

Chromosomal Localization of 5S and 18S rDNA in Five Species of Subgenus *Strobos* and their Implications for Genome Evolution of *Pinus*

QING CAI¹, DAMING ZHANG¹, ZHAN-LIN LIU² and XIAO-RU WANG^{1,*}

¹Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, 100093 Beijing, China and ²Key Laboratory of Resource Biology and Biotechnology in Western China, Northwest University, Ministry of Education, 710069 Xi'an, China

Received: 24 October 2005 Returned for revision: 20 November 2005 Accepted: 5 January 2006 Published electronically: 15 February 2006

• **Background and Aims** Studying the genome structure of pines has been hindered by their large genomes and uniform karyotypes. Consequently our understanding of the genome organization and evolutionary changes in different groups of pines is extremely limited. However, techniques are now available that can surmount these difficulties. The purpose of this study was to exploit some of these techniques to characterize the genome differentiation between the two subgenera of *Pinus*: *Pinus* and *Strobos*.

• **Methods** Double-probe fluorescence *in-situ* hybridization (FISH) was used to localize the 5S and 18S rDNA loci on chromosomes of five species from the subgenus *Strobos*: *P. bungeana*, *P. koraiensis*, *P. armandii*, *P. wallichiana* and *P. strobus*.

• **Key Results** The rDNA FISH pattern varied considerably among the five species, with *P. bungeana* being the most distinct. By comparing the results obtained with those of previous rDNA FISH studies of members of the subgenus *Pinus*, several general features of rDNA loci distribution in the genus *Pinus* can be discerned: (a) species of subgenus *Strobos* generally have more rDNA loci than species of subgenus *Pinus*, correlating with their larger genomes in the subgenus *Strobos*; (b) there is a clear differentiation in 5S and 18S rDNA loci linkage patterns between the two subgenera; (c) variations in the rDNA FISH pattern correlate with phylogenetic relationships among species within the subgenus; (d) *P. bungeana* has fewer 18S rDNA sites than other pines investigated to date, but they give intense signals, and may reflect the primary distribution of the 18S–25S rDNA loci in the genus.

• **Conclusions** The stable differentiation in rDNA FISH pattern between the subgenera suggests that chromosomal rearrangements played a role in the splitting of the two subgenera, and transpositional events rather than major structural changes are likely responsible for the variable rDNA distribution patterns among species of the same subgenus with conserved karyotypes.

Key words: 5S and 18S rDNA, FISH, karyotype, *Pinus*, subgenus *Strobos*.

INTRODUCTION

The genus *Pinus* comprises more than 100 species, which are widely distributed in the northern hemisphere (Mirov, 1967). The genus is divided into two subgenera: *Pinus* and *Strobos* (Little and Critchfield, 1969). Morphological, anatomical and molecular data demonstrate that the two subgenera have considerably diverged (Mirov, 1967; Strauss and Doerksen, 1990; Liston *et al.*, 1999; Wang *et al.*, 1999). Fossil records indicate that the genus split into the two subgenera by the early Cretaceous (Miller, 1977). To date, the genome structure of pines is poorly understood, as is the differentiation between the two subgenera at the genome and chromosome levels.

The genome size of pines has been measured as the amount of DNA in their haploid genomes (C values) (Wakamiya *et al.*, 1993; Hall *et al.*, 2000; Joyner *et al.*, 2001; Grotkopp *et al.*, 2004). Investigations have revealed that members of the subgenus *Strobos* generally have larger genomes (27.36–37.68 pg C⁻¹) than those in the subgenus *Pinus* (19.94–35.26 pg C⁻¹). This variation has been difficult to relate to the karyotypic characters of pines in a phylogenetic framework, because pines show remarkable uniformity in karyotype. All the *Pinus* species have the same chromosome

number, $2n = 24$, composed of 11 pairs of metacentric chromosomes of similar length and a short heterobrachial chromosome (Pederick, 1970; Saylor, 1972, 1983). Fluorescent banding using the fluorochromes chromomycin A (CMA) and 4',6-diamidino-2-phenylindole (DAPI) was applied to some *Pinus* species and was useful for chromosome identification (Hizume *et al.*, 1983, 1989, 1990). However, investigations on the genome organization and structural rearrangements in pines have been limited due to the lack of resolution in the karyotypes. For these applications, *in situ* hybridization (ISH) or fluorescence *in-situ* hybridization (FISH) using an rDNA probe has proved to be an excellent tool in pines (Hizume *et al.*, 1992, 2002; Lubaretz *et al.*, 1996; Jacobs *et al.*, 2000; Liu *et al.*, 2003).

To date, the chromosomal localization of 18S–25S rDNA loci has been reported for 15 *Pinus* species, including *P. elliotii* (Doudrick *et al.*, 1995), *P. sylvestris* (Karvonen *et al.*, 1993; Lubaretz *et al.*, 1996; Hizume *et al.*, 2002), *P. radiata* (Gorman *et al.*, 1992; Jacobs *et al.*, 2000), *P. taeda* (Jacobs *et al.*, 2000), *P. densiflora*, *P. thunbergii* (Hizume *et al.*, 1992; 2002), *P. nigra* (Hizume *et al.*, 2002), *P. tabuliformis*, *P. yunnanensis*, *P. densata*, *P. massoniana*, *P. merkusii* (Liu *et al.*, 2003), *P. resinosa*, *P. banksiana* and *P. strobus* (Nkongolo *et al.*, 2004). Among these pines, only *P. strobus* belongs to the subgenus *Strobos*, the 14 other

* For correspondence. E-mail Xiao-Ru.Wang@niwl.se

pinus are all from the subgenus *Pinus*. Thus, there is a clear need to find out more about the localization of rDNA loci in subgenus *Strobos* to generalize the patterns of rDNA loci distribution in this subgenus and the differentiation between the two subgenera.

