Loblolly Pine Karyotype Using FISH and DAPI Positive Banding

M. N. Islam-Faridi [1,2], C. Dana Nelson [2], T. L. Kubisiak [2], M. V. Gullirmo [1], V. H. McNamara [1], S. Ramakrishnan [1], H. J. Price [1] and D. M. Stelly [1]

INTRODUCTION

The pines (*Pinus*, $2n = 2x = 24$) include many commercially important timber species. *Pinus* spp. 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). The pines have been studied extensively with conventional cytological techniques (Mergen 1958; Borzan and Papes 1978; MacPherson and Filion 1981; Schweizer 1980; Hizume et al. 1990). Molecular cytology, *in situ* hybridization (ISH), coupled with conventional cytological techniques can provide more accurate information about genomes (Heslop-Harrison 1991; Leitch and Heslop-Harrison 1992; Leitch et al. 1992). Well-spread metaphase chromosomes that are free of cell walls and cytoplasmic debris are a prerequisite for ISH. Since the chromosomes of pine are extremely large, well-spread metaphases are very difficult to obtain (Doudrick et al. 1995; Jacobs et al. 2000; Schmidt et al. 2000). We report a modified somatic chromosome preparation technique that was used to improve chromosome spreading and morphology in loblolly (*Pinus taeda* L.) and slash (*P. elliottii* Englm.) pines. Fluorescent ISH (FISH) was then used to locate 18S-28S ribosomal, 5S ribosomal, and telomeric DNA sites in these plant species, and to facilitate the development of karyotypes for each.

MATERIALS AND METHODS

Plant Material: Seeds from an open-pollinated loblolly pine clone (LSG-62, kindly provided by Dr. Tom Byram, Texas Forest Service) were treated with 1% hydrogen peroxide (H_2O_2) to break dormancy and then germinated on moist filter paper in petri dishes at 24C in the dark. Likewise, seeds from an open-pollinated slash pine clone (LA-11) were germinated, except with treating to break dormancy.

Slide Preparation: Healthy roots, 0.06 cm to 1.5 cm long, were excised and pretreated in 0.15% colchicine (Sigma, P-9754) for 7 h at room temperature in the dark and then fixed in 3:1 ethanol (95%) – acetic acid. The roots were treated enzymatically as described by Jewell and Islam-Faridi (1994). The digested root tip was macerated on a cleaned slide in 3:1 ethanol-acetic acid and then squashed under a cover glass in 45% acetic acid. The slides were stored at –80C.

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[1] Department of Soil and Crop Sciences, Texas A&M University College Station, TX 77843;
[2] Southern Institute of Forest Genetics, USDA Forest Service, 23332 MS Highway 67 Saucier, MS 39574.

Probe DNA and Nick Translation: Whole plasmid with 18S-28S (and 5S rDNA) insert was labeled by nick translation using biotin-14-dATP (BioNick Labeling System, Invitrogen Inc.) and digoxigenin-11-dUTP (Dig-Nick Translation Mix, Roche). The *Arabidopsis* type telomere repeat ((TTTAGGG)n , about 300bp, kindly provided by Dr. Tom McKnight ,Texas A&M University) insert was labeled by BioNick labeling system (Invitrogen, Inc.).

In situ Hybridization: A standard *in situ* hybridization technique was followed as published by Islam-Faridi and Mujeeb-Kazi (1995) and Islam-Faridi et al. (2002). The probe hybridization sites were detected with FITC conjugated avidin-dcs (for biotin labeled probe) and/or rhodamine conjugated sheep anti-dig (Roche) followed by rhodamine conjugated anti-sheep (Jackson Immuno Research Lab, Inc.) (for dig labeled probe). The preparations were counterstained with DAPI and mounted with Vectashield (Vector Laboratories Inc., CA).

Microscopy: Digital images were recorded from an Olympus AX-70 Epifluorescence microscope with suitable monochrome filter sets (Chroma Technology, VT), using a 1.3 MP Sensys (Roper Scientific) camera and the MacProbe v4.2.3 digital image system (Applied Imaging, Santa Clara, CA). Images were processed with MacProbe v4.2.3 and Adobe Photoshop 6.0.

Karyotype Analysis: Each chromosome arm was measured three times from its extremity to the centromere after zooming 400% to minimize the error using Optimas v6. The length measurements yielded the following values: a) total chromosome length, b) centromeric indices, c) relative length, d) position of FISH signals and e) luminescence of FISH signals. Six well spread cells were chosen for karyotype analysis from each line. In each cell, chromosomes were numbered arbitrarily from 1 to 24, and paired on the basis of 18S-28S rDNA, 5S rDNA, telomere repeat FISH signals and DAPI positive bands.

