNA gene) with primer pair P3/P4. *Lane M Tenebrio molitor* 142-bp DNA ladder. *Lane 1 A. alba*. **d** Two unit classes revealed by Southern hybridization. Different amounts of genomic DNA were digested with the *Sca*I restriction enzyme and hybridized with a [^{32}P]-labelled longer spacer fragment derived from clone IKS702. Complete DNA digestion revealed two strongly labelled bands of approximately 550 bp and 700 bp that represent short and long unit classes, respectively. Two bands of approximately 1,100 bp and 1,400 bp, which are indicated by *arrows*, correspond to the dimers of the

Discussion

The 5S rRNA gene

PCR amplification and sequencing of the coding region of the 5S rDNA repeat unit of *A. alba* showed that it is 120 bp, beginning with GGG and ending with CTC. A high level of identity (93–100%) was found when these sequences were compared with the corresponding sequences reported for other gymnosperm species (Hori et al. 1985; Melekhovets et al. 1988; Mashkova et al. 1990; Van de Peer et al. 1990; Gorman et al. 1992; Moran et al. 1992; Brown and Carlson 1997; Liu et al. 2003b). In this highly conserved region, the rare mutations observed were mostly base pair transitions. Sequence comparison between gymnosperms and an angiosperm species (*A. thaliana*) also revealed the significant level of conservation of the 5S rDNA coding

```
                      10         20         30         40         50         60
IKS561     106 GAGCTCCACG ACGCGCCTAC GGAGCCGG-G GAACTTACGC CTTTTTCGTC AGCTTTTTTG :60
IKS565     105 .......... .......... .......... .......... .......... .......... :60
IKS560     105 .......... .......... .......CC. .......... .......T.. .AT....... :60
P.radiata  135 .......... CA.....GT. ..T.G.C.C. .......... .......T.. TC.C.C.C.C :60

                      70         80         90        100        110        120
IKS561     166 GTTCCTTTCT TCTCTTTTTT CTTAAGCACA AATACGCGCG GGAGCTCGTC TCCCCCTCCG :120
IKS565     165 .......... ....A..... .......... .......... .......... .......... :120
IKS560     165 .......... .T...G...C .......... ......T... .......C.. .......... :120
P.radiata  195 ...TTC..TC .TC....CC. T..C------ ---------- ----.C.C.T CGTT.T.... :120
```

Fig. 5 Sequence comparison of 120-bp sequences identified in the middle spacer region of silver fir and radiata pine short unit classes. The positions in each spacer are indicated in *italics*. The 75-bp sequence of the short unit class that amplified in the silver fir long unit class is *underlined*. Note the 20-bp insertion characteristic of silver fir clones

sequence (86–91% identity, Fig. 2) as well as preserved intragenic control regions (Box A and Box C). The high level of transitions versus transversions suggests that homogenizing forces were acting strongly across the 5S rDNA transcribing region, thus preserving the ribosome function.

Upstream and downstream spacer regions flanking the 5S rRNA gene involved in regulation and termination of transcription were found to be conserved among the investigated *A. alba* clones. In gymnosperm species, two putative AT- and GA-rich elements upstream of the 5S gene are recognized to be involved in the unwinding of DNA and the transcriptional regulation of the 5S gene (Gorman et al. 1992; Brown and Carlson 1997; Trontin et al. 1999). The upstream regulatory elements recognized as pentanucleotide, TTTAA/TTAAA, and hexanucleotide, AGGAGG/CCTCCT, core sequences in both the short and long spacer class of silver fir located at position 14–29 are slightly different from those described in *P. glauca* (Brown and Carlson 1997), *Larix* (Trontin et al. 1999) and *Pinus* species (Gorman et al. 1992; Moran et al. 1992; Liu et al. 2003b). As these elements are highly conserved among the clones investigated, it seems that such changes did not disturb their regulatory function. The T-rich region located 7–11 bp downstream of the gene, which functions as an RNA