In the present study, the 5S and 18S rDNA loci localization patterns in five species (*P. bungeana*, *P. koraiensis*, *P. armandii*, *P. wallichiana* and *P. strobos*) of subgenus *Strobos* are investigated using DNA probes for the 5S rDNA and 18S rDNA simultaneously to address the following questions: (a) Do the number and location of rDNA loci vary between the two subgenera? (b) Does the difference in rDNA loci number correlate with difference in genome sizes between the two subgenera? (c) Can the variations in FISH patterns be explained within a phylogenetic framework of *Pinus*?

MATERIALS AND METHODS

Chromosome slide preparation

Five species representing three subsections of subgenus *Strobos* were selected for this study (Table 1). Seeds of each species were collected from natural stands or artificial plantations and germinated in Petri dishes on moist filter paper at room temperature. The primary root tips, grown to a length of 1–1.5 cm, were excised and pretreated with 0.05% colchicine for 24 h at 25 °C, fixed in Carnoy's fixative (ethanol:acetic acid, 3:1, v/v) for 24 h, and macerated with a mixture of 1% Y-23 pectolyase (Yakult) and 2% R-10 cellulase (Yakult) at 37 °C for 45 min. Metaphase chromosome spreads were prepared by conventional squashing.

Probe preparation

Genomic DNA of *P. bungeana* was isolated from seed megagametophyte and used as a template for PCR amplification. 18S rDNA sequence was amplified with the primers 5'-CTAGAGCTAATACGTGCAAC-3' and 5'-GAT AAGTTTCAGTGGACTTC-3' (Troitsky *et al.*, 1991). The PCR programme for 18S rDNA amplification consisted of 2 min at 95 °C for initial denaturation followed by 33 cycles of 30 s at 94 °C, 30 s at 55 °C and 2 min at 72 °C. In the last cycle the extension at 72 °C was extended to 8 min. The primers for 5S rDNA amplification were 5'-CGGTGCATTAATGCTGGTAT-3' and 5'-CCCATCCG-TGTACTACTCTC-3' (Amarasinghe and Carlson, 1998). The PCR programme for 5S rDNA amplification was the same as for 18S rDNA, except that the annealing temperature was 60 °C. The fragment sizes of the 18S rDNA and 5S rDNA PCR product were approx. 1.8 kb and 600 bp, respectively. The purified rDNA PCR products were labelled by the random priming method using Klenow fragment (Promega). Biotin-16-dUTP (Roche) was used for labelling 5S rDNA and digoxigenin (DIG)-11-dUTP (Roche) for 18S rDNA labelling.

FISH

The chromosomal slides were treated with 100 µL RNase (100 µg mL⁻¹ in 2× SSC) at 37 °C for 1 h and then soaked

TABLE 1. The five species of subgenus *Strobos* used in the study

Species	Subsection*	Source
<i>Pinus bungeana</i> Zucc.	Gerardianae	Institute of Botany, Chinese Academy of Sciences, Beijing, China
<i>Pinus koraiensis</i> Sieb. et Zucc.	Cembrae	Changbai Mountain, Jilin, China
<i>Pinus armandii</i> Franch.	Strobi	Shengnongjia, Hubei, China
<i>Pinus wallichiana</i> Jackson.	Strobi	Yadong, Tibet, China
<i>Pinus strobos</i> L.	Strobi	North Carolina State University, NC, USA

*The taxonomic system of Little and Critchfield (1969) is followed.

twice for 5 min in 2× SSC. The chromosomes were then digested with proteinase K (1 µg mL⁻¹, Promega) at 37 °C for 10 min. The slides were dehydrated in a series of 70%, 95% and 100% ethanol and then air-dried. A 20-µL hybridization solution, containing 10% dextran sulfate, 50% formamide, 10 ng µL⁻¹ of sheared salmon sperm DNA, 2 ng µL⁻¹ of denatured probes and 2× SSC, was added to each slide. The slides were placed in a moisture chamber, denatured at 85 °C for 13 min, then immediately placed in a hybridization oven and kept at 37 °C overnight for probe hybridization. The slides were then washed with 20% formamide in 0.1× SSC at 42 °C for 10 min, followed by two washes with 2× SSC at 37 °C for 5 min, and 2× SSC at room temperature for 5 min. *In-situ* hybridization was repeated on three to five chromosome spreads for each species.

Before detection, the slides were soaked in detection buffer (5% BSA, 100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100) at 37 °C for 30 min. The 18S rDNA signals were detected using anti-DIG-rhodamine (2 ng mL⁻¹; Roche) and 5S rDNA signals were detected using avidin-FITC (2 µg mL⁻¹; Sigma, USA). The slides were counter-stained with 100 µL DAPI (2 µg mL⁻¹), and observed under a fluorescence microscope (DMRBE, Leica) at 100 × 10 magnification. The FISH images were captured by a cooled CCD colour chromatic camera (Spot Enhanced; Diagnostic Instruments Inc.).

Chromosome length measurement

The arm lengths of chromosomes in the CCD-captured images of ten cells from each of two or three chromosome spreads of each species were measured. The arm ratio was calculated, and the average values were used to construct an ideogram of the karyotype for each species. The chromosomes were assigned consecutively according to their lengths, with the longest being assigned as I and the shortest as XII. For chromosomes of equal length, the chromosome with the longest short arm was assigned first. As well as the length and arm ratios of the chromosomes, secondary constrictions and FISH patterns are also informative characters for karyotyping. The location of rDNA loci on each chromosome is shown on the ideograms. The mapped positions are not precise quantitative representations,

although the relative distances of each rDNA site to the centromere and telomere were considered while constructing the ideogram.

