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Molecular cytogenetics of the genes encoding 18*s*-5.8*s*-26*s* rRNA and 5*s* rRNA in two species of spruce (*Picea*)

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Abstract Molecular cytogenetics is a convenient tool to investigate the organization and evolution of plant genomes. In coniferous trees of the Pinaceae, cytogenetic data is rudimentary since individual chromosomes are difficult to distinguish and karyotypes of related species are poorly differentiated. We determined the chromosomal locations of ribosomal RNA genes in white spruce (*Picea glauca*) and Sitka spruce (*Picea sitchensis*) using fluorescence in situ hybridization. The biotin-labeled DNA probes consisted of the 5*s* ribosomal DNA (rDNA) amplified from white spruce using the polymerase chain reaction and a heterologous 18*s*-5.8*s*-26*s* rDNA sequence. The 5*s* rDNA was present only on chromosome 5 at a single locus and near to an 18*s*-5.8*s*-26*s* rDNA locus in both species. Additional 18*s*-5.8*s*-26*s* rDNA loci were found at interstitial sites on six and four chromosomes of white and Sitka spruce, respectively, providing potentially useful interspecific differences. Progress in karyotyping both species is presented. A molecular analysis of 5*s* rDNA of white spruce revealed the presence of two classes of repeating units, one of 221 bp corresponding to the PCR amplification product, and another of approximately 600 bp. The nucleotide sequence and copy number of the 221-bp class is reported.

Key words *Picea* · 18*s*-5.8*s*-26*s* rDNA · 5*s* rDNA · In situ hybridization

Introduction

Developments in non-isotopic in situ hybridization (ISH) and the physical mapping of repeated DNAs have provided a valuable tool in chromosome identification and in studies of genome organization, chromosome evolution, cytotaxonomy, introgression, and the spatial arrangement of interphase chromatin (for a review, see Jiang and Gill 1994). Gymnosperm plants stand to benefit from the application of ISH methods since karyotypes are remarkably conserved among gymnosperms in comparison to angiosperms (Sax and Sax 1933; Khoshoo 1961). The vast majority of the 210 species in the Pinaceae have diploid chromosome numbers of 2n = 24, most with long metacentric chromosomes of similar size. Since chromosome-banding methods, mainly C-banding, have been developed for only a few conifer species, i.e., species of the pine (*Pinus*) genus (MacPherson and Filion 1981), the lack of chromosome differentiation in the Pinaceae has precluded the karyotyping of most species as well as of any contribution of cytogenetics to forest-tree improvement and genome analysis.

Physical mapping by ISH of the tandem repeated genes encoding the 18*s*-5.8*s*-26*s* ribosomal RNA (18*s*-26*s* rDNA) and 5*s* ribosomal RNA (5*s* rDNA) has provided a number of useful chromosome markers in coniferous trees. In spruce and pine species, the 18*s*-26*s* rDNA has been mapped on 6–8 chromosome pairs (Hizume et al. 1992; Brown et al. 1993; Karvonen et al. 1993; Doudrick et al. 1995; Lubaretz et al. 1996) in comparison to the 1–4 loci typically found in angiosperms. Brown et al. (1993) reported on the preliminary karyotype of white spruce [*Picea glauca* (Moench) Voss] based on the distribution of its seven 18*s*-26*s* rDNA sites. Later, Doudrick et al. (1995) constructed a karyotype of slash pine (*Pinus elliotti* Engelm.) based on eight 18*s*-26*s* rDNA loci, three 5*s* rDNA loci, and fluorochrome banding.

The 5*s* rRNA is a component of all ribosomes except in the mitochondria of certain species. In all higher eukaryotes, 5*s* rRNA is transcribed from hundreds to thousands of genes, each of approximately 120 nucleotides. The 5*s* genes are organized in tandem arrays with

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each coding sequence separated from adjacent ones by a nontranscribed spacer. In plants, non-transcribed spacers of 95–730 bp have been characterized (Hembleden and Werts 1988; Moran et al. 1992) and both sequence and length variation within a species have been documented. ISH of 5*s* sequences in numerous angiosperms, including wheat, rye, barley, pea, tomato and rice (Appels et al. 1980; Ellis et al. 1988; Lapitan et al. 1991; Song and Gustafson 1993; Leitch and Heslop-Harrison 1993), has shown that the tandem arrays are present at one to several chromosome locations, with as many as four sites identified in barley. These loci are usually well separated from the genes encoding the 18*s*-26*s* rRNA, frequently being found on different chromosomes at telomeric or intercalary sites, or less often near the centromere.

The present study is part of an ongoing genome mapping effort in white spruce, a dominant component of the ecology and economy of British Columbia's forests, and the subject of both classical and molecular breeding programs. The 5*s* rDNA structure in white spruce was investigated and its chromosomal location determined by fluorescence ISH (FISH) in this and a related North American species, Sitka spruce [*Picea sitchensis* (Bong.) Carr.]. Progress in constructing molecular karyotypes of both species, based on the chromosomal distribution of the 5*s* rDNA and 18*s*-26*s* rDNAs, is also presented.

