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# Chromosome identification and comparative karyotypic analyses of four *Pinus* species

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**Abstract** Chromosomal landmarks in four *Pinus* species: *P. densiflora*, *P. thunbergii*, *P. sylvestris*, and *P. nigra* were identified by fluorescence *in situ* hybridization (FISH) using hapten- or fluorochrome-labeled probes for the plant telomere repeat, centromeric repeat (*PCSR*), and rDNA. FISH landmarks were located at the interstitial and proximal regions of chromosomes and allowed us to identify nearly all of the homologous chromosomes in each species. A comparative analysis of the FISH karyotypes among the four species showed that the interstitial FISH signals obtained by hybridization with the telomere and rDNA sequences were stable and could be used to identify homologous chromosomes among species. The identification of homologous chromosomes among species facilitated a detailed comparative karyotype analysis. The results suggest that the degree of chromosomal differentiation among the four *Pinus* species is very low and that the proximal regions vary in their DNA sequences. The similarities and differences among FISH karyotypes are discussed in relation to phylogeny.

**Keywords** Chromosome identification · Comparative karyotype analysis · Multicolor FISH · *Pinus*

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## Introduction

The genus *Pinus* is a major constituent of forests in the Northern Hemisphere that supply timber resources. A study of the *Pinus* genome is underway as part of ongoing efforts to improve the growth and quality of wood and the resistance of trees to disease and harmful insects. Consequently, gene linkage maps have been constructed in several species (Remington et al. 1999; Sewell et al. 1999; Costa et al. 2000; Kondo et al. 2000; Kubisiak et al. 2000; Li and Yeh 2001). However, elucidation of the connections between linkage groups and individual chromosomes in different species has been hampered by problems with the identification of chromosomes using cytological methods.

The conventional karyotypes of many *Pinus* species (Saylor 1964, 1972, 1983; Hizume 1988) confirmed that chromosomes comprising a karyotype are similar morphologically. However, the identification of homologous pairs proved difficult, and this prevented precise comparative karyotype analyses of *Pinus* species. Several attempts at chromosomal identification and karyotyping have been reported, including examinations of the small constrictions in the haploid complement (Pederick 1997), C-banding (Borzan and Papes 1978), G-banding (Drewry 1982), and fluorescence banding (Hizume et al. 1983). These procedures were not adopted in many species because of their low reproducibility. Fluorescence banding has been used successfully for chromosomal identification in some *Pinus* species (Hizume et al. 1983, 1989, 1990); of the three Japanese species studied, *P. thunbergii* and *P. luchuensis* showed very similar fluorescence-banding patterns and good correspondence of chromosomes. Fluorescence banding is useful in chromosomal identification, but it is a rather cumbersome and time-consuming procedure. A simple and reproducible method for identifying individual chromosomes in *Pinus* karyotypes is needed.

Recently, fluorescence *in situ* hybridization (FISH), which is routinely used to localize DNA probes and identify chromosomes or chromosomal segments, has

been adapted successfully to the analysis of the *Pinus* species. This technique has promising applications with respect to the identification of chromosome pairs in *Pinus*. Some examples of probes that have been used for FISH analysis of *Pinus* chromosomes are listed here. The 45S rDNA was localized at the interstitial region and CMA-bands in *P. densiflora*, *P. thunbergii* (Hizume et al. 1992), *P. elliottii* var. elliottii (Doudrick et al. 1995), *P. sylvestris* (Lubaretz et al. 1996), *P. radiata*, and *P. taeda* (Jacobs et al. 2000). 5S rDNA has been localized to one, two, or three loci, depending on the *Pinus* species (Doudrick et al. 1995; Lubaretz et al. 1996; Hizume and Kondo 2000; Jacobs et al. 2000). A probe derived from the *Arabidopsis*-type telomere sequence detected the terminal-telomeric, interstitial, and centromeric regions (Fuchs et al. 1995; Hizume et al. 2000; Schmidt et al. 2000). The interstitial and centromeric regions that hybridized with the telomere probe corresponded to DAPI-bands. Recently, two DNA clones of *P. densiflora* were localized at the proximal CMA- or DAPI-bands (Hizume et al. 2001). Chromosomal identification in *Pinus* might be achieved by simultaneous application of these probes in FISH experiments.

We developed a multicolor FISH procedure that could be used to identify individual homologous chromosomes in *Pinus*. Karyotype analysis of four *Pinus* species was carried out using this multicolor FISH technique. The identification of homologous chromosomes by their FISH landmarks enabled karyotype comparisons among *Pinus* species.