RESULTS

Clear, reproducible, unambiguous FISH signals from three to five FISH replicates of each species were registered. Weak signals in centromeric regions were not analysed. Examples of the 18S and 5S rDNA FISH pattern obtained for each species are shown in Fig. 1 and an ideogram of the karyotypes is presented in Fig. 2. The karyotype features of the five pine species are similar to those reported by Saylor (1983): chromosomes I–XI were found in all cases to be metacentric and to have similar lengths, while chromosome XII was always heterobrachial and short. However, the chromosomal distribution patterns of the 5S and 18S rDNA sites varied considerably among the five species.

In *P. bungeana* (Figs 1A and 2A), four pairs of intense 18S rDNA site signals were detected in the interstitial regions of the short arms of chromosomes VI, VIII, XI and XII. Two pairs of 5S rDNA sites were detected, one localized on the short arm of chromosome V and the other together with an 18S rDNA site on the short arm of chromosome XI.

In *P. koraiensis*, nine pairs of 18S rDNA sites were detected on nine pairs of chromosomes, eight on the interstitial regions of the short arms of chromosomes I, II, IV, V, VIII, IX, X and XI and one on the long arm of chromosome XII. Four pairs of 5S rDNA sites were detected: one on the short arm of chromosome III, while the remaining three were localized with 18S rDNA sites on the short arms of chromosomes I, V and XI (Figs 1B and 2B).

In *P. armandii*, ten pairs of 18S rDNA and three pairs of 5S rDNA sites were detected. Seven pairs of 18S rDNA sites were localized in the interstitial regions of the short arms of chromosomes II, IV, V, VI, VIII, X and XI, while the other three were on the long arms of chromosomes IX, XI and XII. A notable feature was that both arms of chromosome XI hosted an 18S rDNA site. Two 5S rDNA sites were detected close to the 18S rDNA sites on the distal regions of the short arms of chromosomes X and XI, respectively. Another 5S rDNA site was situated alone on the short arm of chromosome I (Figs 1C and 2C).

In *P. wallichiana*, ten pairs of 18S rDNA and two pairs of 5S rDNA sites were detected. Similar to the rDNA distribution in *P. armandii*, seven pairs of 18S rDNA were found in the interstitial regions of the short arms of chromosomes II, IV, V, VI, VIII, X and XI and three pairs on the long arms of chromosomes IX, XI and XII. In addition, as in *P. armandii*, chromosome XI of *P. wallichiana* also hosted an 18S rDNA site on each of its arms and a 5S rDNA site in the distal region of the short arm. The other 5S rDNA site was found on the short arm of chromosome I (Figs 1D and 2D).

In *P. strobus*, nine pairs of 18S rDNA and two pairs of 5S rDNA sites were detected. Eight pairs of 18S rDNA sites were found in the interstitial regions of the short arms of chromosomes II, III, IV, V, VI, IX, X and XI and one on the

long arm of chromosome XII. One 5S rDNA site was found close to the 18S rDNA site on the short arm of chromosome XI, and the other on chromosome I (Figs 1E and 2E).

In four of the species examined, except for *P. bungeana*, rDNA sites were detected on ten of the 12 chromosome pairs. Most of the 18S rDNA sites gave clear signals and were positioned in the interstitial regions corresponding to secondary constrictions. No clear, unambiguous 18S signals were observed at the centromere in any case, although weak signals were detected in centromeric regions of two or three pairs of chromosomes in each species. All the 5S rDNA sites were detected at distal positions. Based on the 5S and 18S rDNA distribution patterns and chromosomal morphology, chromosomes XI and XII can be unambiguously identified in all five species. Chromosome XI hosted a 5S and an 18S rDNA site on its short arm in all five species, and a second 18S rDNA site was hosted on the opposite arm of this chromosome in *P. wallichiana* and *P. armandii*. The rDNA FISH pattern of *P. bungeana* differed markedly from that of the other four species, since it contains only four pairs of 18S rDNA sites. The detected signal strength of these sites, however, was noticeably more intense than in the other four pines.

DISCUSSION

Genome research in *Pinus* has not progressed as rapidly as it has in model plants such as *Oryza* (Goff *et al.*, 2002; Bowers *et al.*, 2005; Cheng *et al.*, 2005), *Arabidopsis* (Copenhaver *et al.*, 1998; Arabidopsis Genome Initiative, 2000; Lysak *et al.*, 2003) and *Populus* (Brunner *et al.*, 2004; Sterky *et al.*, 2004). Consequently, our understanding of the genome organization and chromosomal structure among different groups of pines is extremely limited. Karyotype analyses of *Pinus* species have revealed that chromosomal morphology is highly conserved within the genus, and that most of the chromosomes have similar lengths and arm ratios, making precise karyotyping difficult (Saylor, 1972, 1983). In the subgenus *Strobus*, for example, the correct ordering of chromosomes III–VIII has been ambiguous to date (Saylor, 1983). The use of double- or multiple-probe FISH, in combination with other chromosomal characters, has facilitated the identification of homologous chromosomes and the inference of genome evolution among plant species (Brown *et al.*, 1999; Heslop-Harrison, 2000; Marcon *et al.*, 2005; Taketa *et al.*, 2005; Vaio *et al.*, 2005). However, due to the uniformity of the karyotypes among pines, it is still not straightforward to identify precisely all the homologous chromosomes across species, even when the two rDNA markers are used. Among the five species analysed in this study, only chromosomes XI and XII can be aligned with certainty across species. The rDNA distribution patterns of the other ten similar-length chromosomes differed even among species of the same subsection, such as *P. armandii*, *P. wallichiana* and *P. strobus*. This finding is in accordance with the study by Saylor (1983), who found less similarity in karyotypic features among species of the same subsection in subgenus *Strobus* than in subgenus *Pinus*. This makes the alignment of homologous