Materials and methods

Plant material and DNA isolation

Needles and open-pollinated seeds of white spruce were provided by Gyula Kiss, Kalamalka Research Station, Vernon, B.C. Open-pollinated seeds from a Sitka spruce seed-production orchard were obtained from Yousry El-Kassaby of Pacific Forest Products, Ltd., Saanichton, B.C. DNA was extracted from needles according to Wagner et al. (1987) with modifications. Following the addition of sarkosyl to 1% (w/v), the solution was mixed gently and left at room temperature for 20 min. One gram of CsCl per ml was added and the genomic DNA purified by two rounds of CsCl ultracentrifugation.

PCR amplification and cloning

PCR amplification was carried out in a 50-µl reaction containing 5 ng of white spruce DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.2 mM dNTPs, 1 µM primer pair, 2 mM $MgCl_2$ and 1.25 U AmpliTaq DNA Polymerase (Perkin-Elmer/Cetus). 5*s* rDNA primers were chosen on the basis of seven angiosperm sequences reported in Goldsbrough et al. (1982) and included (P1: 5′-GGGTGCGATCA TACCAGCGT-3′ and P2: 5′-GGGTGCAACACTAGGACTTC-3′) and (P3: 5′-GAGTTCTGATGGGATCCGGTG-3′ and P4: 5′-CGCTTGGGCTAGAGCAGTAC-3′). Reactions were initially denatured in a Perkin-Elmer/Cetus DNA Thermal Cycler at 94°C for 3 min, then subjected to 20 cycles of 94°C for 1 min and 55°C for 10 s, and completed by a 10-min 72°C final extension. Reactions were resolved in 2% agarose gels and 1 × TAE, and the products to be cloned were excised and gel purified. Following re-amplification under identical cycling conditions, the reaction mixture was cloned into the *Eco*RV site of pBluescript KS^+ (Stratagene) using the dideoxy T-tailing method (Holton and Graham 1991).

DNA analysis and copy number estimation

Aliquots of genomic DNA (1 µg) were partially digested using *Sca*I at 37°C for 1 h. The enzyme/DNA ratio was varied from 5 U/µg to 0.005 U/µg by serial dilution. Reactions were stopped by adding EDTA to 25 mM, resolved in 1.5% agarose gels and 0.5 × TBE, and then alkali blotted to Hybond N^+ nylon membranes (Amersham). The pWS11 insert, a PCR product amplified using primers P3/P4, was excised from the vector, gel-purified and labeled using ^{32}P-dCTP and a random primer labeling kit (Boehringer-Mannheim). Hybridization was performed at 65°C overnight in 6 × SSC, 20 µg/ml sheared, denatured salmon sperm DNA, 5 × Denhardt's and 0.5% SDS. Membranes were subsequently washed in 2 × SSC for 15 min, 0.2 × SSC at 65°C for 30 min and twice in 0.1 × SSC at 65°C for 30 min before autoradiography. DNA sequencing of both strands of PCR clones was performed by the dideoxy chain-termination method using the T7 Sequencing Kit (Pharmacia) and the T3 and T7 promotor primers.

To estimate 5*s* rDNA copy number, dilutions of pWS11 corresponding to the expected weights of 100, 500, 1000, 2000, 5000, 7500, 10 000 and 20 000 copies were denatured and immobilized on a nylon membrane using a slot blotting apparatus (BIO-RAD). Equivalent molar amounts of pBluescript, as a control for cross-hybridization to vector sequences, and 0.5- and 1.0-µg aliquots of white spruce DNA were also applied. Two replicates of each filter set were prepared. After probing using the pWS11 insert, membranes were washed at high stringency (as above) and the resulting autoradiograms analyzed by densitometry.

Chromosome preparation, probe labeling, and FISH

Seeds of Sitka spruce were stratified for 3 weeks at 4°C to promote uniform germination. This treatment was not necessary for the white spruce seeds. Seeds of both species were germinated in the dark on water-saturated filter papers at 25°C for 6 days. The seedlings were submerged in 0.2% (w/v) aqueous colchicine for 6 h at room temperature and fixed overnight at 4°C in 3 : 1 ethanol : acetic acid. Root tips were rinsed in 0.01 M sodium citrate/citric acid buffer and digested in 2% cellulase (Calbiochem), 1% macerase (Calbiochem) and 2% liquid pectinase (Sigma) for 1 h at 37°C. After squashing in 45% acetic acid, coverslips were removed and the slides were washed in ethanol for 5 min and air dried. Prior to FISH, chromosomes were stained with 0.2 µg/ml of 4′,6-diamidino-2-phenylindole (DAPI) in phosphate-buffered saline, pH 7.4, visualized using a Zeiss UV filter block, and photographed on TMAX 400 film. Chromosomes were de-stained in 3 : 1 methanol : acetic acid for 30 min at room temperature, rinsed three times in methanol and air dried.

pGmR1, supplied by Elizabeth Zimmer, contains a 7.9-kb *Eco*R1 fragment of the 18*s*-26*s* rDNA of soybean [*Glycine max* (L.) Merr.] cloned in pBR325 (Zimmer et al. 1988). pWS11 and pGmR1 were labeled using biotin-14-dATP and a nick translation kit (GibcoBRL). Un-incorporated nucleotides were removed by ethanol precipitation.

