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ORIGINAL PAPER

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# AT-rich sequences containing *Arabidopsis*-type telomere sequence and their chromosomal distribution in *Pinus densiflora*

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**Abstract** Japanese red pine *Pinus densiflora* has 2 $n=24$ chromosomes and after FISH-detection of *Arabidopsis*-type (*A*-type) telomere sequences, many telomere signals were observed on these chromosomes at interstitial and proximal regions in addition to the chromosome ends. These interstitial and proximal signal sites were observed as DAPI-positive bands, suggesting that the interstitial and proximal telomere signal sites are composed of AT-rich highly repetitive sequences. Four DNA clones (PAL810, PAL1114, PAL1539, PAL1742) localized at the interstitial telomere signals were selected from *Alu*I-digested genomic DNA library using colony blot hybridization probed with *A*-type telomere sequences and characterized using FISH and Southern blot hybridization. The AT-contents of these selected four clones were 60.8–76.3%, and repeat units of the telomere sequence and degenerated telomere sequences were found in their nucleotide sequences. Except for two sites of PAL1114, FISH signals of the four clones co-localized with interstitial and proximal *A*-type telomere sequence signals. FISH signals a showed similar distribution pattern, but the patterns of signal intensity were different among the four clones. PAL810, PAL1539 and PAL 1742 showed similar FISH signal patterns, and the differences were only with respect to the signal intensity of some signal sites. PAL1114 had unique signals that appeared on chromosomes 7 and 10. Based on results of the Southern blot hybridization these four sequences are not arranged tandemly. Our results suggest that the interstitial *A*-type telomere sequence signal sites were composed of a mixture of several AT-rich repetitive sequences and that these repetitive sequences contained *A*-type telomere sequences or degenerated *A*-type telomere sequence repeats.

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## Introduction

Telomeres are the structures found at chromosome termini that maintain chromosome structure by protecting and replicating the ends of the chromosome. The telomere is commonly composed of tandem repeats of five to eight nucleotides that are highly conserved in eukaryotes (Zakian 1989). These conserved sequences have been localized at the chromosome ends in nearly all of the species examined using fluorescence in situ hybridization (FISH) (in vertebrate; Meyne et al. 1990, in plants; Fuchs et al. 1995). In the majority of plant species, the telomere repeat unit is the *A*-type (TTTAGGG) (Cox et al. 1993, Fuchs et al. 1995; Hizume et al. 2000; Adams et al. 2001). In some animals and plants, additional telomere signals appear at interstitial, centromeric or pericentromeric locations in addition to the chromosome ends, and this displacement has been taken in some cases as evidence that chromosome rearrangement by fusion has occurred; for example, the telomere signal at the centromere of chromosome 2 in humans (Ijdo et al. 1991) and of chromosomes 1 and 2 of *Arabidopsis thaliana* (Uchida et al. 2002).

In *Pinus* species, FISH results have shown that the *A*-type telomere sequences are localized at both the interstitial and centromeric regions in addition to chromosome ends (Fuchs et al. 1995; Hizume et al. 2000; Schmidt et al. 2000). Interstitial FISH signals of *A*-type telomere sequences are conserved amongst *Pinus* species and are consequently useful for chromosome identification purposes. Comparative karyotype analysis in four

*Pinus* species has been performed on the basis of FISH signals with *A*-type telomere sequence repeats, ribosomal DNAs and centromeric minisatellite DNA (Hizume et al. 2002). All *Pinus* species have the same chromosome number, $x = 12$, and a similar basic karyotype. In this highly conserved karyotype, the interstitial FISH signals of the *A*-type telomere sequence repeats would appear not to be traces of ancestral chromosome fusion. Therefore, an investigation of the nucleotide structure of these additional *A*-type telomere sequence signal sites would provide a good understanding of the chromosome organization and karyotype evolution of *Pinus*

We report here the cloning of DNA sequences that were localized at interstitial and centromeric *A*-type telomere sequence-signaled regions of a Japanese red pine, *P.densiflora*, using the FISH technique. The organization of these *A*-type telomere sequence signal sites on the chromosomes of *P. densiflora* were also compared.