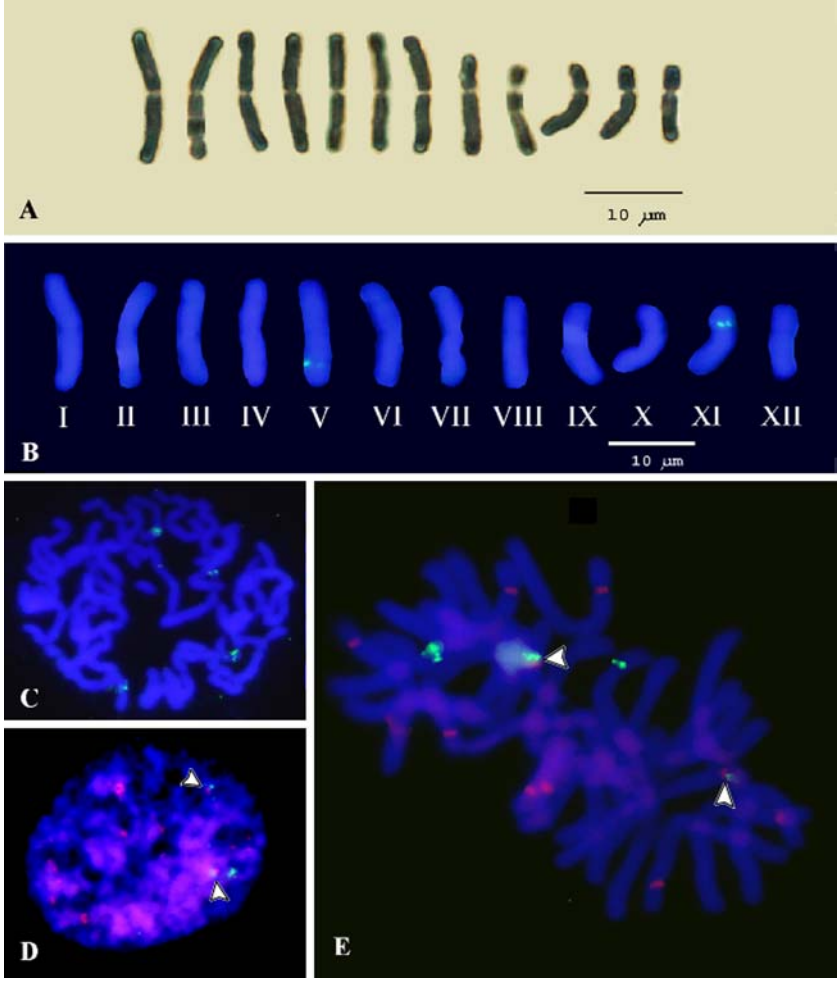

Fig. 6 Haploid chromosome complement of 12 metacentric and submetacentric chromosomes of *A. alba*. **a** Haploid karyogram obtained under the phase-contrast microscope and **b** the same karyogram after fluorescence in situ hybridization (FISH) with digoxigenin-labelled probe IKS702, detected with anti-DIG-FITC and counterstained with DAPI. The *green fluorescent signals* represent the 5S rRNA loci located on the long arm, near the secondary constriction of metacentric chromosome V and on the short arm of submetacentric chromosome XI. **c** Prophase with four green fluorescent signals. **d**, **e** Double FISH with 5S rDNA (*green signals*) and 18S rDNA (*red signals*) probes revealed the proximity of the 5S rDNA loci on chromosome V to 18S rDNA in interphase (*arrowheads*) and metaphase (*arrowheads*). Differences in the FITC signal strength indicate different amounts of 5S rDNA units within and among loci