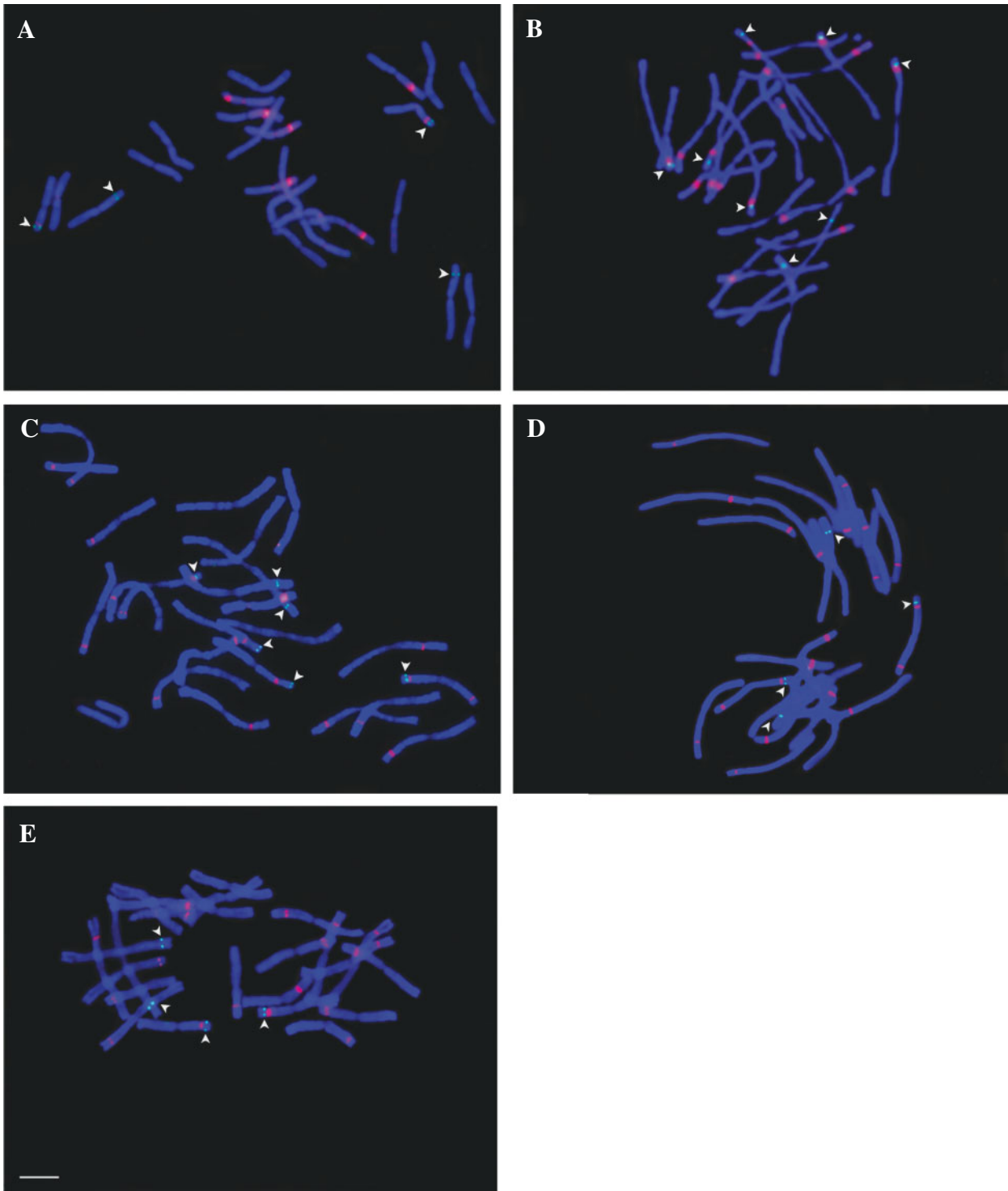


FIG. 1. FISH localization of 18S rDNA (red) and 5S rDNA (green) loci on somatic chromosomes in (A) *P. bungeana*, (B) *P. koraiensis*, (C) *P. armandii*, (D) *P. wallichiana* and (E) *P. strobus*. Arrowheads indicate the 5S rDNA signals. Scale bar = 10 μ m.

chromosomes among pines challenging. Other FISH markers need to be mapped along the chromosomes to facilitate precise identifications (Hizume *et al.*, 2002).

This study reports, for the first time, the physical locations of 5S and 18S rDNA loci in species from the subgenus

Strobus. Combined with the results of previous rDNA FISH analyses of members of the subgenus *Pinus* (Doudrick *et al.*, 1995; Hizume *et al.*, 2002; Liu *et al.*, 2003), several general features of rDNA loci distribution in the genus *Pinus* can be discerned. The number of 5S and 18S rDNA loci vary from