## Materials and methods

### Plant materials and chromosome preparation

Seeds of *Pinus densiflora* Sieb. and Zucc., collected at a natural pine forest on Mt. Tagami, Iyo, Ehime Prefecture, Japan, were germinated in sterilized sand in a pot. The root tips were collected for chromosome analysis from 10-day-old seedlings, treated with 0.05% colchicine at 20°C for 10 h, then fixed in cold ethanol:chloroform:acetic acid (2:1:1) overnight and stored in a freezer. The fixed root tips were incubated in an enzyme mixture containing 2% cellulase Onozuka RS (Yakult, Japan), 0.5% pectolyase Y23 (Kyowa Chemical, Japan), 2× SSC, pH 4.5, at 37°C for 45 min and then rinsed with distilled water. The meristematic cells were squashed in 45% acetic acid under a coverslip on a glass slide and air-dried after the coverslip had been removed by the dry-ice method.

### Cloning and sequencing

Genomic DNA was extracted from young leaves of *P. densiflora* by the CTAB (cetyletrimethylammonium bromide) method (Murray and Thompson 1980). Genomic DNA was digested with *Alu*I and cloned into plasmid vector pUC18. Telomere-containing sequence clones were selected using colony blot hybridization probed with *A*-type telomere sequence repeats $(TTTAGGG)_n$. Four positive clones, PAL810, PAL1114, PAL1539 and PAL1742, were sequenced by the dideoxy chain terminator method using an automated DNA sequencer (ABI310; Applied Biosystems, Foster City, Calif.). The PAL810, PAL1114, PAL1539 and PAL1742 nucleotide sequences have been entered in the DDBJ, EMBL, GenBank nucleotide sequence databases under accession numbers AB175666, AB175667, AB175668 and AB175669, respectively.

### Southern blot hybridization

Three micrograms of restriction endonuclease-digested genomic DNAs was fractionated on 2% agarose gels and blotted onto nylon membranes (Magnagraph; MSI, Westboro, Mass.). The blots were hybridized with digoxigenin (DIG)-labeled cloned DNAs in hybridization buffer [1% blocking reagent (Roche, Indianapolis, Ind.), 0.02% sodium dodecyl sulfate (SDS) and 0.1% N-lauroylsarcosine, in 5× SSC, pH 7.0] at 42°C overnight. The hybridized probe was detected using anti-digoxigenin alkaline phosphatase conjugate and CDP-Star (Roche), and the light emitted by the detection reaction was recorded on X-ray film (RX-U; Fuji Film, Japan).

### Fluorescence in situ hybridization

The plasmid DNAs of PAL810, PAL1114, PAL1539 and PAL1742 were labeled with biotin using the BioNick Labeling System (Invitrogen, Carlsbad, Calif.) and used as the probe for FISH. The hybridization procedure and signal detection followed Hizume et al. (2002). Multicolor FISH was performed on the same slides using the *A*-type telomere sequence repeat $(TTTAGGG)_n$. *Eco*RI-digested wheat 45S rDNA (pTa71; Gerlach and Bedbrook 1979), 5S rDNA amplified by PCR from *Rumex acetosa* genomic DNA and plasmid DNA containing *PCSR* (the proximal CMA band-specific repeat, clone PDCD501; accession no. AB051860; Hizume et al. 2001) were used for comparison purposes following the method of Hizume et al. (2002) for chromosome identification. The hybridization signals were visualized and recorded using a chilled CCD camera (Sensys 1400; Photometrics, Huntington Beach, Calif.), and pseudo-color images were made using IPLAB (Scanalytics, Fairfax, Va.).