FISH was performed following the procedures outlined in Leitch et al. (1994) with several modifications. Paraformaldehyde fixation of chromosome preparations following pepsin treatment was omitted. Hybridization was carried out overnight at 37°C in an Omnigene Hybaid Temperature Cycler programmed according to Heslop-Harrison et al. (1991). Following hybridization, chromosome preparations were washed twice in 50% formamide/2 × SSC for 5 min at 42°C, 2 × SSC for 5 min at 42°C, and 2 × SSC for 5 min at room temperature. Biotin-labeled probes were detected using

FITC-ExtrAvidin (Sigma) diluted 1 : 100 in detection buffer (Leitch et al. 1994). Finally, chromosomal DNA was counterstained for 3–5 s in 0.1 μg/ml of propidium iodide, rinsed in 2 × SSC, and mounted in 50% glycerol in PBS.

In situ hybridized chromosomes were imaged using a BIO-RAD MRC 600 confocal laser scanning system equipped with an argon/krypton laser. FITC and propidium iodide were excited simultaneously using 488-nm and 538-nm laser lines and the resulting green and red emissions were separated to the two photomultipliers using BHS and YHS filter blocks. Images were merged using Adobe Photoshop 3.0 software on a Macintosh Quadra 840 personal computer. After acquiring 5*s* rDNA hybridization images, chromosome preparations were stripped as described by Heslop-Harrison et al. (1992) and re-probed using biotin-labeled pGmR1.

Karyotype analysis

Photographs of DAPI-stained metaphases were digitized using an AGFA Studioscan II flatbed scanner. Chromosomes were randomly numbered 1–24 and the total lengths and the lengths of the short arm (p) and long arm (q) were measured using NIH Image software (developed at the US National Institutes of Health and available on Internet at http://rsb.info.nih.gov/nih-image/). The arm ratio (q/p) was calculated and homologous pairs were identified based on these measurements and the FISH sites of 5*s* rDNA and 18*s*-26*s* rDNA in five well-spread metaphases of each species, and then validated using the hybridization patterns from more than 20 additional cells. Chromosome pairs were ordered from longest to shortest based on the relative length of each pair expressed as a percentage of the diploid cell complement.

Results

Characterization of 5*s* rDNA in white spruce

The 5*s* rDNA was amplified from white spruce genomic DNA using PCR. Two primer pairs based on angiosperm 5*s* coding sequences were designed to provide amplification products from which the sequence of the complete repeating unit could be obtained: primers P1/P2 to amplify the 120-bp coding region only, and primers P3/P4 to amplify the entire repeating unit with the exception of a 12-bp region between the 5′ends of P3 and P4. PCR amplification using P1/P2 produced the expected 120-bp fragment visualized in ethidium bromide-stained agarose gels, while primers P3/P4 gave a major fragment of approximately 210 bp and lesser amounts of 430- and 650-bp fragments (data not shown). Re-amplification of the 430- or 650-bp product produced the 210-bp fragment, consistent with these being dimers and trimers, respectively, of a 5*s* rDNA monomer.

The DNA sequences of two clones from each primer pair were aligned to generate the 221-bp 5*s* rDNA sequence of white spruce shown in Fig. 1 a. By comparison to known plant 5*s* genes, the 5′end of the coding region was identified as GGG (nucleotide +1). Although the 3′end can only be determined with certainty by RNA sequencing, for the purpose of analysis we have assumed the white spruce 5*s* gene to be 120 nucleotides long and terminating with CTT, with the remaining 101 bp comprising the non-transcribed spacer. The predicted 5*s* gene sequence in white spruce could be folded into the generalized secondary structure model proposed for plant 5*s* rRNAs described in Barciszewska et al. (1994).

The 5*s* rDNA sequences of white spruce and radiata pine (*Pinus radiata* D. Don, Moran et al. 1992) were compared. Sequences of the coding region were identical with the exception of nucleotide 120 which in spruce is T and in radiata pine is C. Sequence homology

