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Chromosomal localization of 5S and 18S–5.8S–25S ribosomal DNA sites in five Asian pines using fluorescence in situ hybridization

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Abstract Fluorescence in situ hybridization (FISH) was employed on mitotic metaphase chromosome preparations of five Asian *Pinus* species: *Pinus tabuliformis*, *Pinus yunnanensis*, *Pinus densata*, *Pinus massoniana* and *Pinus merkusii*, using simultaneously DNA probes of the 18S rRNA gene and the 5S rRNA gene including the non-transcribed spacer sequences. The number and location of 18S rDNA sites varied markedly (5–10 pairs of strong signals) among the five pines. A maximum of 20 major 18S rDNA sites was observed in the diploid genome (2n = 24) of *P. massoniana*. The 5S rDNA FISH pattern was less variable, with one major site and one minor site commonly observed in each species. The differentiation of rDNA sites on chromosomes among the five pines correlates well with their phylogenic positions in *Pinus* as reconstructed from other molecular data. *P. densata*, a species of hybrid origin, resembles its parents (*P. tabuliformis* and *P. yunnanensis*), including some components characteristic of each parent in its pattern. However, the species is unique, showing new features resulting possibly from recombination and genome reorganization.

Keywords FISH · Gymnosperm · Hybrid genome · *Pinus* · rDNA organizastion

Introduction

Fluorescence in situ hybridization (FISH) is an excellent tool for chromosome identification and studies of genome organization, chromosome evolution, cytotaxonomy and introgression (Baum and Appels 1992; Jiang and Gill 1994; Castilho and Heslop-Harrison 1995; D'Hont et al. 1998; Zoldos et al. 1999). In plants, physical localization of the tandemly repeated genes encoding the 18S–5.8S–25S ribosomal RNA (18S–25S rDNA) and 5S ribosomal RNA genes (5S rDNA) has provided a group of valuable chromosome landmark markers. The two classes of rDNA are a major type of repetitive sequence in the higher plant genome. In higher eukaryotes, the 5S and 18S–5.8S–25S rRNA genes are relatively independent of each other and they are often organized into separate loci that are located on different chromosomes or at different positions on the same chromosome (Sastri et al. 1992; Badaeva et al. 1996; Lubaretz et al. 1996; Brown and Carlson 1997). In addition, the number of 5S rDNA loci differs from that of the 18S–25S loci in the same individual genome (Appels and Baum 1991; Lubaretz et al. 1996).

In gymnosperms, rDNA loci have been localized on chromosomes of a limited number of species from a few genera of Pinaceae, including *Pinus* (Gorman et al. 1992; Hizume et al. 1992; Karvonen et al. 1993; Doudrick et al. 1995), *Picea* (Lubaretz et al. 1996; Brown and Carlson 1997; Hizume et al. 1999), *Larix* (Lubaretz et al. 1996) and *Pseudotsuga* (Hizume and Akiyama 1992; Amarasinghe and Carlson 1998), together with *Zamia* and *Ceratozamia* of the Cycadeceae family (Tagashira and Kondo 2001). Results from these studies show that the rDNA organization in gymnosperms varies markedly among species. In diploid gymnosperm genomes, the number of major 18S–25S rDNA loci can vary between 3 and 10 pairs (Doudrick et al. 1995; Lubaretz et al. 1996; Brown and Carlson 1997; Tagashira and Kondo 2001). The number of 5S rDNA loci also varies considerably among species: 2–6 sites are commonly observed for Pinaceae species (Doudrick et al. 1995; Lubaretz et al. 1996; Brown and Carlson 1997; Hizume et al. 1999). However, there are exceptions, such as *Pinus radiata,* in which 5S rDNA sites have been detected on all of its 24 chromosomes (Gorman et al. 1992). The marked varia-

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tion in rDNA loci number and location among *Pinus* species could provide valuable information reflecting their evolutionary pathways. To-date, 18S–25S rDNA FISH data is available for only five *Pinus* species, and the localization of both 18S–25S and 5S rDNA has been done in only three species (*Pinus elliotii*, *P. radiata*, *Pinus sylvestris*, see Table 2). From the existing data it is difficult to generalize the patterns of rRNA gene distribution and organization in the *Pinus* genome. More studies involving more species are needed for a better understanding of the rDNA organization and evolution in the genus.