## Results and discussion

### Cloning of *A*-type telomere sequence-containing sequences from genome DNA of *P. densiflora*

*Arabidopsis*-type telomere sequence-containing clones were screened from a genome DNA library constructed from *Alu*I-digested genomic DNA of *P. densiflora* using colony blot hybridization. Four positive clones (PAL810, PAL1114, PAL1539, PAL1742) were selected and sequenced, (Table 1). These sequences were AT-rich (60.8–76.3%), and in all four clones repeat units of *A*-type telomere sequences (TTTAGGG or CCCTAAA) and degenerated telomere sequences (ex: TTTAAGG,

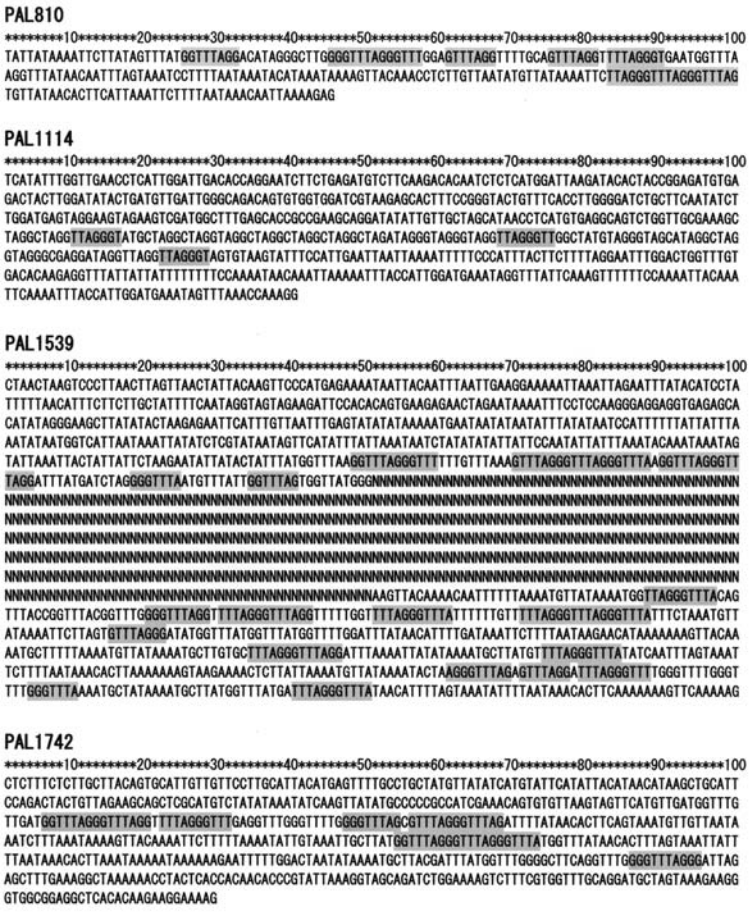


**Fig. 1** Nucleotide sequence of four AT-rich clones (PAL810, PAL1114, PAL1539, PAL1742). *Shaded letters* indicate the repeat unit of *A*-type telomere sequence

**Table 1** Characterization of four AT-rich clones containing *A*-type telomere sequences

| Clone name | Length (bp) | AT contents (%) | Percentage of *A*-type telomere sequence | Accession no. |
|---|---|---|---|---|
| PAL810 | 245 | 73.9 | 24.9 | AB175666 |
| PAL1114 | 640 | 60.8 | 3.4 | AB175667 |
| PAL1539 | approx. 1,700 | 76.3 | 11.6 | AB175668 |
| PAL1742 | 629 | 64.5 | 12.1 | AB175669 |

CCCTTAA) were found. The *A*-type telomere or degenerated *A*-type telomere sequences were scattered throughout the genome, and some were arranged as short tandem repeats (Fig. 1). The amount (expressed in percentage) of *A*-type telomere sequences in the four clones varied, with PAL1114 clearly have less than in the other three clones (Table 1).

## FISH analysis of AT-rich sequences containing *A*-type telomere sequence

When *P. densiflora* chromosome spreads were probed with the four clones (PAL810, PAL1114, PAL1539 and PAL1742), signals were observed on the interstitial and proximal regions of the chromosomes (Fig. 2). Unlike the results when the *A*-type telomere sequences was used as the FISH probe, the terminal end of each chromosome did not show signals. The majority of the interstitial and proximal signal sites overlapped with the signals from *A*-type telomere sequences in multi-probed FISH (Figs. 2, 3).