Fig. 1 a 5*s* rDNA sequence of white spruce derived from two clones of each primer pair. Sequences of primer pairs are given in the Material and methods. Nucleotides in *bold* represent the predicted gene sequence. The recognition sequence of *Sca*I used in Southern analysis is shown. Nucleotides between the 5′ ends of P3 and P4 (*underlined*) are not found in pWS11 used as the Southern and in situ hybridization probe. GenBank accession number: U63826. **b** Comparison of putative regulatory elements found upstream of the 5*s* rDNA transcription intiation site. Sequences of radiata pine (*Pinus radiata*) and a representative angiosperm, yellow lupin (*Lupinus luteus*), were taken from Moran et al. (1992) and Rafalski et al. (1982), respectively. Nucleotides −1 to −50 are shown and correspond to white spruce nucleotides 171–221 in **a**. *Lower case* letters indicate nucleotide substitutions and *dashes* denote nucleotide identity. Putative regulatory elements identified in angiosperms referred to in the Discussion are *underlined* in the lupin sequence

a

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                                                                    60
GGGTGCGATCATACCAGCGTTAATGCACCGGATCCCATCAGAACTCCGCAGTTAAGCGCG

                  ScaI                                              120
CTTGGGCTAGAGTAGTACTGGGATGGGTGACCTCCCGGGAAGTCCTAGTGTTGCACCCTT

                                                                    180
CCCCCCTTTTGCATGGCTCCGCGATGGATCGGGGCGCTTTTAAGCCCTCCCCCGCGGCTC

                                                          221
GGTGATGTCCATTCAAGAGGGGGAGGGGGCCTGATCCTTGC
```

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          -50          -40          -30          -20          -10
White spruce   CCGCGGCTCGGTGATGTCCATTCAAGAGGGGGAGGGGGCCTGATCCTTGC
Radiata pine   g--t-------c-g-c---c--g----------------ga----t----
Lupin          gacaa-ac----a--tagggc-t-tagcattagatc-cgcgt-ca-----
```

between these coniferous species extended into the non-transcribed spacer where nucleotides −1 to −50 were 80% conserved (Fig. 1 b). In contrast, only 32% identity from nucleotide −1 to −50 was found between white spruce and an angiosperm (lupin).

Figure 2 shows the Southern hybridization of pWS11, a P3/P4 clone, to white spruce genomic DNA following partial digestion with *Sca*I. This restriction enzyme has a single recognition site within the 5*s* rDNA coding sequence and is expected to generate a ladder of hybridizing fragments characteristic of DNA sequences organized in tandem arrays. Our interpretation of the hybridization pattern is that two 5*s* rDNA size classes are present in white spruce. Five clearly resolved multimers of the 221-bp 5*s* rDNA repeat, designated array A, were observed among the partial digest products in lane 2. In nearly complete digests (lane 3), most array A members were digested to the monomer form. An additional prominent ladder of fragments based on an approximately 600-bp repeating unit (array B) was observed in lane 3. Only faint hybridization to multimers of array B was observed in lane 2 where most higher-molecular-weight partial-digest fragments were not clearly resolved. A wide range of PCR reaction and cycling conditions was tested to amplify array-B repeating units but failed to produce the corresponding products. Fragments of array A and array B accounted for 43% and 55%, respectively, of the total signal in lane 3 of Fig. 2, as determined by densitometry. Additional hybridization was seen to a 1.9-kb incomplete digestion product and a fragment of approximately 1.0 kb. The latter fragment was not a multimer of either array A or B and could represent DNA flanking the 5*s* array.

The copy number of the 5*s* genes in white spruce was estimated by slot-blot hybridization experiments to be 1170 ±82 copies per haploid genome (data not shown) based on a haploid genome size of 8.5×10^9 bp (Dhillon 1987). This value underestimates 5*s* rDNA copy number given that chloroplast and mitochondrial DNA sequences were not excluded from preparations of genomic DNA.