## Materials and Methods

### Plant materials and chromosome preparation

The seeds of *Pinus densiflora* Sieb. & Zucc., *P. thunbergii* Parl., *P. sylvestris* L., and *P. nigra* Arn. were collected in a natural pine forest on Mt. Tagami Iyo (Ehime Prefecture, Japan), in Masaki (Ehime Prefecture, Japan), in Tribec-Drazovce (Slovak Republic), and in Oravsky Biely Potok (West Highland Tatras, Slovak Republic), respectively. The seeds were sown in sterilized sand in a pot, and 10–14 days later the primary root tips were collected for chromosomal analysis. The root tips were treated with 0.05% colchicine at 20 °C for 10 h, fixed in a chilled solution of ethanol:chloroform:acetic acid (2:1:1) overnight, and then stored in a freezer. Fixed root tips were macerated in an enzyme mixture containing 3% Cellulase Onozuka RS (Yakult), 1.25% pectolyase Y23 (Seishin), and 5 m*M* EDTA in 2× SSC (pH 4.5). The meristematic cells were squashed under a coverslip on a glass slide and air-dried after coverslip removal by the dry-ice method.

### Fluorescence in situ hybridization

The *Arabidopsis*-type telomere sequence repeats $(TTTAGGG)_n$ were polymerase chain reaction (PCR)-amplified using $(TTTAGGG)_5$ and $(CCCTAAA)_5$ primers in the absence of template DNA (Ijodo et al. 1991; Cox et al. 1993). The amplified telomere sequences were labeled with biotin using the BioNick Labeling System (BRL, Gaithersburg, Md.). *Eco*RI-digested wheat 45S rDNA (pTa71; Gerlach and Bedbrook 1979) and 5S rDNA, amplified by PCR from *Rumex acetosa* genomic DNA, were labeled with digoxigenin (DIG) using DIG-High Prime (Roche, Indianapolis, Ind.). Plasmid DNA containing *PCSR* (the proximal CMA-band-specific repeat, clone PDCD501; accession no. AB051860; Hizume et al. 2001) was FITC-labeled using FITC-High Prime (Roche). The probes were dissolved in 2× SSC, 10% dextran sulfate, and 50% formamide. The FISH procedure has been described previously (Shibata et al. 2000). Chromosomal DNA was denatured at 80 °C for 1 min in 70% formamide, 2× SSC. The hybridized probes were detected with Strepetavidin-Cy5 (Amersham Biosciences) and anti-digoxigenin rhodamine conjugate (Roche). The slides were counterstained with DAPI (4,6-diamino-2-phenylidole). The hybridization signals were visualized and recorded using a chilled CCD camera (Sensys 1400, Photometrics), and pseudocolor images were made using IPLab (Scanalytics). Complementary chromosomes were arranged in order of decreasing length.