The FISH results using clones PAL810, PAL1539 and PAL1742 were similar, although some differences were observed in signal intensity. With PAL810, two interstitial signals of chromosome 6 were stronger than the other signals; with PAL1539, proximal signals of chromosome 7 was stronger than other signals; with PAL1742, one interstitial signal of chromosome 5 was stronger than the others (Fig. 2).

When PAL1114 was used as the probe, signals were not observed on the proximal telomere sequences signal sites of chromosomes 7 and 9 and interstitial regions of chromosomes 1, 3, 4, 5 and 11 (Arrowheads, Figs. 2, 3). Conversely, two unique signals appeared on the interstitial region, one on the short arm of chromosome 7

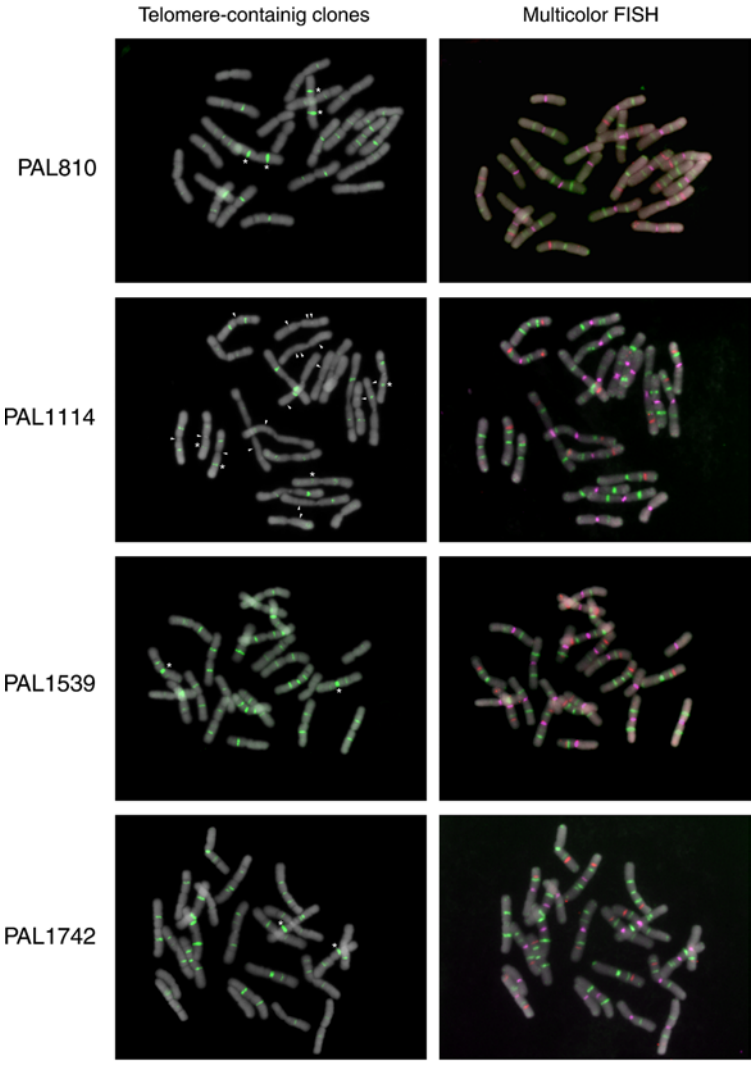


**Fig. 2** FISH images of somatic chromosomes probed with AT-rich clones (PAL810, PAL1114, PAL1539 and PAL1742) and multicolor FISH on the same chromosomes of *Pinus densiflora*. The *red signal* corresponds to the 45S and 5S rDNA probes, *magenta* represents the PCSR probe, and *green* corresponds to the *A*-type telomere sequence probe. *Asterisks* indicate characteristic signals of each clone. *Arrowheads* in PAL1114 indicate the sites without signals indicating a telomere-containing sequence. *Bar*: 10 μm

and one on the long arm chromosome 10 (Asterisks, Figs. 2, 3). These two signals did not overlap with *A*-type telomere signals or any other FISH signals observed with the three other *A*-type telomere-containing sequence signals.

