CHROMOSOMAL LOCATIONS OF THE RIBOSOMAL DNA GENES IN SHORTLEAF PINE

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ABSTRACT.—A reference karyotype (i.e., chromosome-specific description of a species' chromosomal complement) is a pre-requisite for advanced genetic and genomic studies. The Southern Institute of Forest Genetics has initiated a project to develop reference karyotypes for each of the major southern U.S. pine species, including shortleaf pine, using AT-rich chromosomal banding and fluorescent *in situ* hybridization (FISH). About half of the project has been completed to date, including the development of karyotypes for loblolly pine and slash pine, with the remaining experiments being directed towards shortleaf pine and longleaf pine. Preliminary FISH results for rDNA genes in shortleaf pine show that there are seven major, and as many as eight medium-to-minor centromeric 18S-28S rDNA sites. In addition, one major and one minor 5S rDNA sites were observed and most of the chromosomes showed AT-rich bands. A complete shortleaf pine karyotype is being developed for comparison with other pine and conifer species.

INTRODUCTION

The genus *Pinus* ($2n = 2x = 24$), originally confined almost entirely to the northern hemisphere, includes many economically and ecologically important species. Loblolly pine (*Pinus taeda* L.), slash pine (*P. elliottii* var. *elliottii* Englm.), shortleaf pine (*P. echinata* Mill.), and longleaf pine (*P. palutris* Mill.) are the four *Pinus* species most commonly planted in the southern U.S. All *Pinus* species studied have 12 pairs of chromosomes with 10 or 11 pairs of long metacentric chromosomes and one pair of short sub-metacentric chromosomes (Sax and Sax 1933). Conventional cytogenetics has been used in studying the pines (Mergen 1958, Borzan and Papes 1978, MacPherson and Filion 1981, Hizume and others 1990). However, combining molecular cytology, *in situ* hybridization (ISH), and conventional cytological techniques provides more accurate information about genomes (Heslop-Harrison 1991, Leitch and Heslop-Harrison 1992, Leitch and others 1992, Hizume and others 2002, Doudrick and others 1995). We recently completed a reference karyotype and cytomolecular map for loblolly pine (Islam-Faridi and others 2007), as part of our institute's southern pine karyotyping project at the Southern Institute of Forest Genetics. In this paper, we present some preliminary data from our investigation of shortleaf pine.

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MATERIALS AND METHODS

Plant Material

Seeds from an open-pollinated shortleaf pine clone, WO-5, were treated with 1 percent hydrogen peroxide to break dormancy and then germinated in the dark on moist filter paper in petri-dishes at 24 ˚C. Germinated seedlings were transferred to potting mix in pots and allowed to grow in a greenhouse prior to harvesting of root tips for cytogenetic analysis.

Slide Preparation

Actively growing roots tips, about 1.5 cm long, were excised and pretreated in 0.15 percemt colchicine (Sigma, P-9754) for 7 h at room temperature in the dark and then fixed in 2:1:1 ethanol (95 percent)-acetic acid-double distilled water. The fixed roots were treated enzymatically as described by Jewell and Islam-Faridi (1994). The digested root tips were macerated on a cleaned slide in 3:1 ethanol-acetic acid and squashed in 45 percent acetic acid with a cover glass (Islam-Faridi and others 2007). Finally, chromosome spreads were stored at -80 ˚C until used for fluorescence *in situ* hybridization (FISH).

Probe DNA and Nick Translation

Whole plasmids containing 18S-28S rDNA or 5S rDNA inserts were labeled by nick translation using biotin-16-dUTP (Biotin-Nick Translation Mix, Roche Diagnostics).

Fluorescent *In situ* Hybridization

A standard *in situ* hybridization technique was followed (Islam-Faridi and Mujeeb-Kazi 1995). Probe hybridization sites were detected with Cy3 fluorochrome conjugated

streptavidin. The chromosome preparations were also counterstained with 4'-6-Diamidino-2-phenylindole (DAPI, 4 µg/ml) and mounted by Vectashield (Vector Laboratories) to prevent fluorochrome bleaching.

Microscopy

Digital images were recorded from an Olympus AX-70 epi-fluorescence microscope with suitable monochrome filter sets (Chroma Technology) using a 1.3 MP Sensys (Roper Scientific) camera and a MacProbe v4.2.3 digital image system (Applied Imaging). Images were processed with MacProbe v4.2.3 and Adobe Photoshop CS 8.

RESULTS AND DISCUSSION

We modified a technique for preparing pine chromosomes that consistently provides a high number of metaphase chromosome spreads in various pine species, including shortleaf pine. When our modified technique was employed, a single root tip yielded as many as 650 metaphase cells with as many as 40 of these containing well separated chromosomes that are ideal for DAPI and FISH analysis.