## Results and discussion

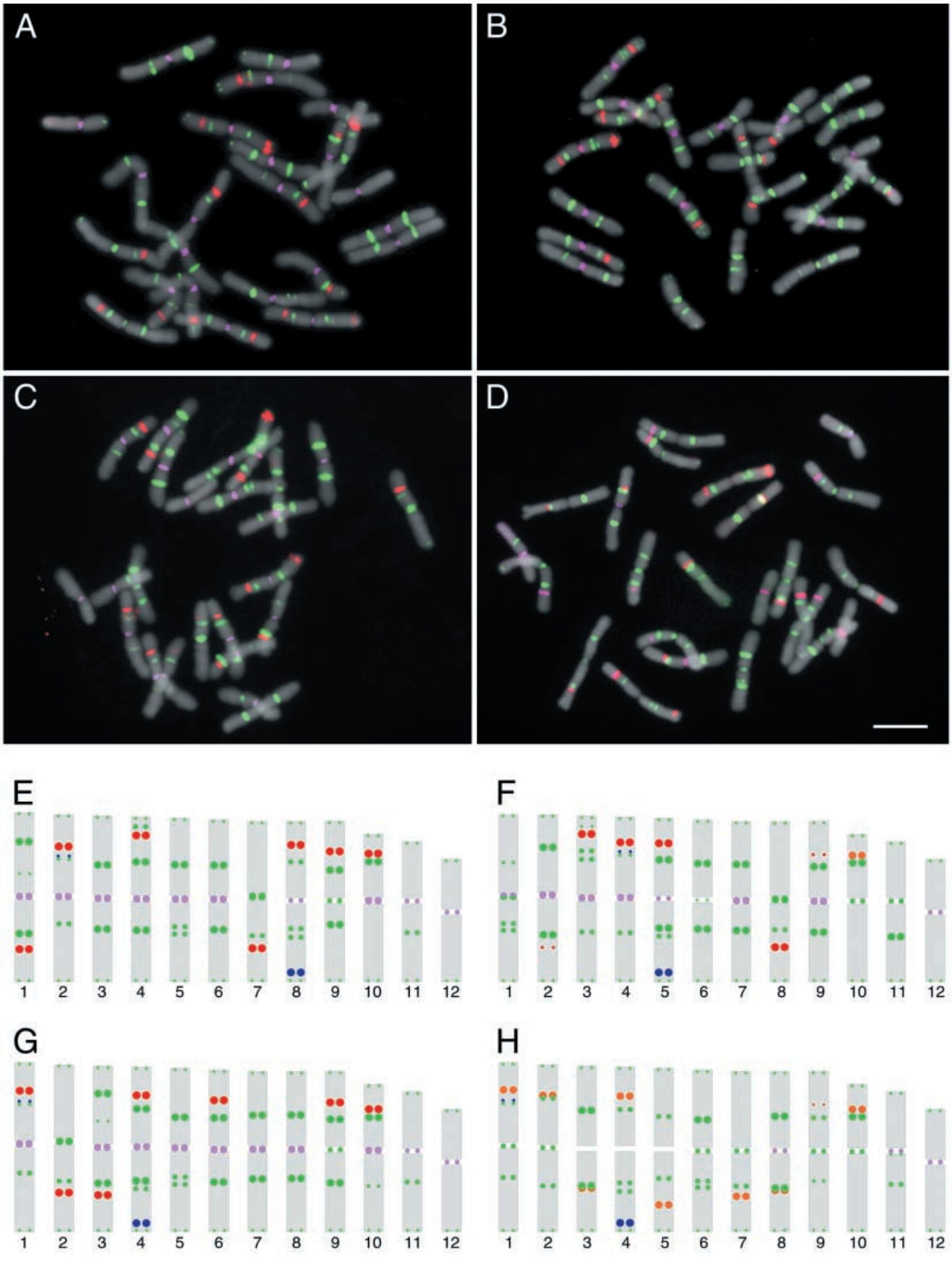
### Multicolor FISH landmarks on *Pinus* chromosomes

The somatic chromosomes of four Eurasian *Pinus* species, *P. densiflora*, *P. thunbergii*, *P. sylvestris* and *P. nigra*, were analyzed by multicolor FISH using probes consisting of the *Arabidopsis*-type telomere sequence, *PCSR*, and a mixture of 45S and 5S rDNA. Pseudocolor images were synthesized for each chromosome by overlaying the FISH signals from each probe on the DAPI-fluorescence images (Fig. 1A–D).

In all four species, the FISH signals from the telomere probe appeared at the interstitial regions of nearly all chromosomes, the proximal or centromeric regions of several chromosomes, and at all chromosome ends (green signals in Fig. 1A–D). This finding corroborates previous studies of *Pinus* species (Fuchs et al. 1995; Hizume et al. 2000; Schmidt et al. 2000). The telomere sequence signals at the interstitial and centromeric regions coincided with DAPI bands (data not shown), as shown previously for *P. elliottii* var. elliottii (Schmidt et al. 2000).

Since the 45S and 5S rDNA were both DIG-labeled in this multicolor FISH, it was necessary to perform a pilot experiment using these probes with distinct labels to differentiate the signals (data not shown). Two 5S rDNA loci of different sizes were detected in all of the species (blue signals in Fig. 1E–H). A strong 5S rDNA signal was observed near the terminal regions of two metacentric chromosomes and a weak signal was observed at the interstitial regions of two other metacentric chromosomes in every species studied. The 5S rDNA signals were good markers for two pairs of long metacentric chromosomes. Intense 45S rDNA signals were observed at all secondary constrictions, and weak signals appeared at some interstitial and proximal regions.