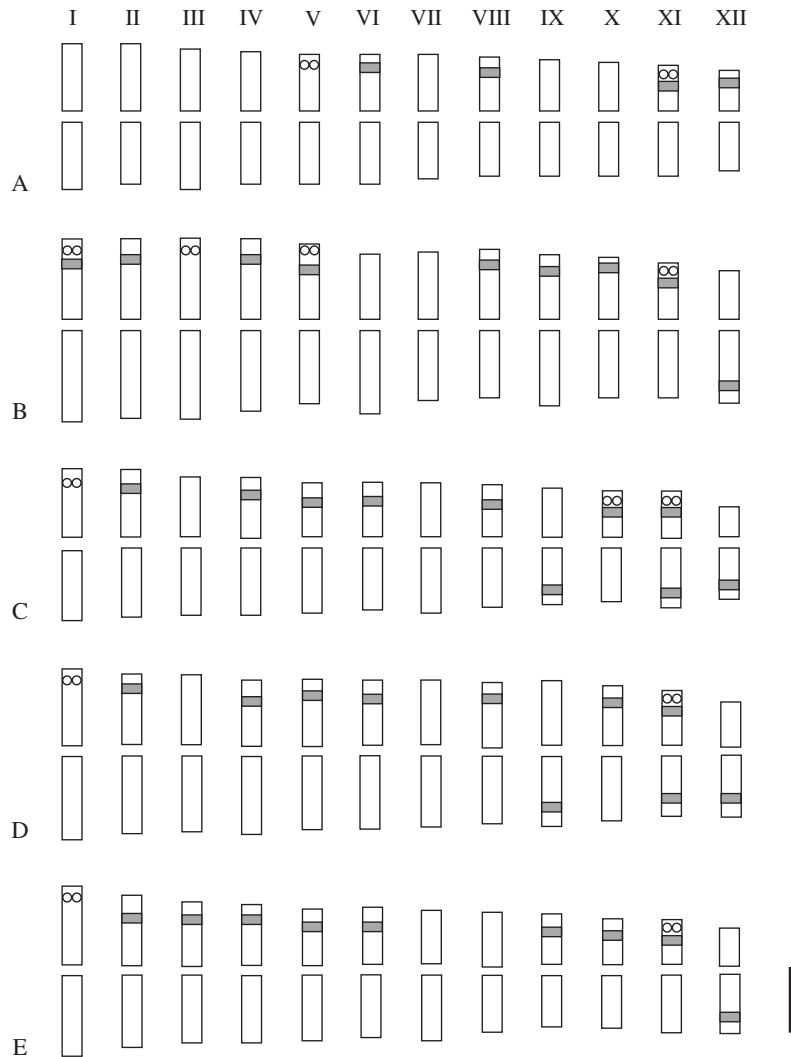


FIG. 2. Ideogram of (A) *P. bungeana*, (B) *P. koraiensis*, (C) *P. armandii*, (D) *P. wallichiana* and (E) *P. strobus* karyotypes. The open dots represent signals of 5S rDNA and the solid bands represent strong 18S rDNA signals. Scale bar = 10 μ m.

one to four pairs, and from seven to ten pairs, respectively (Table 2), significantly more than the one or two pairs of 5S rDNA loci and one to five pairs of 18S–25S rDNA loci in diploid angiosperms (Castilho and Heslop-Harrison, 1995; Linares *et al.*, 1996; de Melo and Guerra, 2003; Vaio *et al.*, 2005). The difference is, presumably, partly related to the evolutionary divergence between angiosperm and gymnosperm genomes, and partly to the much larger genomes of gymnosperms (Leitch *et al.*, 2005). Pines have large genomes, with estimates of approx. 10^{10} bp for the haploid genome (Ohri and Khoshoo, 1986; Wakamiya *et al.*, 1993; Murray, 1998; Elsik and Williams, 2000). These huge genomes are largely (approx. 90%) composed of repetitive DNA, together with many high-copy retroelement families (Kamm *et al.*, 1996; Kossack and Kinlaw, 1999; Elsik and Williams, 2000). 18S–25S rDNA is one of the major components of the repetitive DNA in the genome (Bobola *et al.*, 1992). Thus, it is not surprising to discover a high number of rDNA sites in *Pinus*.

Within the genus, there is a tendency for the genomes to be larger in subgenus *Strobus* than in subgenus *Pinus* (Hall *et al.*, 2000; Joyner *et al.*, 2001; Grotkopp *et al.*, 2004). Genome size seems to have remained stable or increased from the ancestral genome size over evolutionary time in the subgenus *Strobus*, while it has decreased in most subsections of the subgenus *Pinus* (Grotkopp *et al.*, 2004). Interestingly, more rDNA loci were observed in subgenus *Strobus* than in subgenus *Pinus*, except for *P. bungeana* (Table 2). rDNA FISH results can vary among studies, mainly due to differences in experimental procedures including the homology of the probes and the hybridization and washing stringency. The present results were compared with previous results on five species of subgenus *Pinus* obtained under the same experimental conditions (Liu *et al.*, 2003). The comparison indicates that there are nine or ten pairs of 18S rDNA loci and two to four pairs of 5S rDNA loci in species of subgenus *Strobus* as compared with five to ten pairs of 18S rDNA loci, excluding the

TABLE 2. The number of 18S–25S and 5S rDNA sites in the diploid genome of Pinus

Species	18S–25S rDNA	5S rDNA	Reference
Subgenus <i>Strobos</i>			
<i>Pinus bungeana</i> Zucc.	8 strong	4 strong	Present study
<i>Pinus koraiensis</i> Sieb. et Zucc.	18 strong	8 strong	Present study
<i>Pinus armandii</i> Franch.	20 strong	6 strong	Present study
<i>Pinus wallichiana</i> Jackson	20 strong	4 strong	Present study
<i>Pinus strobus</i> L.	18 strong	4 strong	Present study
<i>Pinus strobus</i> L.	10–14	–	Nkongolo et al. (2004)
Subgenus <i>Pinus</i>			
<i>P. banksiana</i> Lamb.	10–14	–	Nkongolo et al. (2004)
<i>Pinus elliotii</i> Englem.	16 strong	2 strong, 4 weak	Doudrick et al. (1995)
<i>Pinus radiata</i> D. Don	12 strong, 8 weak	2 strong, 2 weak	Jacobs et al. (2000)
<i>Pinus sylvestris</i> L.	>16, 14 strong	2 strong, 2 weak	Hizume et al. (2002); Karvonen et al. (1993); Lubaretz et al. (1996)
<i>Pinus densiflora</i> Sieb. et Zucc.	14 strong	2 strong, 2 weak	Hizume et al. (1992, 2002)
<i>Pinus thunbergii</i> Parl.	10 strong, 2 weak	2 strong, 2 weak	Hizume et al. (1992, 2002)
<i>Pinus nigra</i> Arnold	16	2 strong, 2 weak	Hizume et al. (2002)
<i>Pinus taeda</i> L.	12 strong, 8 weak	2 strong, 2 weak	Jacobs et al. (2000)
<i>Pinus tabuliformis</i> Carr.	14 strong, 10 weak	2 strong, 2 weak	Liu et al. (2003)
<i>Pinus densata</i> Mast.	10 strong, 8 weak	2 strong, 1 weak	Liu et al. (2003)
<i>Pinus yunnanensis</i> Franch.	16 strong, 4 weak	2 strong, 2 weak	Liu et al. (2003)
<i>Pinus massoniana</i> Lamb.	20 strong	2 strong	Liu et al. (2003)
<i>Pinus merkusii</i> Jungh. et de Vriese	12 strong, 4 weak	2 strong, 2 weak	Liu et al. (2003)