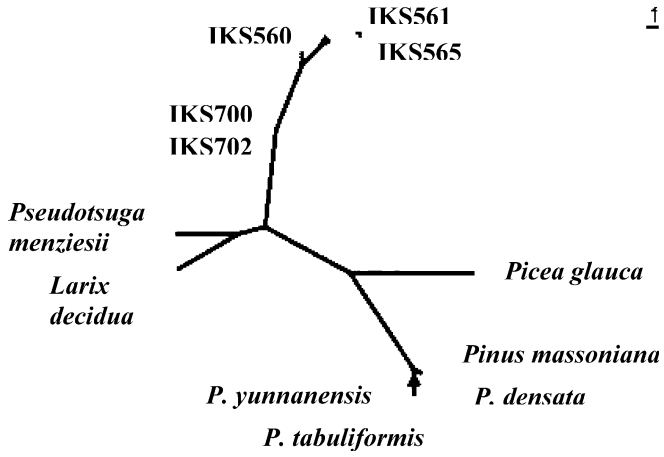

Fig. 7 Unrooted phenetic tree (parsimony tree: tree length 88, constancy index = 0.8, retention index = 0.85) generated from assembled upstream and downstream 78-bp unambiguously aligned sequences of 5S rDNA of six gymnosperm species taken from the National Centre for Biotechnology Information data base and Amarasinghe and Carlson (1998), and *A. alba* clones IKS560, IKS561, IKS565, IKS700 and IKS702

polymerase III termination signal, was also conserved among the clones, with only one transition (T → C) mutation observed in the long unit class sequence.

5S rDNA NTS

PCR amplification, sequence analysis and Southern hybridization revealed two size classes of 5S rDNA spacers in the *A. alba* genome: a short spacer consisting of 427–437 bp and a long spacer of 585–586 bp. The presence of two size classes of 5S rDNA spacers seems to be a common feature in Pinaceae, since two size classes were revealed in species of the subgenus *Pinus* (Gorman et al. 1992; Moran et al. 1992), *P. glauca* (Brown and Carlson 1997), *L. decidua* and *L. kaempferi* (Trontin et al. 1999). Scoles et al. (1988) and Sastri et al. (1992) clearly showed that duplications and deletions are the main sources of 5S rDNA unit size variations. In *L. decidua*, the difference in short and long spacer classes appeared as a consequence of duplication events that occurred in the middle spacer region, close to both the upstream and downstream spacer regions. In *P. radiata*, for example, the difference is mainly due to a 330-bp insertion in the middle spacer region (Moran et al. 1992). In contrast to *Larix* species, in which two spacer classes are highly divergent, parsimonious analysis in silver fir showed that both unit classes have the same rate of evolution. The length difference between the short and long spacer regions of *A. alba* occurred due to the amplification of four 54–68 bp tandem repeats (A, B, C and D, see Fig. 3a) in the long spacer class. A high similarity score (59–73%) was obtained when these four tandem elements were compared to the 75-bp sequence of the short spacer class (see Fig. 3a, b). Interestingly, this 75-bp sequence characteristic of both the short and long spacer classes of silver fir also forms part of a 100-bp silver fir–radiata pine common element. These 100-bp common elements, recognized as TC-rich regions, of the short spacer classes of silver fir and radiata pine showed 66% identity. A 20-bp insertion (part of the 75-bp sequence), characteristic of silver fir, into the 100-bp silver fir–radiata pine common element suggests that this change appeared after and/or during differentiation of the two species. This could provide additional evidence for our interpretation of the evolution of the long spacer class from the short one. In addition, it is interesting that larch and radiata pine also share a 63–71 bp common element (Trontin et al. 1999), but this element is located upstream of the TC-rich element common to radiata pine and silver fir. On the basis of this, it seems that larch, silver fir and radiata pine share ancestral sequence of a hypothetical common ancestor.

The chromosomal location of the 5S rDNA repeat units

FISH of *A. alba* metaphase chromosomes, using the long unit spacer class as a probe, revealed two loci of 5S rDNA positioned on chromosomes V and XI. The question is whether each of these loci is composed of a single 5S rDNA unit class or whether composite tandem arrays of both unit classes are present at both loci. FISH could not fully answer this question due to the high sequence similarity between the two spacer classes. However, the position and behaviour of 5S rDNA loci in interphase nuclei is very informative and may indicate whether interlocus exchanges between the two 5S rRNA loci take place (Cronn et al. 1996; Fulneček et al. 2002; Shibata and Hizume 2002). Interlocus gene conversion as a mechanism for interlocus homogenization is recognized in species where multigene families are terminally located and associated during interphase, when they are decondensed and, therefore, active. Such homogenization is a common feature of 18S–26S rDNA, i.e. nucleolar organizer regions (Cronn et al 1996; Volkov et al. 1999) that often associate during interphase. The intercalary position of both 5S rDNA loci in the silver fir karyotype as well as the absence of any associations among the loci during interphase, as shown by FISH, suggest that interlocus recombination does not occur in silver fir. Using FISH, different unit size classes of 5S rDNA loci positioned on different chromosomes were shown not to associate in interphase nuclei in *Nicotiana* and *Allium* species (Fulneček et al. 2002; Shibata and Hizume 2002).