**Fig. 1** FISH images of somatic chromosomes (**A–D**), and ideograms of FISH karyotypes (**E–H**), of *Pinus densiflora* (**A**, **E**), *P. thunbergii* (**B**, **F**), *P. sylvestris* (**C**, **G**), and *P. nigra* (**D**, **H**). **A–D** The *red* signal corresponds to the 45S and 5S rDNA probes, *magenta* represents the *PCSR* probe, and *green* corresponds to the telomere sequence probe. *Bar*: 10 μm. **E–H** The corresponding colors of each dot are: 45S rDNA signal (*red*), 5S rDNA signal (*blue*), proximal *PCSR* signal (*magenta*), telomere sequence signal (*green*), and 45S rDNA signal with *PCSR* signal (*orange*). *Bar*: 10 μm ▶


A
B
C
D
E
F
G
H
1 2 3 4 5 6 7 8 9 10 11 12

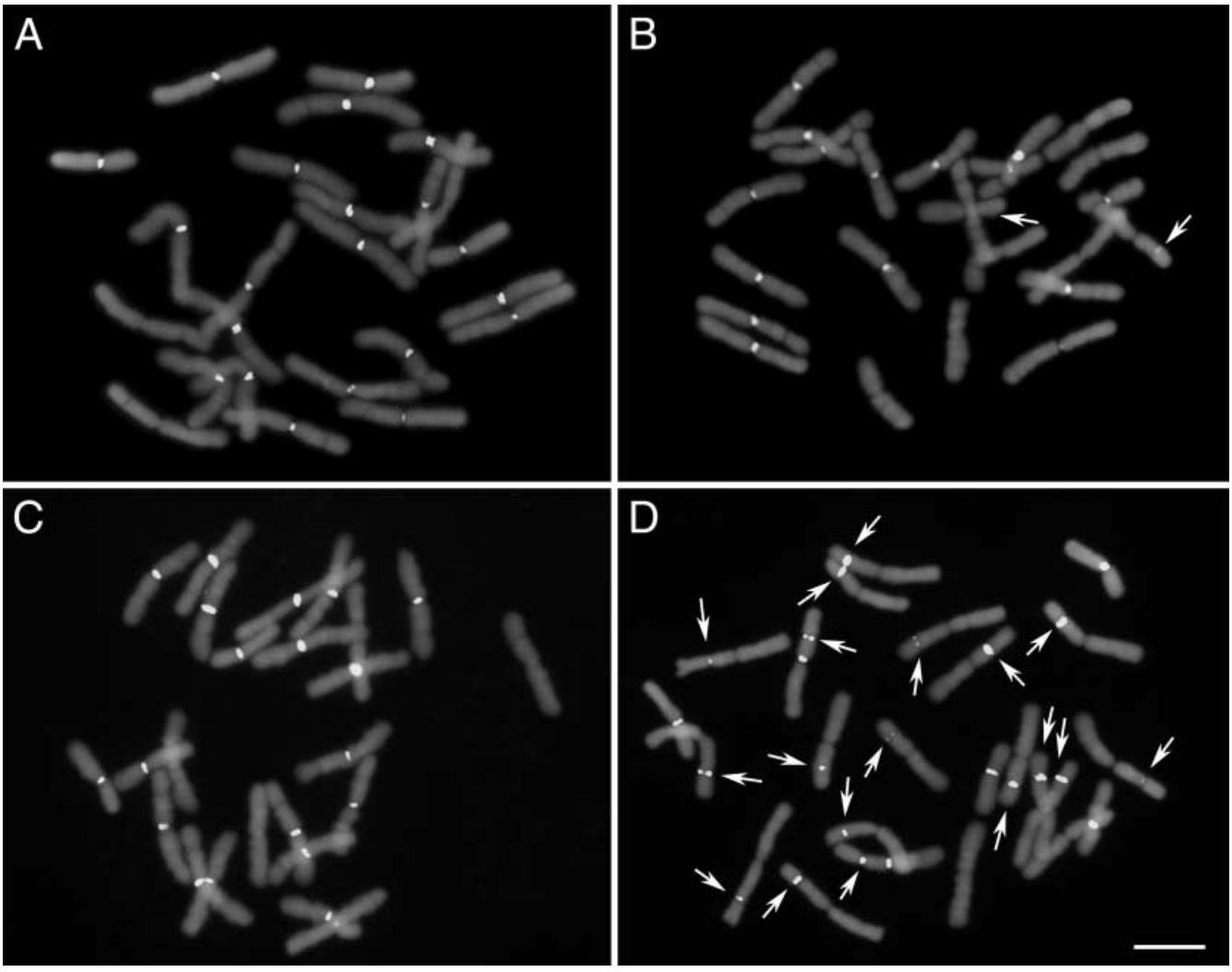



**Fig. 2A–D** FISH signals from the *PCSR* probe with the same metaphase plate as shown in Fig. 1A–D. *P. densiflora* (**A**), *P. thunbergii* (**B**), *P. sylvestris* (**C**), and *P. nigra* (**D**). *Arrows* indicate non-proximal *PCSR* signals. *Bar*: 10 μm