In the present study we report rDNA FISH results for five Asian pines from the subgenus *Pinus* (Diploxylon pines). We included *Pinus densata* in this study due to its hybrid nature. Previous morphological, allozyme and chloroplast (cp) DNA data all support the hybrid origin of the species, with *Pinus tabuliformis* and *Pinus yunnanensis* being its putative parents (Wang and Szmidt 1994; Wang et al. 2001). We anticipated that rDNA FISH analysis of the three species would help to identify their homologous chromosomes and, thus, provide additional important evidence for the genetic origins of the hybrid genome. *Pinus massoniana* is a close sister species to *P. tabuliformis* and *P. yunnanensis*. *Pinus latteri* is a species recognized by the Flora of China (Anonymous 1978) that has a very limited distribution in the extreme southeast of China neighboring Vietnam, Laos and Burma. This species has been regarded as *Pinus merkusii*, or a variety of it (*P. merkusii* var. *tonkinensis*) by some authors (see Flora of China, Anonymous 1978). For convenience, we regard *Pinus latteri* as *P. merkusii* in further discussions in this report. *P. merkusii* is the most divergent species among the Asian diploxylon pines (Liston et al. 1999; Wang et al. 1999). Our aim was to characterize the rDNA loci in these five pines through simultaneous use of 18S and 5S rDNA FISH probes. The rDNA organization pattern should shed new light upon the genome evolution and phylogenetic relationships of this group of pines.

Materials and methods

Chromosome slide preparation

Five pine species were selected for this study and seeds of each species were collected from natural stands (Table 1). The seeds were germinated in Petri dishes on moist filter paper at 25 °C. The root tips, grown to a length of 1 cm, were excised and pretreated with 0.1% colchicine for 10 h, fixed in Carnol's Fixative (ethanol:acetic acid = 3:1, v/v) for 24 h, and macerated with a mixture of 1% pectolyase Y-23 (Yakult, Japan) and 2% cellulase R-10 (Yakult, Japan) at 37 °C for 25 min. The chromosome spreads were prepared by conventional squashing.

Probe preparation

Genomic DNA of *P. densata* was isolated from seeds by the CTAB method and used as a template for PCR amplification of rDNA probes. 18S rDNA was amplified with the primers 5′-CTAGAGCTAATACGTGCAAC-3′ and 5′-GATAAGGTTCAGTGGACTTC-3′ (Troitsky et al. 1991). The PCR program 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′-CCCATCCGTGTACTACTCTC-3′ (Amarasinghe and Carlson 1998). The PCR program for 5S rDNA amplification was almost the same as for 18S rDNA, except the annealing temperature was 60 °C. The PCR products were separated in 1.5% agarose gels and the rDNA bands were cut out, recovered and purified using a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). The purified rDNA preparations were re-amplified by the same PCR primers and then labeled by the random priming method using a DIG DNA Labeling and Detection Kit (Roche) following the manufacturer's instructions. Biotin-16-dUTP (Roche) was used for labeling 18S rDNA and digoxigenin (DIG)-11-dUTP (Roche) for 5S rDNA labeling.

In situ hybridization

The procedures of Leitch et al. (1994) for in situ hybridization were adopted in this study with minor modifications. The chromosomal slides were treated with 100 µl of RNase (100 µg/ml in 2 × SSC) at 37 °C for 1 h and then soaked twice for 5 min in 2 × SSC. The chromosomes were then digested with proteinase K (1 µg/ul) at 37 °C for 10 min, followed by 10-min fixation in 4% paraformaldehyde. The slides were dehydrated in a series of 70%, 95% and 100% ethanol and then air-dried. A 20-µl hybridization solution was added to each slide, consisting of 10% dextran sulfate, 0.1% SDS, 50% formamide, 10 ng/µl of sheared salmon sperm DNA, 2 ng/µl of denatured probes and 2 × SSC. The slides were placed in a moisture chamber, denatured at 85 °C for 13 min, then immediately put into a hybridization oven and hybridized at 37 °C overnight. The slides were then washed with 20% formamide in 0.1 × SSC at 42 °C for 10 min, followed by washing with 2 × SSC at 37 °C for 2 × 5 min, and 2 × SSC at room temperature for 2 × 5 min. Using the above conditions, a 85% hybridization and washing stringency is achieved. In situ hybridization was repeated on 3–5 chromosome spreads for each species.