Regarding the position of the two 5S rDNA repeat units in the silver fir genome, at least two different scenarios of chromosomal evolution could be proposed. The first scenario, in which both 5S rDNA repeat units were together in the composite tandem arrays and present at both chromosomal 5S rDNA loci, would imply the primary existence of only one chromosomal locus inside which duplication and deletions gradually

led to the evolution of the long spacer unit. Translocation or transposition of this composite tandem array to another chromosome could have led to the establishment of the second chromosomal locus in the silver fir genome. This scenario is, however, not very likely as Southern hybridization did not reveal such a composite 5S rDNA unit.

The second scenario implies the existence of two chromosomal loci each bearing short 5S rDNA unit size arrays at the beginning. If this were the case, duplications and deletions probably have occurred in one 5S rDNA locus, leading to the evolution of the long unit classes, which were accumulated due to weak selection forces and fixed through generations. We could not, however, exclude the presence of a small amount of short spacer class in the long spacer's locus, despite the fact that Southern hybridization did not detect composite tandem arrays.

The Southern hybridization results, which confirmed the presence of two 5S rDNA unit classes as well as a double-ladder pattern and no composite tandem arrays, the position of 5S rDNA loci on chromosomes and their behaviour in interphase, which indicates a lack of interlocus exchange, rather support the second scenario, suggesting that the long and the short 5S rDNA units are organized tandemly at separate chromosomal loci. This interpretation also supports our hypothesis that short spacer units are more ancient in origin or, in other words, that the long spacer units were derived from the short ones. In addition, the preservation of control regions upstream and downstream of the 5S rDNA gene in long spacer units, despite large deletions occurring in this region, indicates that 5S rDNA genes are active at both loci.

Phylogeny of *A. alba* and related gymnosperms

The phylogenetic position of silver fir among other gymnosperm species in our study was based on the comparative sequence analysis of conserved upstream and downstream (78-bp) sequences of the 5S rRNA genes. The middle spacer region appeared to be too variable and unsuitable for sequence comparison. Phenetic analysis indicates that both silver fir unit classes have the same rate of evolution. The genetic distance between the short and long unit classes occurred, because the long unit class sequences containing deletions were included in the phylogenetic analysis. Although we are aware that regions containing deletions and insertions cannot be used for the accurate estimation of genetic distance and phylogenetic reconstruction, using standard algorithms, we included such sequences in our calculation in order to avoid the loss of valuable information, such as deletions characteristic of the silver fir long unit class. An unrooted phenetic tree, even when short sequences were used, clearly showed that silver fir is genetically distant from all the species included in this study, but is more closely related to *Larix* and *Pseudotsuga* than to *Pinus* and *Picea*. Our results partially agree with those obtained by Wang et al. (2000), based on the nuclear, chloroplast and mitochondrial sequence data of the Pinaceae family. Wang et al. (2000) showed that *Abies* is more closely related to *Cedrus*, *Keteleeria*, *Pseudolarix* and *Tsuga*, which form the Cédrées group, than to *Larix*, *Picea*, *Pinus* and *Pseudotsuga*, which belong to the Pinées group as classified by the number and position of resin canals in the central vascular cylinder of the young taproot. The central position of *Abies*, *Larix* and *Pseudotsuga* species in the phylogenetic trees obtained by Wang et al. (2000), however, suggests that these species are more related than *Picea* and *Pinus*, which is in agreement with our results. Unfortunately, studies of 5S rDNA sequences to date have only been performed on a limited number of gymnosperms, which prevents the extension of this phylogenetic study based on 5S rDNA spacer sequence to other species belonging to different subfamilies.

A more detailed study of 5S rDNA unit structure and organization has to be extended to other species in order to elucidate the evolution of species not only of the genus *Abies* but also in the subfamily Abietoideae. Preliminary results obtained by PCR amplification show the presence of both, short and long spacer units of similar size in other fir species.