The *PCSR* signals appeared at the proximal and interstitial regions or secondary constrictions in the chromosomes of every species (magenta signals in Figs. 1 and 2). The *PCSR* signals in *P. densiflora* and *P. sylvestris* were observed only on the proximal regions of ten chromosome pairs (Fig. 2A, C). In *P. thunbergii*, eight pairs of chromosomes had proximal *PCSR* signals, and a weak *PCSR* signal appeared at the secondary constriction of chromosome 10 (Fig. 2B). *P. nigra* had *PCSR* signals in the proximal regions of three chromosome pairs and in the secondary constrictions or interstitial regions of nine chromosome pairs (Fig. 2D). In four species, all of the proximal *PCSR* signals, except for those in chromosome 1 of *P. thunbergii*, coincided with the appearance of weak 45S rDNA signals. Our results with *P. densiflora* corroborate those previously reported (Hizume et al. 2001). All of the non-proximal *PCSR* signals in *P. thunbergii* and *P. nigra* coincided with 45S rDNA signals (Fig. 1B, D).

The homologous chromosomes in all four species showed unique FISH signal patterns and nearly all were clearly distinguishable. All of the homologous chromosomes in *P. thunbergii* and *P. nigra* could be identified. Chromosomes 3 and 6 of *P. densiflora* and chromosomes 7 and 8 of *P. sylvestris* were difficult to distinguish because of similarities in signal patterns and chromosome shapes.

Some intraspecific variation in FISH signals was observed for *P. densiflora*, *P. thunbergii*, and *P. sylvestris*. The centromeric *PCSR* signal present in chromosome 10 of *P. densiflora* was replaced with a telomere signal in one of the ten examined chromosomes, and with respect to chromosome 7, two of the ten chromosomes examined had weak interstitial telomere signals on the short arm. In *P. thunbergii*, the centromeric telomere signal on chromosome 11 was replaced with a *PCSR* signal on two of eight chromosomes. In *P. sylvestris*, the interstitial telomere signal of the long arm of chromosome 10 was absent on one of eight chromosomes, and on chromosome 9 the centromeric telomere signal was replaced with a