Before detection, the slides were soaked in Washing Buffer (0.2% Tween 20 in 4 × SSC) at room temperature for 5 min, and then in Detecting Buffer (5% BSA in Washing Buffer) at 37 °C for 30 min. The DIG-labeled signals (5S rDNA sites) were detected using anti-DIG-rhodamine (Roche), and the biotin-labeled signals (18S rDNA sites) were detected using avidin-FITC (Sigma). The slides were counter-stained with 100 µl (2 µg/ml) of DAPI, and observed under a Leica fluorescent microscope (Leica, DMRBA)

Table 1 *Pinus* species included in the study. All species belong to the subgenus *Pinus* Sect. *Pinus*, Subsect. *Sylvestres*

Species	Origin	Altitude (m a.s.l.)
P. tabuliformis Carr.	Songshan, Beijing, China	1,000
P. densata Mast.	Linzhi, Tibet, China	2,800
P. yunnanensis Franch.	Dali, Yunnan, China	1,500
P. massoniana Lamb.	Zhangping, Fujian, China	600
P. latteri Mason (*P. merkusii* Jungh. et de Vriese)	Baisha, Hainan, China	700

using lenses 100 × 10. The microphotographs were taken using 0.32 coefficient of magnification with Kodak Ektachrome 400 film. The images were scanned into a computer using a ScanWit 2720S (Acer) scanner at 2,700 dpi resolution. The scanned images were adjusted using Photoshop 6.0 (Adobe Systems Inc.) to enhance the contrast.

Chromosome length measurement

Chromosome arm lengths were measured on the scanned computer images. Ten cells from each of 2–3 chromosome spreads of each species were measured. The arm ratio was calculated for each chromosome and the average values were used to construct the ideogram. The chromosomes were assigned consecutively according to their lengths, with the longest being assigned as I and the shortest as XII. In case of equal length, the chromosome with longer short arm was assigned first. Apart from chromosome length and arm ratio, the constrictions and FISH patterns are also informative characters for the karyotyping. The presence of rDNA loci on each chromosome is indicated on the ideogram. However, the mapped positions are not precise quantitative representations, although the relative distance of a rDNA site to the centromere and telomere was considered while constructing the ideogram.

Results

The sizes of the PCR-amplified 18S rDNA and 5S rDNA fragments were approximately 1.8 kb and 700 bp, respectively, similar to those reported previously in conifers (Karvonen et al. 1993; Amarasinghe and Carlson 1998). In situ hybridizations using 18S and 5S probes revealed signals of different intensity. The weak signals can be distinguished from background noise by their band-like appearance while the noise signals are usually dot-like. In addition, only the reproducible signals from 3 to 5 FISH replicates of each species were registered. An example of 18S and 5S FISH pattern in each pine is shown in Fig. 1 and an ideogram of the karyotypes is presented in Fig. 2. The chromosome number of all the five pines was the same, 2n = 24, with a submedian centromere karyotype in which chromosomes XI and XII were markedly shorter than the others (Figs. 1 and 2), corresponding to observations by Saylor (1964). However, the chromosomal distribution pattern of the 18S rDNA varied markedly among the different pine species.

In *P. tabuliformis* the 18S rDNA sites were distributed among eight pairs of chromosomes and a total of 12 pairs of signals were detected: seven pairs of strong signals in the interstitial regions of the long arms of chromosomes I, II and VI, and the short arms of chromosomes IV, V, IX and X, and five pairs of weak signals in the centromeric regions of chromosomes I, II, IV, V and XII (Figs. 1a and 2a).

In *P. densata* seven pairs of the chromosomes were found to carry 18S rDNA sites. Five pairs of strong sites were localized in the interstitial regions of the short arms of chromosomes IV, VI, IX and X, and the long arm of chromosome II. Four other pairs of signals were weak and were found at the centromeric regions of chromosomes I, IV and XII and the interstitial area of the long-arm of chromosome I (Figs. 1c and 2b).