Various patterns of DAPI bands occurred near or around the centromere of most shortleaf pine chromosomes. Some of the centromeric DAPI bands appeared at both sides of the centromere, while others were clearly on one side or the other. Intercalary (the area between a centromere and a telomere) DAPI bands were also observed in some chromosomes. Similar results have been obtained for loblolly pine (Islam-Faridi and others 2007).

In shortleaf pine, we observed 13 major and 17 medium-to-minor 18S-28S rDNA signals (Fig. 1). All major and one medium (Fig. 1a and 1b, arrowheads) signals are located at intercalary positions, representing seven homologous loci, with one locus containing a major and a medium signal. A similar observation was also reported and has been observed for slash pine (Doudrick and others 1995, Islam-Faridi and others unpublished). In contrast, loblolly pine shows 14 major signals, two each for the seven homologous loci (Jacobs and others 2000, Islam-Faridi and others 2007). This observation suggests that the shortleaf pine and slash pine homologues with the medium signal lack several hundreds to thousands of copies of the highly repetitive 18S-28S rDNA gene. The remaining medium-to-minor intensity signals are located at or near centromeric positions. Taken together, these observations indicate that the shortleaf pine karyotype is more similar to slash pine than it is to loblolly pine.

The longest chromosome (i.e., chromosome 1) in shortleaf pine can easily be identified by its 5S rDNA signal (Figs. 1a and 1b, arrows), which appears to be the major 5S rDNA site. In addition, a minor 5S rDNA site was observed (Fig. 1a, insert). This second 5S rDNA site appeared toward the end of a different chromosome, which also showed a major intercalary 18S-28S rDNA site located on the opposite arm. Chromosome 2 of the loblolly pine reference karyotype has the same distinguishing characteristics (Islam-Faridi and others 2007).

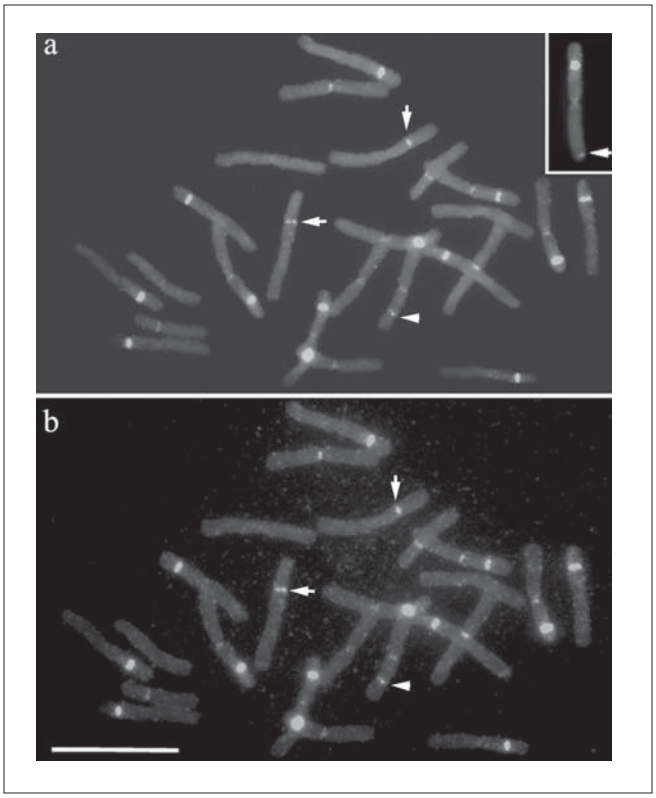

Figure 1.—FISH with 18S-28S rDNA and 5S rDNA probes on somatic metaphase chromosome spread of shortleaf pine, clone WO-5. The major 5S rDNA site is located on chromosome 1 (arrows, a and b). Also shown is a medium intercalary 18S-28S rDNA signal (arrowheads, a and b). The second 5S rDNA site is shown in the insert of a (arrow). Bar = 10 µm.

Further studies including use of an Arabidopsis–type telomere repeat sequence (A-type TRS) probe are being carried out to develop a comprehensive shortleaf pine karyotype for comparison with our loblolly pine reference karyotype (Islam-Faridi and others 2007) and two slash pine karyotypes (Doudrick and others 1995, Islam-Faridi and others unpublished). Cytogenetic analyses including karyotype comparisons are useful in identifying structural rearrangements (i.e., large translocations and/or inversions) within and between species which can be used to infer evolutionary relationships, to inform gene conservation efforts, and to guide interspecies breeding projects.

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