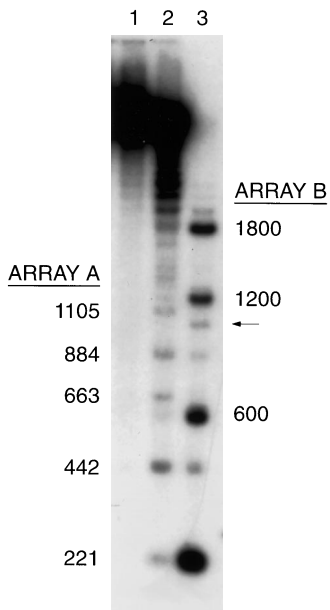


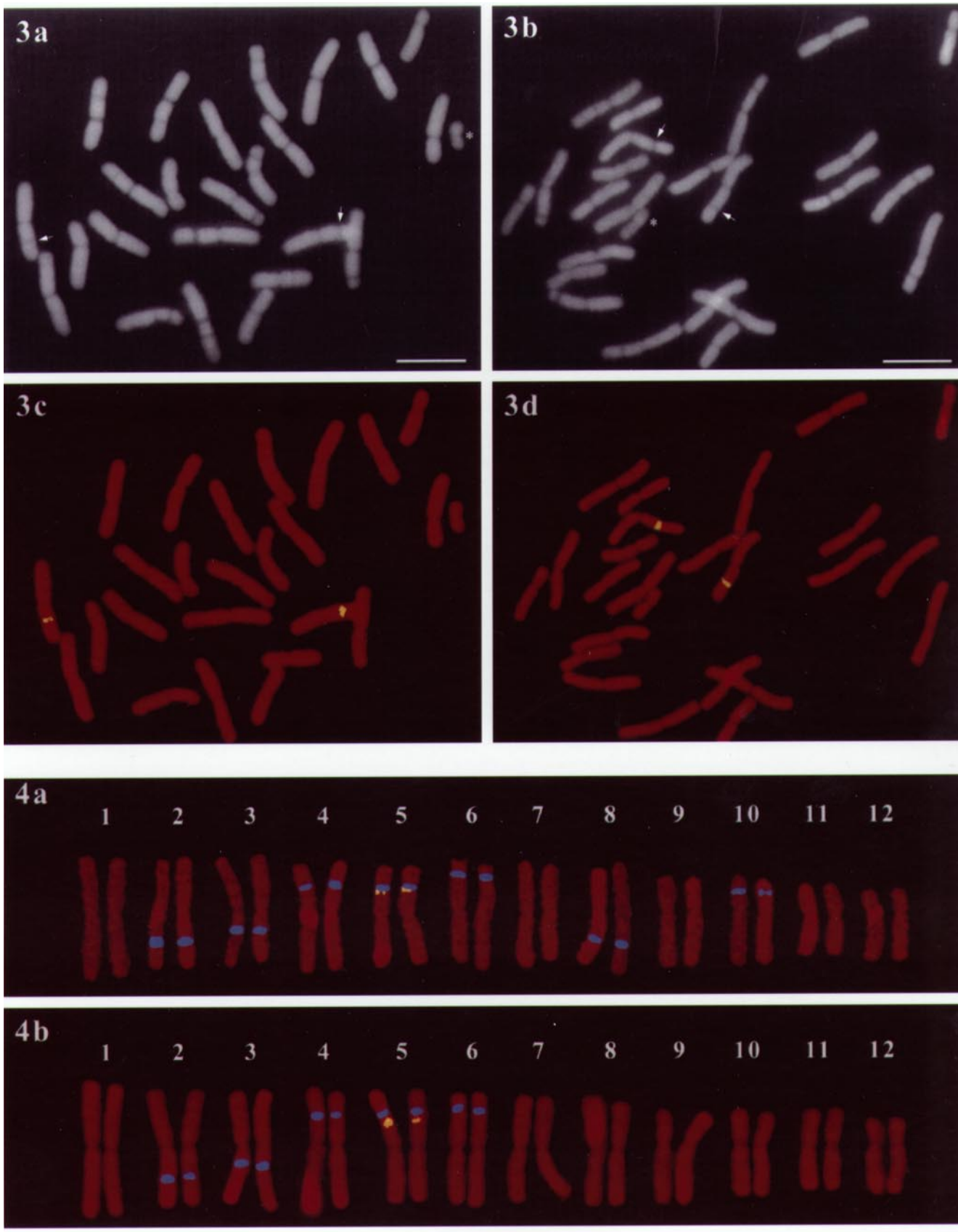
Fig. 2 Southern hybridization of white spruce DNA and ^{32}P-labeled pWS11. Genomic DNA (1 μg) was digested using *Sca*I (*lane 1*, 0.31 U; *lane 2*, 1.25 U; *lane 3*, 5 U) for 1 h at 37°C and resolved in 1.5% agarose and 0.5 × TBE. *Array A* consists of tandem repeated units of 221 bp with the nucleotide sequences shown in **Fig. 1 a**. *Array B* consists of repeating units of approximately 600 bp. A band of approximately 1000 bp which is not a multimer of either array class is shown by the *arrow*. Molecular weights are in base pairs

Cytogenetic analysis of white spruce and Sitka spruce

Metaphase chromosome spreads prepared from root tips of white and Sitka spruce and stained using the AT-specific fluorescent dye DAPI are shown in Figs. 3 a and b. DAPI clearly revealed the positions of primary constrictions, and the chromosome complements of both species were composed of eight long metacentric or submetacentric chromosome pairs and four smaller submetacentric pairs. A single metacentric B-chromosome was observed in many root tip cells of white and Sitka spruce. B-chromosomes of both species have been previously described (Moir and Fox 1972; Teoh and Rees 1977).

No DAPI-positive bands corresponding to AT-rich regions of the genome were observed in either species. Secondary constrictions appeared as DAPI-negative regions distinct from the primary constriction and were found at intercalary sites on 14 white spruce chromosomes and ten Sitka spruce chromosomes. Two chromosome pairs in both species were distinguished readily by a DAPI-negative region either proximal to the centromere or in a subtelomeric location. The remaining DAPI-negative regions were found at the approximate midpoint of the chromosome arm on each chromosome.