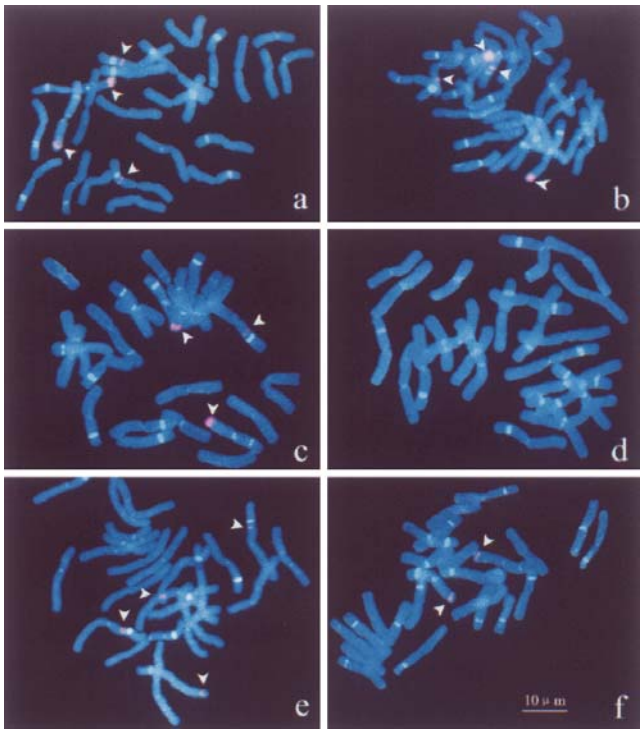

Fig. 1a–f FISH localization of 18S rDNA (*green*) and 5S rDNA (*red*) on chromosomes of (**a**) *P. tabuliformis*, (**b** and **d**) *P. yunnanensis* (**d** hybridized only with 18S rDNA), (**c**) *P. densata*, (**e**) *P. merkusii* and (**f**) *P. massoniana*. *Arrowheads* show the 5S rDNA signals

In *P. yunnanensis* eight pairs of the chromosomes were found to host 18S rDNA sites. Eight pairs of strong signals were found in the interstitial regions of the short arm of chromosomes IV, V, VI, IX and X, the long arm of chromosome II and the centromere regions of chromosomes VII and XII. Two pairs of weak signals were detected at the centromere regions of chromosomes II and IV (Figs. 1b, d and 2c).

In *P. massoniana* ten pairs of strong 18S rDNA signals were detected on nine pairs of chromosomes. Sites were observed in the interstitial regions of the short-arms of chromosomes I, V, VI, IX and X, the long arm of chromosome II, and the centromere regions of chromosomes VII and XII. Chromosome IV carried two sites, one in the interstitial region of its short arm and the other at the centromeric region (Figs. 1f and 2d).

In *P. merkusii*, 18S rDNA signals were visualized on seven pairs of the chromosomes. Six strong pairs of signals were found in the interstitial regions of the short-arms of chromosomes I, II, IV and IX, and the centromere region of chromosomes VII and XII. Two pairs of weak signals were located at the centromeric region of chromosomes I and V (Figs. 1e and 2e).

Unlike the 18S rDNA FISH pattern, the 5S rDNA locations were relatively constant among the five pines (Figs. 1 and 2). A major pair of 5S rDNA signals was detected in the terminal region of the long arm of chromosome IV of *P. tabuliformis*, *P. densata P. yunnanensis*