Another AT-rich repetitive sequence that we have cloned and mapped with FISH (PDCD159, Hizume et al. 2001) gave signals at the proximal DAPI-positive bands of chromosomes 7 and 9. At these sites, *A*-type telomere sequence signals and three *A*-type telomere-containing sequences signals (PAL810, PAL1539, PAL1742) were also present. The nucleotide content of PDCD159 is 61.7%, and PDCD159 does not contain *A*-type telomere sequence or degenerated *A*-type telomere sequence and shows no sequence similarity with the *A*-type telomere-containing sequences reported in this paper. On the basis of these results, the DAPI-positive bands observed at the interstitial and proximal regions were composed of several AT-rich sequences, with some of them containing *A*-type telomere sequences in their nucleotide sequences.

## Southern blot analysis of AT-rich sequences containing *A*-type telomere sequences

In general, fluorescence-positive bands on chromosomes are composed of tandemly arranged repetitive sequences. Four *A*-type telomere sequence-containing clones and *A*-type telomere repeats were used as probes for Southern blot analysis (Fig. 4). The banding patterns of these four sequences showed that all of these sequences were a partial sequence of long, dispersed repetitive sequences. Their banding patterns were different from that of the *A*-type telomere sequence repeats, indicating that these probes detected mainly unique sequences in genome DNA similar to the results of the FISH experiments. When PAL810 was used as the probe, the signal intensity was very weak, which might be due to a low-copy number or the accumulation of mutations in the nucleotide sequence. The signal patterns of PAL1539 and PAL1742 were similar to each other, suggesting that these sequences might be partial sequences of the same long repeat unit or sequences

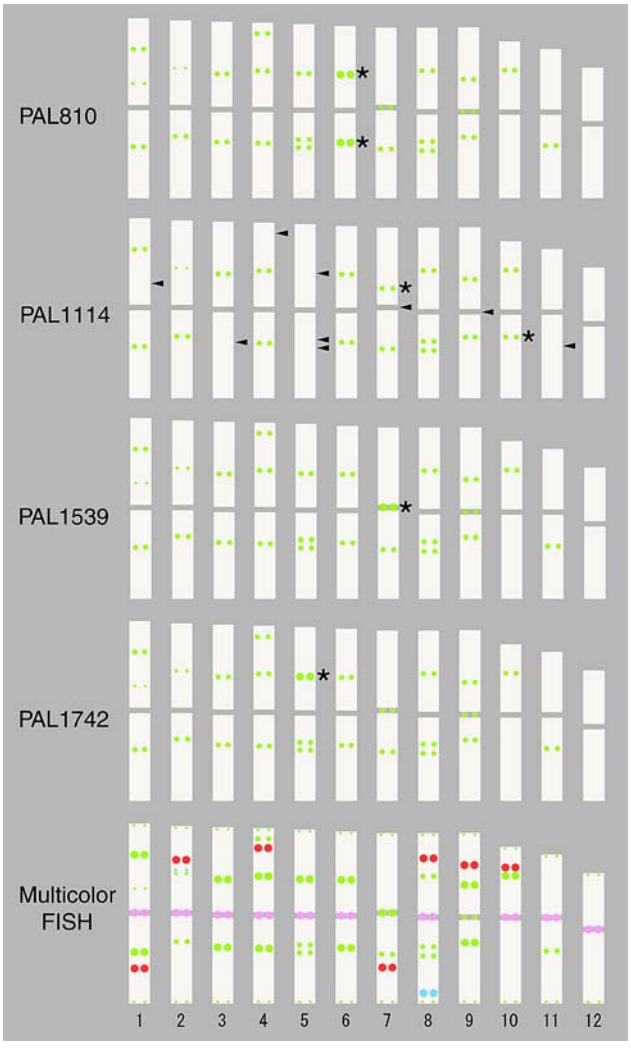


**Fig. 3** Ideograms of FISH karyotypes probed with PAL810, PAL1114, PAL153, PAL1742 (signals are shown as *green dots*) and multicolor FISH. The corresponding colors of each dot in multicolor FISH are: *red* 45S rDNA signal, *blue* 5S rDNA signal, *magenta* proximal PCSR signal, *green* telomere sequence signal . *Asterisks* indicate characteristic signals of each clone. *Arrowheads* in PAL1114 indicate the sites without signals of telomere-containing sequence