Figs. 3–4 Cytogenetic analysis of white spruce (2n = 24) and Sitka spruce (2n = 24). **Fig. 3** DAPI staining and chromosome localization of 5*s* rDNA in white spruce (*a*, *c*) and Sitka spruce (*b*, *d*). Preparations were stained using DAPI to reveal chromosome morphology (*a*, *b*), then de-stained and hybridized in situ using biotin-labeled pWS11 (*c*, *d*). The 5*s* rDNA locus (*yellow*) was mapped adjacent to an 18*s*-26*s* rDNA locus that stained poorly using DAPI (*arrows* in *a*, *b*). Chromosomal DNA was stained using propidium iodide (*red*). B-chromosomes are denoted by *asterisks*. The bar represents 10 μm. **Fig. 4** The mitotic chromosomes of white spruce (*a*) and Sitka spruce (*b*) following sequential FISH using pWS11 (*yellow*) and pGmR1 (*blue*)


3a
3b
3c
3d
4a
1 2 3 4 5 6 7 8 9 10 11 12
4b
1 2 3 4 5 6 7 8 9 10 11 12

The chromosomal location of 5*s* rDNA in white and Sitka spruce was determined by FISH of biotin-labeled pWS11 (Fig. 3 b,d). Fluorescent signals were found on both chromatids of one pair of metacentric chromosomes very near to a DAPI-negative region in all analyzed metaphases of both species. Amplification of the hybridization signal failed to reveal additional pWS11 hybridization sites (data not shown). The relative position of the 5*s* rDNA locus, calculated as the distance from the centromere expressed as a percentage of chromosome arm length, was estimated as 46.8 ±1.9 and 47.3 ±2.1 for white and Sitka spruce, respectively. The chromosome on which the 5*s* rDNA was mapped was ranked consistently as the fifth largest in both species' complements.

After pWS11 hybridization, chromosome preparations were stripped and re-probed using pGmR1, an 18*s*-26*s* rDNA clone from soybean. These results are shown in Fig. 4 a and b after merging the propidium iodide counterstain, pWS11 and pGmR1 hybridization images, and pairing and aligning homologous chromosomes. Fourteen pGmR1 hybridization sites were observed in white spruce as previously reported (Brown et al. 1993) while ten sites were found in Sitka spruce.In each species, sites of probe hybridization were coincident with the secondary constrictions revealed as DAPI-negative regions. The somatic chromosomes of white and Sitka spruce were identified based on (1) relative lengths, (2) arm ratios, and (3) the presence and location [a, defined according to Lubaretz et al. (1996) as the distance from the centromere expressed as a percentage of the chromosome arm] of genes encoding 18*s*-26*s* rDNA and 5*s* rDNA. Tables 1 and 2 summarize the results obtained in white spruce and Sitka spruce,

Table 1 The relative lengths and arm ratios of mitotic chromosomes and the position of the 18*s*-26*s* rDNA loci in white spruce (2n =24)

Number	Relative length ±SD	Arm ratio ±SD	a ±SD*	
			18*s*-26*s* rDNA	5*s* rDNA
1	10.01 ± 0.11	1.08 ± 0.05		
2	9.28 ± 0.10	1.10 ± 0.09	51.6 ± 2.7	
3	9.25 ± 0.23	1.03 ± 0.02	35.8 ± 1.3	
4	9.24 ± 0.36	1.21 ± 0.05	p 57.4 ± 2.4	
5	8.88 ± 0.17	1.05 ± 0.04	52.5 ± 2.8	46.8 ± 1.9
6	8.50 ± 0.32	1.09 ± 0.05	72.8 ± 3.6	
7	8.37 ± 0.18	1.04 ± 0.03		
8	8.33 ± 0.22	1.29 ± 0.06	q 49.8 ± 1.1	
9	7.78 ± 0.28	1.72 ± 0.07		
10	7.46 ± 0.18	1.33 ± 0.10	p 54.6 ± 3.1	
11	6.81 ± 0.18	1.17 ± 0.08		
12	6.10 ± 0.09	2.02 ± 0.17		

* a =distance from centromere, expressed as a percentage of chromosome arm length; p or q is denoted only for chromosomes with clearly distinguished arm sizes

Table 2 The relative lengths and arm ratios of mitotic chromosomes and the position of the 18*s*-26*s* rDNA and 5*s* rDNA loci in Sitka spruce (2n =24)

Number	Relative length ±SD	Arm ratio ±SD	a ±SD*	
			18*s*-26*s* rDNA	5*s* rDNA
1	10.35 ± 0.64	1.07 ± 0.04		
2	9.57 ± 0.24	1.05 ± 0.02	52.7 ± 4.6	
3	9.40 ± 0.34	1.12 ± 0.06	35.8 ± 2.3	
4	9.37 ± 0.31	1.17 ± 0.10	p 56.3 ± 2.2	
5	9.07 ± 0.38	1.08 ± 0.04	54.8 ± 4.2	47.3 ± 2.1
6	8.87 ± 0.24	1.08 ± 0.06	73.6 ± 2.0	
7	8.75 ± 0.26	1.05 ± 0.03		
8	8.42 ± 0.19	1.27 ± 0.08		
9	7.65 ± 0.22	1.82 ± 0.11		
10	6.93 ± 0.44	1.51 ± 0.13		
11	6.58 ± 0.27	1.20 ± 0.10		
12	6.01 ± 0.34	1.81 ± 0.10		

* a =distance from centromere, expressed as a percentage of chromosome arm length; p is denoted only for chromosomes with clearly distinguished arm sizes

respectively. Although variation between homologous chromosomes, in total length among the larger chromosomes in particular, was observed frequently, the effects of differential chromosome condensation or random distortion introduced through squashing did not prevent the pairing of homologous chromosomes.