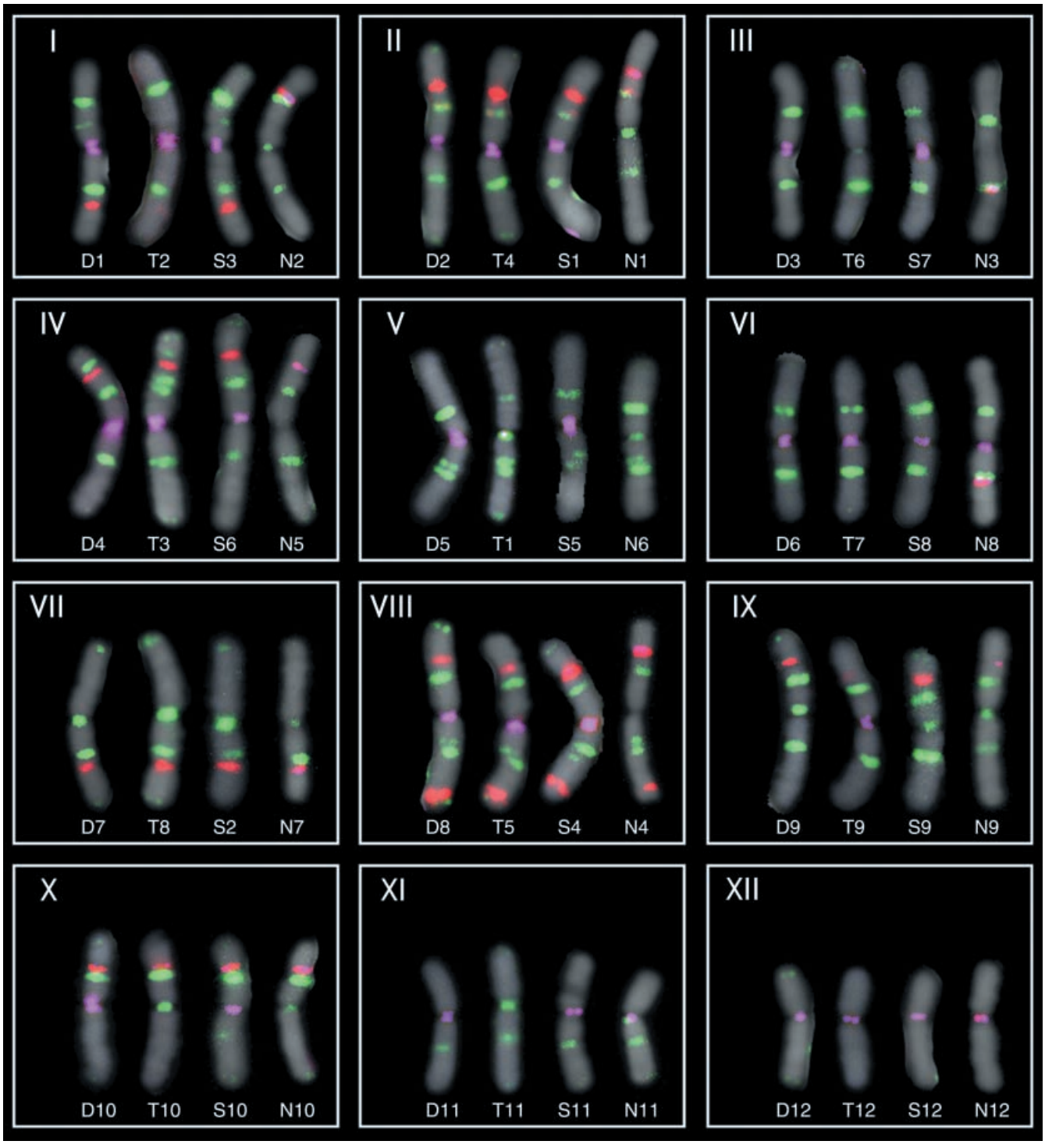



**Fig. 3** Comparison of FISH chromosomes probed with 45S rDNA and 5S rDNA (*red*), *PCSR* (*magenta*) and telomere sequence (*green*) probes in four *Pinus* species. The chromosome groups are numbered *I–XII*. The *P. densiflora* chromosome was selected as a standard, and the chromosomes of the other species were identified based on interstitial FISH signals. The *letters* (*D, T, S, N*) appended to each chromosome number identify *P. densiflora, P. thunbergii, P. sylvestris*, and *P. nigra*, respectively

*PCSR* signal on one of eight chromosomes. The signal strength from the 45S rDNA probe in the interstitial region of the short arm of chromosome 9 varied from strong to very weak among the different chromosomes. The consensus FISH signal patterns for each species are illustrated schematically in Fig. 1E–H.

We compared the FISH signal patterns of the four species and found conspicuous interspecific differences with respect to their centromeric regions. *P. densiflora*, *P. thunbergii*, and *P. sylvestris* had *PCSR* or telomere

**Table 1** Chromosome groups with observed interspecies differences

| | *Pinus sylvestris* | *P. thunbergii* | *P. nigra* |
|---|---|---|---|
| *Pinus densiflora* | IV, X | I, III, IV, V, IX, X, XI | I, II, III, IV, V, VI, VIII, X, XI |
| *P. sylvestris* | | I, III, IV, V, IX, X, XI | I, II, III, IV, V, VI, VIII, X, XI |
| *P. thunbergii* | | | I, II, III, IV, V, VI, VIII, IX, XI |

signals in the centromeric regions of all chromosomes. Chromosomes 3, 4, and 5 in *P. nigra* showed no FISH signals in the centromeric region. The interstitial signal patterns for each chromosome were generally stable, suggesting that these regions would make good landmarks for the determination of homologous or homoeologous chromosomes among *Pinus* species.

## Chromosomal comparisons among four *Pinus* species using FISH landmarks

Based on interstitial FISH signal patterns, nearly all of the homologous chromosomes could be distinguished by FISH karyotyping. The *P. densiflora* karyotype was selected as the standard, and the chromosomes of the other three species were classified into 12 groups (I–XII; Fig. 3). Groups I–IX had long metacentric chromosomes and were difficult to identify by chromosomal morphology. The other groups were easier to identify; group X members had the shortest metacentric chromosomes, and the members of groups XI and XII had short submetacentric chromosomes. We conclude that in the *Pinus* species examined, chromosomes within the same FISH chromosomal group correspond to homologous or homoeologous chromosomes.