derived from a common ancestral one. While some of the PAL1114 FISH signals co-localized with those of other three telomere-containing sequences, the Southern signals of PAL1114 were unique, indicating that the PAL1114 telomere-containing sequence and other three telomere-containing sequences were not arranged in the same repeat unit.

The results of this study indicate that interstitial and proximal DAPI-positive bands are composed of AT-rich *A*-type telomere sequence-containing sequences. The origin and mechanism of generation of these AT-rich sequences are unknown. We speculate that there might be a possibility that some mobile element acts in transferring or amplifying of these AT-rich sequences at specific sites on the chromosome arms. Some transposable elements, such as *Athila* in *A. thaliana* and the *Tbv* retrotransposon family in sugar beet, tend to accumulate in centromeric regions (The Arabidopsis Genome Initiative 2000; Schmidt and Heslop-Harrison 1998). However, there has been no report of these transposable elements being associated with fluorescent bands on chromosome arms, although they may be present in such small numbers as to be undetectable with present-day banding methods. In *Allium cepa*, a dispersed repetitive sequence, W1-41, and the *En*/*Spm*-transposable element-like sequence intermingle with tandemly arranged repetitive sequences in terminal heterochromatic regions (Pich and Schubert 1998; Shibata and Hizume 2003). In *P. densiflora*, Hizume et al. (2001) has shown the co-localization of two tandemly arranged repetitive sequences (45S rDNA and the microsatellite *PCSR*) at secondary constrictions and proximal CMA bands . Therefore, there remains the possibility that interstitial and proximal DAPI-positive bands are composed of a mixture of the unknown tandemly arranged repetitive sequences and AT-rich sequences reported in this study.

Further investigation of amplification and translocation mechanisms of repetitive sequences could provide important clues into chromosome evolution and chromosome structure in the genus *Pinus*.

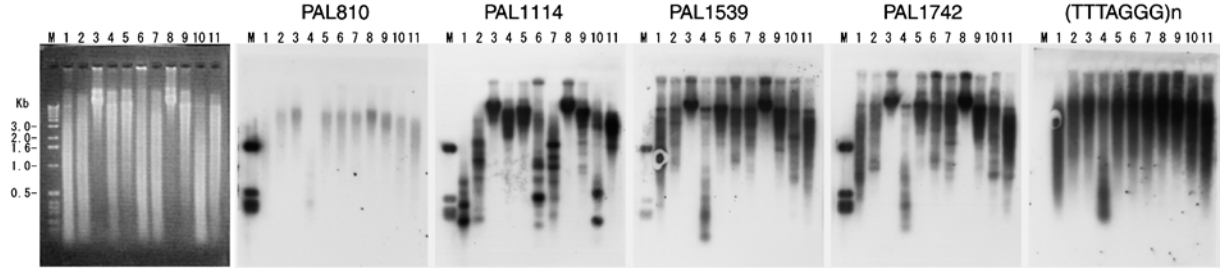


**Fig. 4** Southern blot hybridization of restriction enzyme-digested genomic DNA of *P. densiflora* probed with *A*-type telomere sequence-containing clones (PAL810, PAL1114, PAL1539 and PAL1742) and the *A*-type telomere sequence $(TTTAGGG)_n$. The restriction enzymes used in each lanes are: *Alu*I (*1*), *Afa*I (*2*), *Bam*HI (*3*), *Dra*I (*4*), *Eco*RI (*5*), *Fok*I (*6*), *Hae*III (*7*), *Hap*II (*8*), *Hin*dIII (*9*), *Mbo*I (*10*) and *Ssp*I (*11*). *M*: 1-kb DNA ladder marker (Invitrogen)

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