The chromosome complements of both species were divided into two groups: group A, made up of chromosomes with 18*s*-26*s* rDNA or 5*s* rDNA loci, and group B, made up of those chromosomes lacking rDNA loci. In white spruce, group A was composed of seven pairs corresponding to chromosomes 2, 3, 4, 5, 6, 8, and 10 in Fig. 4 a. Chromosome 5 was the only pair with a 5*s* rDNA locus. The locations of 18*s*-26*s* rDNA loci proximal to the centromere ($a = 35.8 \pm 1.3$) or in a subtelomeric location ($a = 72.8 \pm 3.6$) were unique and identified chromosomes 3 and 6, respectively. The smallest chromosome with an 18*s*-26*s* rDNA locus was chromosome 10 and was distinguished easily from the second smallest chromosome of group A (chromosome 8) by size alone. Additionally, the 18*s*-26*s* rDNA locus on chromosome 8 was found on the long arm whereas that on chromosome 10 was located on the short arm. Chromosome 4 was indistinguishable from chromosome 2 based on relative length; however, the difference in arm ratios and the location of the 18*s*-26*s* rDNA locus on the short arm of chromosome 4 was usually suffficient for identification. In many spreads, one homologue of chromosome 2 had a much more extensive secondary constriction resulting in a higher average arm ratio for the pair. Where noted, this polymorphism also helped to identify one of the chromosome-2 homologues. Group B included chromosomes 1, 7, 9, 11 and 12. Chromosome 1 is the longest of the group and is distinguished easily from the next longest, chromosome 7, by relative length. The arm ratios and relative lengths of chromosomes 9, 11 and 12 were sufficient to identify these chromosomes from others in group B as well as from all chromosomes of group A in the absence of FISH data.

The same procedure was followed for Sitka spruce to identify the five chromosomes of group A (chromosomes 2, 3, 4, 5, 6) and the seven chromosomes of group B (chromosomes 1, 7–12). Chromosomes 3, 5 and 6 were distinguished by the same criteria used in white spruce. Distinguishing chromosomes 2 and 4 from each other was possible in most metaphases of Sitka spruce based on differences in arm ratios (1.05 vs 1.17) and the location of the 18*s*-26*s* rDNA locus on the short arm of chromosome 4. Chromosome 1 was clearly the longest chromosome of group B. While chromosomes 7 and 8 were of similar relative lengths, differences in arm ratios (1.05 vs 1.27) permitted the identification of each. The arm ratios and relative lengths of chromosomes 9, 10, 11 and 12 were sufficient to identify these chromosomes from others in group B in all metaphases. Lastly, although the chromosomes of white and Sitka spruce appear very similar at this level of resolution, the absence of 18*s*-26*s* rDNA loci on the long arm of chromosome 8 and the short arm of chromosome 10 in Sitka spruce was noteworthy.

Discussion

Two size classes of 5*s* rDNA, consisting of 221-bp and approximately 600-bp repeating units (arrays A and B, respectively), were observed in white spruce. PCR using primers designed to amplify entire repeats was successful in amplifying members of array A only, possibly arising from sequence differences in the annealing sites of primers P3 and/or P4 in members of array B. Southern analysis with pWS11 revealed considerably more hybridization to dimers and trimers of array B than to array A. Five of the six nucleotides of the *Sca*I restriction site (AGTACT) comprised the 3′end of primer P4, and *Sca*I is not sensititive to the methylation of nucleotides in its recognition sequence. Therefore, dimers and trimers or larger multimers in complete digests could have arisen only by altered restriction sites, suggesting that alterations in the P4 annealing site prevented amplification of array-B repeats. While PCR was useful in generating homologous ISH probes in a short time, a thorough investigation of 5*s* gene family diversity in white spruce should rely on ultracentrifugation methods (Lawrence and Appels 1986) and genomic DNA cloning.

The 50 nucleotides of the nontranscribed spacers immediately upstream of the 5*s* rDNA coding region in white spruce and radiata pine were 80% identical. Regulatory functions for spacer elements conserved in *Neurospora* and a number of angiosperm plants have been proposed (Venkateswarlu et al. 1991). By analogy, the AT- rich region from -30 to -26 (TTC/GAA) in white spruce and radiata pine may play a role in the transcription of the 5*s* gene although the strict AT composition reported among angiosperms, and indicated in the lupin sequence shown in Fig. 1 b for comparison, was not observed. A GC-rich region centered around -13 has also been implicated in transcriptional regulation, and in both white spruce and radiata pine a tandem duplication of AGGGGG was found from -24 to -13. The conservation of nucleotides -27 through -13 in these two Pinaceae genera is suggestive of a regulatory role; however, sequence data from other conifers is lacking.