weak signals in centromeric regions, and one or two pairs of 5S rDNA loci in species of subgenus *Pinus* (Liu et al., 2003) (Table 2). Thus, more chromosomes contained rDNA loci in subgenus *Strobos* than in subgenus *Pinus*. Three to five pairs of chromosomes in subgenus *Pinus* have no rDNA loci, while only two pairs of chromosomes have no rDNA loci in all the species of subgenus *Strobos* examined except for *P. bungeana*. Another difference between the two subgenera is that weak 18S–25S rDNA signals have been detected from the pericentromeric regions of most chromosomes in subgenus *Pinus* (Hizume et al., 2001; Liu et al., 2003), while in subgenus *Strobos* such signals were detected in only two or three pairs of chromosomes, and nearly all the distinct 18S rDNA signals originated from the interstitial regions. The weak 18S–25S rDNA signals in the centromeric regions reflect the presence of homologous sequences. However, these sequences seem to be nonfunctional (Hizume et al., 2001). It has been suggested that these weak signals might be the remnants of primary sites of 18S–25S rDNA that once existed at the centromeres but were later moved to distal sites by chromosomal rearrangements (Liu et al., 2003). If this is true, the variation in the presence of weak 18S rDNA signals in centromeric regions between the subgenera should reflect, to some degree at least, genome rearrangements accompanying their divergence.

Double-rDNA FISH facilitates visualization of the relative positions of the two classes of rDNA loci on chromosomes. In subgenus *Pinus*, all the 5S sites are situated on chromosomes that contain one 18S–25S site. The two rDNA sites are either situated on the same arm, with the 5S site in the interstitial region and the 18S–25S site toward the terminal region, or on different arms of the same chromosome (Liu et al., 2003). However, in subgenus *Strobos* the relative

positions of the two rDNA sites examined were reversed, with the 5S site toward the terminal region and the 18S site toward the interstitial region. In addition, one 5S site in subgenus *Strobos* was observed on a chromosome with no 18S–25S rDNA site. Among the pines that have been examined by double-rDNA FISH, the 5S and 18S–25S rDNA loci linkage pattern seems to be a stable character for each subgenus. Such a stable differentiation in rDNA distribution patterns between the subgenera is more likely caused by chromosome inversion and/or translocation, suggesting that chromosomal rearrangements played a role in the splitting of the two subgenera.

The variations in rDNA distribution have phylogenetic implications, since the closeness of taxa is correlated to the similarity of their rDNA FISH patterns (Hizume et al., 2002; Liu et al., 2003). Among the species of subgenus *Strobos* examined here, the 18S rDNA site patterns of the two Asian species of subsection *Strobi*, *P. armandii* and *P. wallichiana*, are most similar, while the North American *P. strobus*, which is in the same subsection, diverges from them less in this respect than *P. koraiensis* of subsection *Cembrae* (Fig. 2). These relationships agree well with the rDNA- and chloroplast DNA-based phylogeny (Liston et al., 1999; Wang et al., 1999). The most unique rDNA pattern was found in *P. bungeana*. It showed the fewest 18S rDNA sites, but the signal strength of these sites was noticeably more intense than those of other pines, indicating that high copy numbers of the 18S–25S rDNA repeats are present within each site. These sites may represent the primary sites of 18S–25S rDNA before some of them moved out and became dispersed among more chromosomal sites in the speciation of pines. Molecular phylogeny supports a close-to-basal position for *P. bungeana* in subgenus *Strobos* (Liston et al., 1999; Wang et al., 1999).

It would be interesting to examine *P. gerardiana*, from the same subsection as *P. bungeana*, and pines of the subsection *Balfourianae* (which lies at the basal position of the subgenus *Strobus*) to test the hypothesis that the condensed pattern of 18S–25S rDNA sites found in *P. bungeana* reflects the primary distribution of these loci in the *Pinus* genome. Variations in rDNA FISH pattern among angiosperms of the same ploidy level have been attributed to chromosomal rearrangements, transpositional events and gene silencing (Moscone *et al.*, 1999; de Melo and Guerra, 2003; Marcon *et al.*, 2005). The structural similarity among the karyotypes of pines within each subgenus suggest that major chromosomal structural rearrangements (e.g. translocation/inversion) are not frequent among species of the same subgenus. Mechanisms such as transposition of rDNA and amplification of cryptic minor rDNA sites by unequal crossover have been proposed for the dispersed genomic organization of rDNA in angiosperms and fungi (Drouin and de Sá, 1995; Dubcovsky and Dvořák, 1995; Rooney and Ward, 2005). Such mechanisms are likely to be involved in the origin of the variation in the rDNA loci distribution among closely related *Pinus* species.

ACKNOWLEDGEMENTS

We thank Dr Bailian Li, North Carolina State University, for seeds of *P. strobus*, and Dr Alfred E. Szmidt, Kyushu University, for critically reading the manuscript and making valuable comments on it. This study was supported by grants from the Natural Science Foundation of China (NSFC 30325006, 30121003).