Acknowledgements The authors thank M. Gradečki from the Forestry Research Institute 'Jasrebarsko' for preparing the plant material used in the cytogenetic study and K. Vlahoviček for sequence analysis and suggestions in the interpretation of the phylogeny results. This work was supported by research grant no. 119112 from the Ministry of Science, Education and Sport of the Republic of Croatia. Jasna Puizina acknowledges the support of the Gregor Mendel Institute, Vienna, Austria.

References

Amarasinghe V, Carlson JE (1998) Physical mapping and characterization of 5S rRNA genes in Douglas-fir. J Hered 89:495–500

Basten CJ, Ohta T (1992) Simulation study of a multigene family, with special reference to the evolution of compensatory advantageous mutations. Genetics 132:247–252

Brown GR, Carlson JE (1997) Molecular cytogenetics of the genes encoding 18S–5.8S–26S rRNA and 5S rRNA in two species of spruce (*Picea*). Theor Appl Genet 95:1–9

Campell BR, Song Y, Posch TE, Cullis CA, Town CD (1992) Sequence and organization of 5S ribosomal RNA-encoding genes of *Arabidopsis thaliana*. Gene 112:225–228

Cronn RC, Zhao X, Paterson AH, Wendel JF (1996) Polymorphism and concerted evolution in a tandemly repeated gene family: 5S ribosomal DNA in diploid and allopolyploid cottons. J Mol Evol 42:685–705

Doudrick RL, Heslop-Harrison JS, Nelson CD, Schmidt T, Nance WL, Schwarzacher T (1995) Karyotype of slash pine (*Pinus elliottii* var. *elliottii*) using patterns of fluorescence in situ hybridization and fluorochrome banding. J Hered 86:289–296

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Farjon A (1990) Pinaceae. Koelltz Scientific, Königstein, p 340

Fulnaček J, Lim KY, Leitch AR, Kovařik A, Matyášek R (2002) Evolution and structure of 5S rDNA loci in allotetraploid *Nicotiana tabacum* and its putative parental species. Heredity 88:19–25

Gorman SW, Teasdale RD, Cullis CA (1992) Structure and organization of the 5S rRNA genes (5S DNA) in *Pinus radiata* (Pinaceae). Plant Syst Evol 183:223–234

Gottlob-McHugh SG, Lévesque M, MakKenzie K, Olson M, Yarosh O, Johnson DA (1990) Organization of the 5S rRNA genes in the soybean *Glycine max* (L.) Merill and conservation of the 5S rDNA repeat structure in higher plants. Genome 33:486–494

Hayes JJ, Tullius TD (1992) Structure of the TF-IIIA-5S DNA complex. J Mol Biol 227:407–417

Hizume M, Shibata F, Kondo K, Hoshi Y, Kondo T, Ge S, Yang QE, Hong DY (1999) Identification of chromosomes in two Chinese spruce species by multicolor fluorescence in situ hybridization. Chrom Sci 3:37–41

Hori H, Lim B-L, Osawa S (1985) Evolution of green plants as deduced from 5S rRNA sequences. Proc Natl Acad Sci USA 82:820–823

Liepelt S, Bialozyt R, Ziegenhagen B (2002) Wind-dispersed pollen mediates postglacial gene flow among refugia. Proc Nat Acad Sci USA 99:14590–14594

Liu T-S (1971) A monograph of the genus *Abies*. National Taiwan University, Taipei, p 608

Liu Z-L, Zhang D, Hong D-Y, Wang X-R (2003a) Chromosomal localization of 5S and 18S–25S ribosomal DNA sites in five Asian pines using fluorescent in situ hybridization. Theor Appl Genet 106:198–204

Liu Z-L, Zhang D, Wang X-Q, Ma X-F, Wang X-R (2003b) Intragenomic and interspecific 5S rDNA sequence variation in five Asian pines. Am J Bot 90(1):17–24

Lubaretz O, Fuchs J, Ahne R, Meister A, Schubert I (1996) Karyotyping of tree *Pinaceae* species via fluorescent in situ hybridization and computer-aided chromosome analysis. Theor Appl Genet 92:411–416