Insertions into or duplications within the non-transcribed spacer, if successfully amplified, would give rise to more than one 5*s* rDNA repeat class. In radiata pine, for example, the two size classes of 5*s* rDNA differ by a 330-bp insertion of a predominantly CT-microsatellite (Moran et al. 1992). In estimating 5*s* rDNA copy number, the existence of two repeat classes was not accounted for. Insertion of an unrelated sequence into the 101-bp spacer of array A would not affect the total

copy number estimate since pWS11 would hybridize in equal molar amounts to either repeat class in genomic DNA samples. On the other hand, duplication of spacer sequences would have resulted in an overestimation of 5*s* rDNA copy number.

In genomes containing more than one non-transcribed spacer length, the members of each class are usually restricted to a discrete chromosomal site in concurrence with the theory of concerted evolution (Dover et al. 1982). However, in white and Sitka spruce, all 5*s* rDNA length variants mapped to a single locus on chromosome 5. Since the estimated lengths of array A ($0.43 \times 1170 \times 0.221 = 111$ kb) and array B ($0.55 \times 1170 \times 0.6 = 386$ kb) in white spruce are most likely within the sensitivity of our ISH procedure, it appears that both variant classes are found at the same locus, either in adjacent arrays or interspersed with one another to some extent.

Substantial improvements in our FISH procedures have been made since a preliminary karyotype of white spruce was reported (Brown et al. 1993). These included (1) improved chromosome spreading, (2) the use of pepsin to obtain cleaner chromosome preparations, (3) the enhanced reproducibility of chromosomal DNA denaturation and hybridization conditions using a thermal cycler, (4) the improved detection sensitivity using avidin conjugates as opposed to immunological methods, and (5) the use of computer software for chromosome analysis. We attribute any differences in the white spruce karyotype reported here to that in Brown et al. (1993) as arising from these factors. However, chromosome spreading was facilitated by using more-condensed metaphase chromosomes in our study and the accuracy of chromosome measurements was most likely reduced. As a consequence, the ordering of chromosomes 2–4 and 5–8 in white and Sitka spruce, as well as the designation of the short and long arms of chromosomes 1–3 and 5–7, were considered tentative and may change as less-condensed chromosome preparations are studied. Additionally, the distinction between chromosomes 2 and 4 in both species relies on differences in arm ratios which may not be unequivocal in all metaphases. Therefore, new physical markers to distinguish these chromosomes are necessary before unambiguous karyotypes of white and Sitka spruce are available.

Detailed analyses of genome evolution and organization in spruce and other conifer genera will become possible through the broader application of ISH methods. To-date, physical mapping of the 18*s*-26*s* rDNA and 5*s* rDNA in other spruces has been reported only in Norway spruce [*P. abies* (L.) Karst, Lubaretz et al. 1996] and we have presented the white and Sitka spruce data in a similar fashion to facilitate comparisons. Among the three species, the homoeology of chromosomes 1 and 9–12 can be inferred by morphology, together with the size and the absence of rDNA loci. The 5*s* rDNA in Norway spruce was mapped adjacent to an 18*s*-26*s* rDNA locus, as in white and Sitka spruce, but was assigned to the long arm of chromosome 2. Similar discrepancies in chromosome and arm assignments of four of the remaining 18*s*-26*s* rDNA loci in Norway spruce were evident. This may be due to the limited sample size (five metaphases of each species) on which chromosome numberings were based in this report and in Lubaretz et al. (1996) as well as the more-condensed chromosomes used in this study and the related difficulties mentioned above. However, the distinctive 18*s*-26*s* rDNA loci on chromosomes 3 and 6 in white and Sitka spruce were absent in Norway spruce suggesting genuine rearrangements between homoeologues. Both loci were absent from the karyotype of black spruce [*P. mariana* (Mill) B.S.P.] as well (Nkongolo and Klimazewska 1994) although these results were based on observations of secondary constrictions which depend on the expression of the 18*s*-26*s* rDNA genes for their cytological appearance. ISH with an 18*s*-26*s* rDNA probe would establish more clearly the similarities and differences in black spruce.

The evolution of gymnosperm genomes has been proposed to have proceeded via numerous small exchanges rather than the large scale alteration of chromosome organization commonly seen in angiosperm plant evolution (Khoshoo 1961). This can be tested by interspecific comparisons of marker orders in genetically defined linkage groups and, at the chromosome level, by molecular cytogenetics. Integrating both approaches to genome analysis in conifers cannot make use of cytogenetic stocks, such as chromosome addition lines, but could be achieved by ISH or through the identification and segregation analysis of polymorphic restriction sites or flanking tandem repeated DNAs of known chromosomal location. The single 5*s* rDNA locus in spruce would simplify the identification of segregating alleles and make it a good candidate locus to bridge the genetic and physical maps of chromosome 5.

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