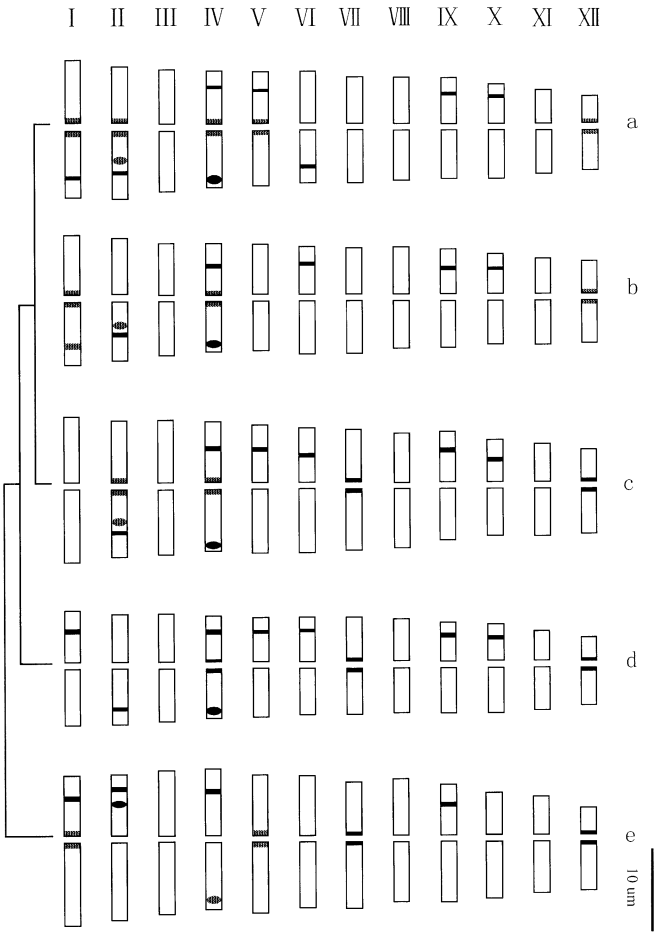

Fig. 2 Ideogram of (**a**) *P. tabuliformis*, (**b**) *P. densata*, (**c**) *P. yunnanensis*, (**d**) *P. massoniana* and (**e**) *P. merkusii*. *Solid dots and bands* represent strong signals of 5S and 18S rDNA, respectively. The *shadowed dots and bands* represent the weak signals. The phylogenetic relationships were simplified from Wang et al. (1999)

and P. massoniana. In *P. merkusii*, however, this site was much weaker and a strong 5S rDNA site was detected on the short arm of chromosome II in close proximity to the 18S rDNA site on the same arm. In *P. tabuliformis* and *P. yunnanensis* an additional pair of weak 5S signals was found in the interstitial region of the long arm of chromosome II, closer to the centromere than the 18S rDNA site on the same arm. Interestingly, in *P. densata* the weak signal was observed only on one homologue of chromosome II, giving a total of three 5S rDNA sites in its diploid genome.

Discussion

The distribution patterns of rDNA loci among the chromosomes vary markedly between angiosperms and gymnosperms. In diploid angiosperms the number of major 18S–25S rDNA sites typically ranges between 1 and 5 pairs (e.g. Ricroch et al. 1992; Castilho and Heslop-Harrison 1995; Linares et al. 1996; Zhang and Sang 1999). In gymnosperms, 6–8 pairs are commonly found (Table 2). Our studies of the five *Pinus* species detected 5–10 pairs of major 18S–25S rDNA sites in each of the diploid genomes, slightly higher than the corresponding numbers reported in *Picea*, which ranged between 5 and 7 pairs in the diploid genomes (Table 2). These results show that gymnosperms harbor more rDNA sites than angiosperms. The difference is, presumably, related to the evolutionary divergence between angiosperm and gymnosperm genomes, and partly to the much-larger genome size in gymnosperms. The estimated haploid genome size of *Picea* species is 8.5×10^9 bp (Dhillon 1987). Pines have even larger genomes than other conifers, with estimates of approximately 10^{10} bp for the haploid nucleus (Miksche 1985; Ohri and Khoshoo 1986; Wakamiya et al. 1993; Murray 1998; Elsik and Williams 2000). This huge genome is largely (about 86%) composed of repetitive DNA, together with many high-copy retro-element families (Miksche 1985; Kamm et al. 1996; Kossack and Kinlaw 1999; Elsik and Williams 2000). rDNA is one of the major components of the repetitive DNA in the genome. In *Picea mariana* and *Picea rubens* rDNA comprises approximately 4% of the genome (Bobola et al. 1992). Thus, it is not surprising to discover a higher number of rDNA sites in Pinaceae in general and in *Pinus* in particular.

In angiosperms the 5S rDNA arrays tend to be localized at one or a few sites on one to three homologous pairs of chromosomes (Murata et al. 1997; Benabdelmouna et al. 2001; Hasterok et al. 2001). Unlike 18S–25S rDNA, the 5S rDNA loci pattern in gymnosperms does not differ much from that in angiosperms and 1–2 pair(s) of main sites are commonly observed in *Pinus* and *Picea* species (Table 2). Our results from the five pines are consistent with the 5S rDNA patterns of most Pinaceae species.

Use of double- or multiple-probe FISH facilitates the identification of homologous chromosomes and the inference of changes in genome structure among species. It also helps to clarify genetic maps and to assign linkage groups to physically marked chromosomes. By comparing the rDNA FISH patterns among *P. sylvestris*, *Picea abies* and *Larix decidua* Lubaretz et al. (1996) identified homologous chromosomes among the three species. Analysis of the linkage pattern of the 18S–25S and 5S rDNA in the five pines we studied revealed one or two chromosomes in each species that harbored loci for both classes of rDNA. There were two types of linkage patterns. In one, the 18S–25S and 5S rDNA were located in opposite arms of the same chromosome, with 5S rDNA in the long arm and the 18S–25S rDNA in the short arm interstitial region. In the other, both rDNA loci were on the same chromosome arm, with the 5S rDNA site closer to the centromere. The former pattern is more common as it was observed in *P. tabuliformis*, *P. densata*, *P. yunnanensis* and *P. massoniana,* while the latter was observed only in *P. merkusii*. Chromosome IV carries loci for both genes in all the five species, although the 5S site in *P. merkusii* is much weaker. Thus, these chromosomes are