*P. densiflora* and *P. sylvestris* were very similar in their FISH signal patterns, and the only differences were observed in chromosome groups IV and X. The *P. thunbergii* FISH signal patterns were similar to those of *P. densiflora* and *P. sylvestris*, but some differences were observed in the FISH signal patterns in the proximal regions of chromosome groups III, V, IX, X, and XI, and in the interstitial regions of chromosome groups I and IV. Chromosome 1 (i.e., chromosome group V) of *P. thunbergii* was unusual in that it displayed a combination of *PCSR* and telomere signals in the centromeric region. On the other hand, the homologous chromosomes of chromosome group V from other species had either *PCSR* signals or telomere signals.

The FISH signal patterns of *P. nigra* differed significantly from those of the other three species. Three chromosome pairs from chromosomes 3, 4, and 5 had no centromeric signals, and a weak signal from the telomere sequence was observed close to the centromeric region of chromosome 11. The 45S rDNA loci on chromosomes 2, 3, and 8 were localized very close to the distal side of the interstitial telomere signals and were unique to *P. nigra*.

The chromosome groups and interspecific differences are shown in Table 1. In spite of differences in FISH signal patterns, the chromosome shapes and the positions of interstitial FISH signals from either the rDNA or telomere sequence probes were highly conserved among the four species examined. The fact that homologous chromosomes of different species were related suggests that the longitudinal organization of chromosomes is not significantly altered during species differentiation.

The comparative analysis of the FISH karyotypes suggests evolutionary relationships for the four species studied. *P. densiflora* and *P. sylvestris* are very closely related to each other, and both are close to *P. thunbergii*. *P. nigra* appears to be more distantly related to these three species.

Reliable neighbor-joining tree analysis of these four species has been deduced by sequence analysis of four chloroplast DNA regions (Wang et al. 1999). A single clade containing the four species could be segregated into two clades: one contained *P. nigra*, and the second included the other three species. The second clade segregated into the two sister species *P. densiflora* and *P. sylvestris*, and the other clade segregated into several species, including *P. thunbergii*. These results confirm that the FISH karyotypes are good indicators of phylogenetic relationships in *Pinus*.

Karyotype analysis in the *Pinus* subgenus using FISH probing with 45S and 5S rDNA and the fluorescence banding pattern of DAPI and CMA has been reported for three American species (*P. elliottii* var. elliottii, Doudrick et al. 1995; *P. radiata* and *P. taeda*, Jacobs et al. 2000). We assume that interstitial and centromeric DAPI bands correspond to telomere signals and that centromeric CMA bands correspond to *PCSR* signals in all *Pinus* species. Thus, we can compare the FISH and fluorescence-banding karyotypes of the three American species with our multicolor FISH karyotypes of the four Eurasian species. The American species showed significantly different FISH and fluorescence banding patterns from those of the Eurasian species. These three American species and four Eurasian species were classified as *Pinus*, subgenus *Pinus* according to the systematic system of Little and Critchfield (1969). The four Eurasian species were further classified into subsection *Sylvestris*; *P. elliottii* and *P. taeda* were both classified into subsection *Australes*; *P. radiata* was placed in subsection *Oocarpae*. The American species had fewer interstitial DAPI bands than *Sylvestris* members, and it was difficult to find homologous chromosomes between these species and subsection *Sylvestris* members. However, the chromosome 2 signal and banding patterns in *P. elliottii* var. elliottii showed some similarity to those in chromosome group VIII of subsection *Sylvestris*. This similarity suggests the existence of homoeologous chromosomes among different subsections. Karyotypic differences and similarities between species subsections may prove to be

an important clue to understanding species differentiation.

In the study reported here, we performed comparative karyotypic analysis using FISH landmark patterns in four species that belonged to subsection *Sylvestris*, subgenus *Pinus*. There are more than 100 known *Pinus* species. Therefore, further analyses of other species of this subgenus, and the subgenus *Strobus* are needed to accurately assess the reliability of FISH karyotype analysis in phylogenetic studies on *Pinus*. This study strongly indicates that comparative FISH karyotype analysis following the identification of homologous chromosomes reveals the relationship between karyotype and species in the genus *Pinus*.

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