Mashkova TD, Barciszewska MZ, Joachimiak A, Nalaskowska M, Barciszewski J (1990) Molecular evolution of plants as deduced from changes in free energy of 5S ribosomal RNAs. Int J Biol Macromol 12:247–250

Melekhovets YF, Troitsky AV, Valiejo-Roman KM, Bobrova VK, Antonov AS (1988) Nucleotide sequences of cytosolic 5S ribosomal RNAs from two gymnosperms, *Gnetum gnemon* and *Ephedra kokanica*. Nucleic Acid Res 16:4155

Moran GF, Smith D, Bell JC, Appels R (1992) The 5S RNA genes in *Pinus radiata* and the spacer region as a probe for relationships between *Pinus* species. Plant Syst Evol 183:209–221

Rushforth KD (1987) Conifers. Facts on File, New York, p 232

Sastri DC, Hilu K, Appels R, Lagudah ES, Playford J, Baum BR (1992) An overview of evolution in plant 5S DNA. Plant Syst Evol 183:169–181

Schlötterer C, Tautz D (1994) Chromosomal homogeneity of Drosophila ribosomal DNA arrays suggests intrachromosomal exchanges drive concerted evolution. Curr Biol 4:777–783

Schwarzacher T, Heslop-Harrison JS (2000) Practical in situ hybridization. Bios Scientific, Oxford, p 203

Scoles GJ, Gill BS, Xin Z-Y, Clarke BC, McIntyre CL, Chapman C, Appels R (1988) Frequent duplication and deletion events in the 5S RNA genes and the associated space regions of the Triticae. Plant Syst Evol 160:105–122

Shibata F, Hizume M (2002) Evolution of 5S rDNA units and their chromosomal localization in *Allium cepa* and *Allium schoenoprasum* revealed by microdissection and FISH. Theor Appl Genet 105:167–172

Siljak-Yakovlev S, Cerbah M, Coulaud J, Stoian V, Brown SC, Zoldoš V, Jelenić S, Papeš D (2002) Nuclear DNA content, base composition, heterochromatin and rDNA in *Picea omorika* and *Picea abies*. Theor Appl Genet 104:505–512

Smith GP (1976) Evolution of repeated DNA sequences by unequal crossover. Science 191:528–535

Soltis DE, Soltis PS, Doyle JJ (1998) Molecular systematics of plants: DNA sequencing. Kluwer, Boston, p 574

Swofford DL (1998) PAUP. Phylogenetic analysis using parsimony (*and or other methods). Version 4.0. Sinauer, Sunderland

Szymanski M, Specht T, Barciszewska MZ, Barciszewski J, Erdmann VA (1998) 5S rRNA data bank. Nucleic Acid Res 26:156–159

Toress-Ruiz RA, Hemleben V (1994) Pattern and degree of methylation in ribosomal RNA genes of *Cucurbita pepo* L. Plant Mol Biol 26:1167–1179

Trontin J-F, Grandemange C, Favre J-M (1999) Two highly divergent 5S rDNA unite size classes occur in composite tandem array in European larch (*Larix decidua* Mill.) and Japanese larch [*Larix kaempferi* (Lamb.) Carr]. Genome 42:837–848

Van de Peer Y, De Baere R, Cauwenbergh J, De Watcher R (1990) Evolution of green plants and their relationship with other photosynthetic eukaryotes as deduced from 5S ribosomal RNA sequences. Plant Syst Evol 170:85–96

Vendramin GG, Degen B, Petit RJ, Anzidei M, Madaghiele A, Ziegenhagen B (1999) High level of variation at *Abies alba* chloroplast microsatellite loci in Europe. Mol Ecol 8:1117–1126

Vidaković M (1982) Četinjače. Sveučilišna naklada Liber, Zagreb, p 710

Volkov RA, Borisjuk NV, Panchuk II, Schweizer D, Hemleben V (1999) Elimination and rearrangement of parental rDNA in the allotetraploid *Nicotiana tabacum*. Mol Biol Evol 16:311–320

Wang X-Q, Tank DC, Sang T (2000) Phylogeny and divergence times in Pinaceae: evidence from three genomes. Mol Biol Evol 17:773–781