Table 2 18S–5.8–25S rDNA and 5S rDNA sites in the diploid genome of various Pinaceae species

Species	18S–25S rDNA	5S rDNA	References
Picea abies (L.) Karst.	12	2	Lubareta et al. 1996
Picea crassifolia Komar.	10 or 12	2 strong and 2 weak signals	Hizume et al. 1999
Picea koraiensis Nakai	12 or 14	2 strong and 2 weak signals	
Picea glauca (Moench) Voss.	14	2	Brown and Carlson 1997
Picea sitchensis (Bong) Carr.	10	2	
Pseudotsuga menziesii (Mirb.) Franco	12	2	Hizume and Akiyama 1992; Amarasinghe and Carlson 1998
Larix decidua Mill.	6	2	Lubareta et al. 1996
Pinus elliotii Engelm.	16	2 strong and 4 weak signals	Doudrick et al. 1995
Pinus radiata D. Don	>10	>24	Cullis et al. 1988; Gorman et al. 1992
Pinus sylvestris L.	>16, 14	4	Karvonen et al. 1993; Lubareta et al. 1996
Pinus densiflora Sieb. et Zucc.	14	–	Hizume et al. 1992
Pinus thunbergii Parl.	12	–	
Pinus tabuliformis Carr.	14 strong and 10 weak signals	2 strong and 2 weak signals	Present study
Pinus densata Mast.	10 strong and 8 weak signals	2 strong and 1 weak signals	Present study
Pinus yunnanensis Franch.	16 strong and 4weak signals	2 strong and 2 weak signals	Present study
Pinus massoniana Lamb.	20 strong signals	2 strong signals	Present study
Pinus latteri Mason (*P. merkusii* Jungh. et de Vriese)	12 strong and 4 weak signals	2 strong and 2 weak signals	Present study

likely homologous. Similarly, chromosome II of *P. tabuliformis*, *P. yunnanensis* and *P. densata*, characterized by the existence of a major 18S–25S site and a minor 5S site on the long arm, are probably homologous.

The 18S rDNA hybridization revealed weak signals in the centromeric region of many chromosomes in each pine. This result correlates with the finding in *Pinus densiflora* showing that the centromeric region contains 18S and 25S rDNA sequences (Hizume et al. 2001). The weak signals observed in this study would indicate low copy numbers of 18S–25S rDNA repeats in this region. Another hypothesis that could account for these weak signals is that these sites are remnants of primary sties of 18S–25S rDNA that once existed at the centromeres but later moved out to distal sites by large-scale chromosome rearrangements.

Divergence and speciation are often accompanied by reorganization in the chromosomes. However, the karyotypes of *Pinus* species are highly conserved (Khoshoo 1961; Saylor 1964, 1972; Pederick 1970) and it is difficult to differentiate closely related species, such as species of the same Section, by karyotyping. In contrast, rDNA FISH gives much higher resolution for detecting chromosome variations among closely related species. Our results and other studies (Gorman et al. 1992; Hizume et al. 1992; Doudrick et al. 1995; Lubaretz et al. 1996) have shown that within the genus *Pinus* the number and positions of major rDNA sites on chromosomes vary among species. This variation should have phylogenetic implications, since the more similar rDNA FISH patterns are likely to be in the most closely related the taxa. Among the five Asian diploxylon pines *P. merkusii* appeared to be the most divergent. It had a major 5S and 18S–25S rDNA site on the same arm of chromosome II, similar to the pattern observed in *Picea* (Lubaretz et al. 1996; Brown and Carlson 1997; Hizume et al. 1999). In contrast, the major 5S and 18S–25S rDNA site were on opposite arms of chromosome IV in all the other four pines. This indicates that chromosome organization differs in *P. merkusii*. Phylogenetic studies based on cpDNA RFLP (Wang and Szmidt 1993), cpDNA sequences (Wang et al. 1999) and rDNA ITS sequences (Liston et al. 1999) have all demonstrated *P. merkusii* to have a position distinct from the other Asian diploxylon pines. Laser flow cytometry analysis has shown that *P. merkusii* also has a larger genome than other Asian diploxylon pines (Joyner et al. 2001). All these data consistently indicate a pronounced divergence of *P. merkusii* from the other Asian members of the subsection *Sylvestres*. Its unique position is attributed to early separation and prolonged isolation from other Asian pines (Szmidt et al. 1996). *Pinus massoniana* is a close sister species to *P. tabuliformis* and *P. yunnanensis*. Their rDNA FISH patterns share more similarity to each other than with *P. merkusii*. These findings correlate well with the DNA sequence based phylogeny, and demonstrate that important phylogenetic information can be obtained by FISH analysis.