RESULTS AND DISCUSSION

Our modified chromosome preparation yielded well-spread metaphase chromosomes in loblolly and slash pines (Fig. 1). A relatively high mitotic index was observed for both pine species. A single root yielded as many as 731 mitotic divisions, mostly metaphases. Chromosome morphology was sharp and clear after *in situ* hybridization, as evidenced in the following photomicrographs. Strong DAPI positive bands occurred in various patterns near or around the centromeres of 23 chromosomes (11 pairs + one) in loblolly pine and 22 chromosomes (11 pairs) in slash pine. Distinct DAPI positive bands appeared at both sides of the primary constrictions (i.e., centromeres) in some chromosomes. In both species numerous light or weaker DAPI bands appeared interstitially in either or both arms of the chromosomes.

Light to strong telomere repeat FISH (TR-FISH) signals appeared at or near the centromeric sites in nine or possibly 10 pairs of chromosomes in both species (Figs. 1a and c). TR-FISH signals also appeared interstitially and pairs of snake-eyed signals

appeared near the end of most of the chromosomes. TR-FISH signals are found to be associated with DAPI positive bands but not all DAPI positive bands are associated with TR-FISH signals. DAPI positive bands located at the end of chromosomes are found to be associated with TR-FISH signals. This is the first report of this phenomenon.

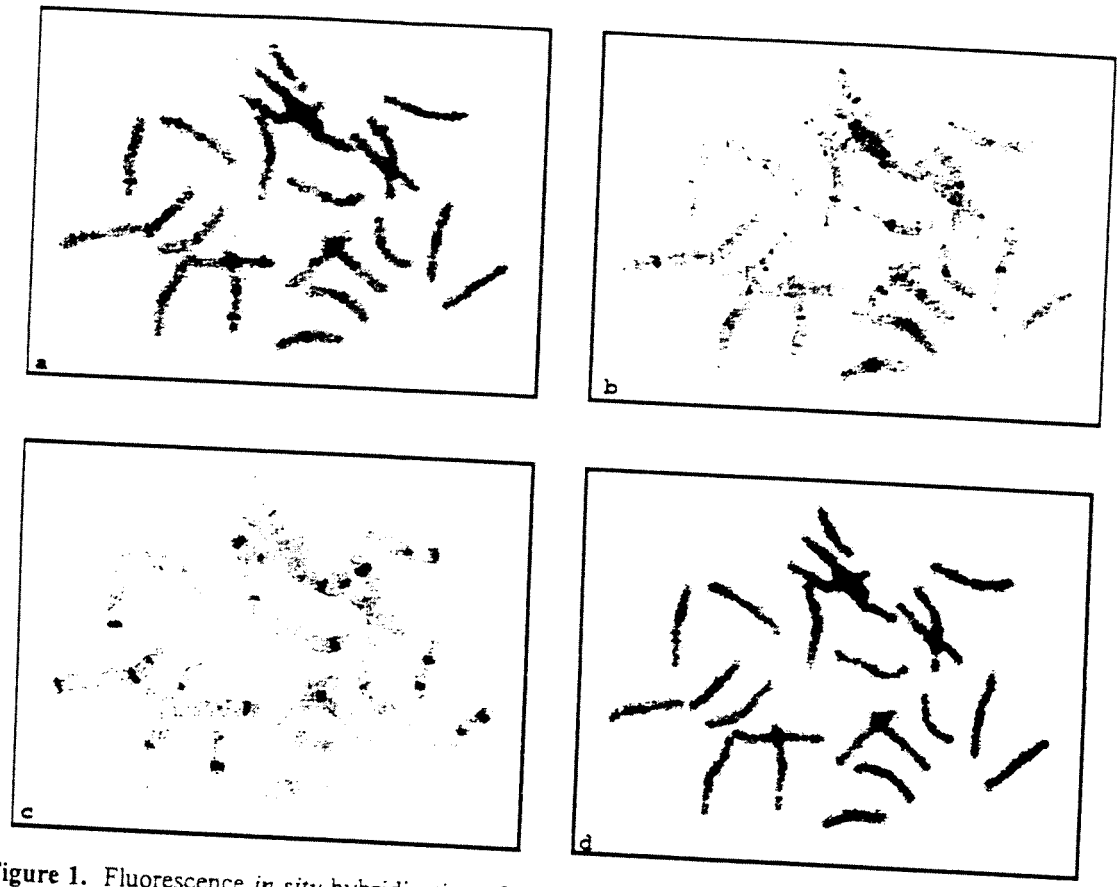

Figure 1. Fluorescence *in situ* hybridization of 18S-28 rDNA and telomere repeat probes to a mitotic metaphase spread of (**a**) loblolly pine (LSG-62). Individual signals from 18S-28S rDNA and telomere repeat are represented in Figures 1b and c, respectively; (**b**) FISH of 18S-28S rDNA sites (image from Cy3 filter followed by inversion); (**c**) FISH of telomere repeat sites (image from FITC filter followed by inversion) and (**d**) gray scale followed by inverted image of DAPI stained chromosome.