LITERATURE CITED

- Amarasinghe V, Carlson JE. 1998. Physical mapping and characterization of 5S rRNA genes in Douglas-fir. *Journal of Heredity* **89**: 495–500.
- Arabidopsis Genome Initiative. 2000. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**: 796–815.
- Bobola MS, Smith DE, Klein AS. 1992. Five major nuclear ribosomal repeats represent a large and variable fraction of the genomic DNA of *Picea rubens* and *P. mariana*. *Molecular Biology and Evolution* **9**: 125–137.
- Bowers JE, Arias MA, Asher R, Avise JA, Ball RT, Brewer GA, *et al.* 2005. Comparative physical mapping links conservation of microsynteny to chromosome structure and recombination in grasses. *Proceedings of the National Academy of Sciences of the USA* **102**: 13206–13211.
- Brown SE, Stephens JL, Lapitan NL, Knudson DL. 1999. FISH landmarks for barley chromosomes (*Hordeum vulgare* L.). *Genome* **42**: 274–281.
- Brunner AM, Busov VB, Strauss SH. 2004. Poplar genome sequence: functional genomics in an ecologically dominant plant species. *Trends in Plant Science* **9**: 49–56.
- Castilho A, Heslop-Harrison JS. 1995. Physical mapping of 5S and 18S–25S rDNA and repetitive DNA sequences in *Aegilops umbellulata*. *Genome* **38**: 91–96.
- Cheng CH, Chung M-C, Liu S-M, Chen S-K, Kao F-Y, Lin S-J, *et al.* 2005. A fine physical map of the rice chromosome 5. *Molecular Genetics and Genomics* **274**: 337–345.
- Copenhaver GP, Browne WE, Preuss D. 1998. Assaying genome-wide recombination and centromere functions with *Arabidopsis* tetrads. *Proceedings of the National Academy of Sciences of the USA* **95**: 247–252.
- 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. *Journal of Heredity* **86**: 289–296.
- Drouin G, de Sá MM. 1995. The concerted evolution of 5S ribosomal genes linked to the repeat units of other multigene families. *Molecular Biology and Evolution* **12**: 481–493.
- Dubcovsky J, Dvořák J. 1995. Ribosomal RNA multigene loci: nomads of the Triticeae genomes. *Genetics* **140**: 1367–1377.
- Elsik CG, Williams CG. 2000. Retroelements contribute to the excess low-copy-number DNA in pine. *Molecular Genetics and Genomics* **264**: 47–55.
- Goff SA, Ricke D, Lan T-H, Presting G, Wang R, Dunn M, *et al.* 2002. A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). *Science* **296**: 92–100.
- Gorman SW, Teasdale RD, Cullis CA. 1992. Structure and organization of the 5S rRNA genes (5S DNA) in *Pinus radiata* (Pinaceae). *Plant Systematics and Evolution* **183**: 223–234.
- Grotkopp E, Rejmanek M, Sanderson MJ, Rost TL. 2004. Evolution of genome size in pines (*Pinus*) and its life-history correlates: supertree analyses. *Evolution* **58**: 1705–1729.
- Hall SE, Dvorak WS, Johnston JS, Price HJ, Williams CG. 2000. Flow cytometric analysis of DNA content for tropical and temperate New World pines. *Annals of Botany* **86**: 1081–1086.
- Heslop-Harrison JS. 2000. Comparative genome organization in plants: from sequence and markers to chromatin and chromosomes. *Plant Cell* **12**: 617–635.
- Hizume M, Ohgiku A, Tanaka A. 1983. Chromosome banding in the genus *Pinus*. I. Identification of chromosomes in *P. nigra* by fluorescent banding method. *Botanical Magazine* **96**: 273–276.
- Hizume M, Ohgiku A, Tanaka A. 1989. Chromosome banding in the genus *Pinus*. II. Interspecific variation of fluorescent banding patterns in *P. densiflora* and *P. thunbergii*. *Botanical Magazine* **102**: 25–36.
- Hizume M, Arai M, Tanaka A. 1990. Chromosome banding in the genus *Pinus*. III. Fluorescent banding pattern of *P. luchuensis* and its relationships among the Japanese diploxylon pines. *Botanical Magazine* **103**: 103–111.
- Hizume M, Ishida F, Murata M. 1992. Multiple locations of the rRNA genes in chromosomes of pines, *Pinus densiflora* and *P. thunbergii*. *Japanese Journal of Genetics* **67**: 389–396.
- Hizume M, Shibata F, Maruyama Y, Kondo T. 2001. Cloning of DNA sequences localized on proximal fluorescent chromosome bands by microdissection in *Pinus densiflora* Sieb. & Zucc. *Chromosoma* **110**: 345–351.
- Hizume M, Shibata F, Matsusaki Y, Garajova Z. 2002. Chromosome identification and comparative karyotypic analyses of four *Pinus* species. *Theoretical and Applied Genetics* **105**: 491–497.
- Jacobs MD, Gardner RC, Murray BG. 2000. Cytological characterization of heterochromatin and rDNA in *Pinus radiata* and *P. taeda*. *Plant Systematics and Evolution* **223**: 71–79.
- Joyner KL, Wang X-R, Johnston JS, Price HJ, Williams CG. 2001. DNA content for Asian pines parallels New World relatives. *Canadian Journal of Botany* **79**: 192–196.
- Kamm A, Doudrick RL, Heslop-Harrison JS, Schmidt T. 1996. The genomic and physical organization of Ty1-copia-like sequences as a component of large genomes in *Pinus elliottii* var. *elliottii* and other gymnosperms. *Proceedings of the National Academy of Sciences of the USA* **93**: 2708–2713.
- Karvonen P, Karjalainen M, Savolainen O. 1993. Ribosomal RNA genes in Scots pines (*Pinus sylvestris* L.): chromosomal organization and structure. *Genetica* **88**: 59–68.
- Kossack DS, Kinlaw CS. 1999. IFG, a gypsy-like retrotransposon in *Pinus* (Pinaceae), has an extensive history in pines. *Plant Molecular Biology* **39**: 417–426.
- Leitch IJ, Soltis DE, Soltis PS, Bennett MD. 2005. Evolution of DNA amounts across land plants (Embryophyta). *Annals of Botany* **95**: 207–217.
- Linares C, Gonzalez J, Ferrer E, Fominaya A. 1996. The use of double fluorescence *in situ* hybridization to physically map the positions of 5S rDNA genes in relation to the chromosomal location of 18S–5.8S–26S rDNA and a C genome specific DNA sequence in the genus *Avena*. *Genome* **39**: 535–542.