Comparison of the 5S rDNA sites in eight *Pinus* species showed that *P. radiata* differed distinctly from the others by having 5S rDNA sites on all chromosomes, as compared to 1–3 pairs of sites in other pines (Table 2). *P. elliottii* had three pairs while all the analyzed Asian diploxylon pines had 1–2 pairs of 5S rDNA sites. Ac-

cording to the classification by Little and Critchfield (1969), *P. radiata* belongs to the subgenus *Pinus*, section *Pinus,* subsection *Oocarpae*, and *P. elliottii* to the subsection *Australes*. These two subsections are distant from the subsection *Sylvestres,* to which all the Asian diploxylon pines belong, according to rDNA ITS-based phylogeny (Liston et al. 1999). However, a different methodology employing radioactive probe hybridization was used in the *P. radiata* study (Cullis et al. 1988; Gorman et al. 1992). In addition, the hybridization and washing stringency used in that study were lower than the ones employed in the present study. Thus, it is difficult to conclude if this difference in 5S rDNA sites is characteristic of each subsection or arises from differences in detection techniques. More species need to be surveyed using comparable methods to determine the variation in 5S rDNA patterns across the genus.

Allozyme and cpDNA data have shown that *P. densata* originated through hybridization between *P. tabuliformis* and *P. yunnanensis* (Wang and Szmidt 1994; Wang et al. 2001). It has a diploid genome with 2n = 24, as do its parents. Thus, the evolution of *P. densata* occurred not through polyploidy but through homoploid hybrid speciation without duplication in chromosome numbers. Careful analysis of the rDNA FISH patterns showed certain shared similarities between *P. densata*, *P. tabuliformis* and *P. yunnanensis*. Chromosomes I, VII and XII of *P. densata* resemble the corresponding chromosomes of *P. tabuliformis*. The chromosome VI of *P. densata*, however, has the same FISH pattern as the corresponding chromosome of *P. yunnanensis*. For chromosomes II, IV, IX and X, the three species had the same rDNA FISH pattern, thus these chromosomes are likely homologous chromosomes among the three species. Apart from these shared similarities, *P. densata* differed from both parents in several respects, and the number of chromosomes bearing rDNA sites in the hybrid is not simply the sum of the numbers of sites in its parents. We noticed that the 5S rDNA site on the two homologues of chromosome II in *P. densata* is not symmetric. One homologue gave a clear, but weak, signal while the other appeared to give no signal. In addition, chromosome V of *P. densata* differed from the corresponding chromosomes in both *P. tabuliformis* and *P. yunnanensis* as the major 18S rDNA site on the short arm of the two parental species is missing from *P. densata*. The loss of a site in the hybrid genome could have resulted from events such as chromosome rearrangement, unequal crossing-over, gene conversion or retro-transpositional events (Leitch and Heslop-Harrison 1993; Castilho and Heslop-Harrison 1995; Taketa et al. 1999; Hasterok et al. 2001). In addition, genome dormancy, transcriptive inactivity and suppression have also been invoked to explain changes in rDNA sites and reductions in signal intensity (Maluszynska and Heslop-Harrison 1993; Lewis et al. 1996; Taketa et al. 1999; Singh et al. 2001). All these findings indicate that the genome of *P. densata* has undergone changes since its speciation through hybridization. *P. densata* as a hybrid is not simply the result of the addition/combination of the parental genomes. In the course of evolution, the hybrid genome appears to have undergone structural reorganization and recombination and, thus, formed unique characteristics as a new species. For more information on the extent of genome reorganization and, thus, better understanding of the homoploid nature of its hybrid speciation, more markers along the chromosomes and linkage maps, both genetic and physical, would be of great value.

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