Seven intercalary 18S-28S rDNA sites have been identified both in loblolly and slash pines (Figs. 1a and b, 2a). Eight or possibly nine distinct 18S-28 rDNA sites appeared near or around the centromeric regions in slash pine and as many as nine sites were observed in loblolly pine.

None of these 18S-28 rDNA sites were associated with the DAPI positive bands or proximal TR-FISH sites. To date, this is the largest number of 18S-28S rDNA sites ever reported for a pine species. We hypothesize that the higher number of 18S-28S rDNA sites in this study as compared to previous reports (Doudrick et al. 1995; Jacobs et al.

chromosome preparation. One major 5S rDNA site was observed on a long metacentric chromosome and two minor sites were observed on two other different metacentric chromosomes in both loblolly and slash pines.

We have clearly categorized 10 large pairs of metacentric chromosomes, one smaller pair (11 th pair) of sub-metacentric chromosomes, and the smallest pair (12 th pair) of sub-metacentric chromosome in both species. This is in contrast to previous reports of 11 pairs of long metacentric chromosomes and one pair of short sub-metacentric chromosomes.

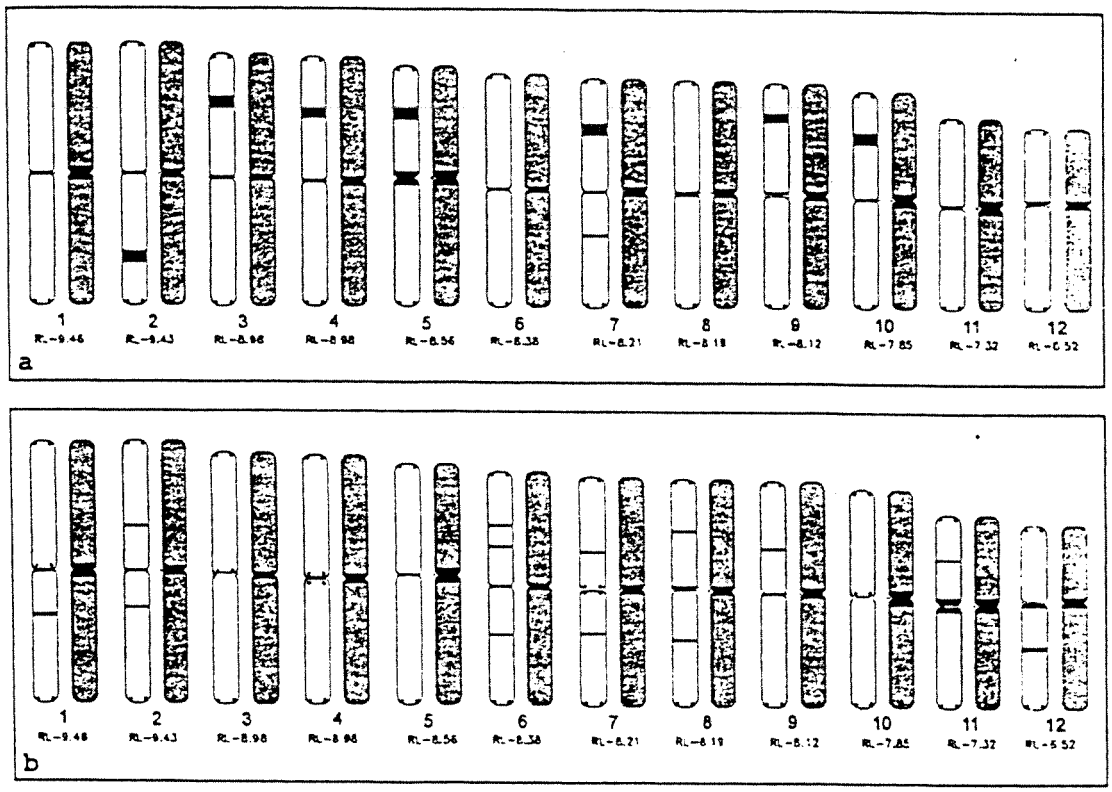

Figure 2. Ideogram of FISH based karyotype of loblolly pine (LSG-62). (a) The banded (Cy3 fluorochrome) signal corresponds to 18S-28S rDNA sites (b) shaded (FITC fluorochrome) signal corresponds to telomere repeat sites. Shaded ideogram represents DAPI positive bands (dark).

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