- Liston A, Robinson WA, Pinero D, Alvarezbuyla ER. 1999. Phylogenetics of *Pinus* (Pinaceae) based on nuclear ribosomal DNA internal transcribed spacer region sequences. *Molecular Phylogenetics and Evolution* **11**: 95–109.
- Little Jr EL, Critchfield WB. 1969. Subdivisions of the genus *Pinus* (pines). USDA Forest Service, Washington D.C. Miscellaneous Publication No. 1144.
- Liu Z-L, Zhang D, Hong D-Y, Wang X-R. 2003. Chromosomal localization of 5S and 18S-5.8S-25S ribosomal DNA sites in five Asian *Pinus* species using fluorescence *in situ* hybridization. *Theoretical and Applied Genetics* **106**: 198–204.
- Lubaretz O, Fuchs J, Ahne R, Meister A, Schubert I. 1996. Karyotyping of three Pinaceae species via fluorescent *in situ* hybridization and computer-aided chromosome analysis. *Theoretical and Applied Genetics* **92**: 411–416.
- Lysak MA, Pecinka A, Schubert I. 2003. Recent progress in chromosome painting of *Arabidopsis* and related species. *Chromosome Research* **11**: 195–204.
- Marcon AB, Barros ICL, Guerra M. 2005. Variation in chromosome numbers, CMA bands and 45S rDNA sites in species of *Selaginella* (Pteridophyta). *Annals of Botany* **95**: 271–276.
- de Melo NF, Guerra M. 2003. Variability of the 5S and 45S rDNA sites in *Paspiflora* L. species with distinct base chromosome numbers. *Annals of Botany* **92**: 309–316.
- Miller CN. 1977. Mesozoic conifers. *Botanical Review* **43**: 217–280.
- Mirov NT. 1967. *The genus Pinus*. New York: Ronald Press.
- Moscone EA, Klein F, Lambrou M, Fuchs J, Schweizer D. 1999. Quantitative karyotyping and dual-color FISH mapping of 5S and 18S-25S rDNA probes in the cultivated *Phaseolus* species (Leguminosae). *Genome* **42**: 1224–1233.
- Murray BG. 1998. Nuclear DNA amounts in gymnosperms. *Annals of Botany* **82** (Suppl. A): 3–15.
- Nkongolo KK, Kim NS, Michae P. 2004. Detection and physical mapping of the 18S-5.8S-26S rDNA and the pKFJ660 probe with microsatellite sequences derived from the rice blast fungus (*Magnaporthe grisea*) in conifer species. *Hereditas* **140**: 70–78.
- Ohri D, Khoshoo TN. 1986. Genome size in gymnosperms. *Plant Systematics and Evolution* **153**: 119–132.
- Pederick LA. 1970. Chromosome relationships between *Pinus* species. *Silvae Genetica* **19**: 171–180.
- Rooney AP, Ward TJ. 2005. Evolution of a large ribosomal RNA multi-gene family in filamentous fungi: birth and death of a concerted evolution paradigm. *Proceedings of the National Academy of Sciences of the USA* **102**: 5084–5089.
- Saylor LC. 1972. Karyotype analysis of the genus *Pinus*—subgenus *Pinus*. *Silvae Genetica* **21**: 155–163.
- Saylor LC. 1983. Karyotype analysis of the Genus *Pinus*—subgenus *Strobos*. *Silvae Genetica* **32**: 119–124.
- Sterky F, Bhalerao RR, Unneberg P, Segerman B, Nilsson P, Brunner AM, et al. 2004. A *Populus* EST resource for plant functional genomics. *Proceedings of the National Academy of Sciences of the USA* **101**: 13951–13956.
- Strauss SH, Doerksen AH. 1990. Restriction fragment analysis of pine phylogeny. *Evolution* **44**: 1081–1096.
- Taketa S, Ando H, Takeda K, Ichii M, von Bothmer R. 2005. Ancestry of American polyploid *Hordeum* species with the I genome inferred from 5S and 18S-25S rDNA. *Annals of Botany* **96**: 23–33.
- Troitsky AV, Melekhovets Y, Rakhimova GM, Bobrava VK, Valiejo-Roman KM, Autonov AS. 1991. Angiosperm origin and early stages of seed plant evolution deduced from rRNA sequence comparisons. *Journal of Molecular Evolution* **32**: 253–261.
- Vaio M, Speranza P, Valls JF, Guerra M, Mazzella C. 2005. Localization of the 5S and 45S rDNA sites and cpDNA sequence analysis in species of the Quadrifaria group of *Paspalum* (Poaceae, Paniceae). *Annals of Botany* **96**: 191–200.
- Wakamiya I, Newton RJ, Johnston JS, Price HJ. 1993. Genome size and environmental factors in the genus *Pinus*. *American Journal of Botany* **80**: 1235–1241.
- Wang X-R, Tsumura Y, Yoshimaru H, Nagasaka K, Szmidt AE. 1999. Phylogenetic relationships of Eurasian pines (*Pinus*, Pinaceae) based on chloroplast *rbcl*, *matK*, *rpl20-rps18* spacer, and *trnV* intron sequences. *American Journal of Botany* **86**: 1742–1753.