Development of Reference Karyotypes for Longleaf and Shortleaf Pines using Fluorescence *in situ* Hybridization†

N. Islam-Faridi[1], M. Abdul Majid[2], H. Banda[3], C. D. Nelson[4]

Abstract: The Southern Institute of Forest Genetics is developing reference karyotypes for each of the major southern U. S. pine species— loblolly (*Pinus taeda*), slash (*P. elliottii* var. *elliottii*), shortleaf (*P. echinata*) and longleaf (*P. palustris*)— using 18S-28S rDNA, 5S rDNA, and *Arabidopsis*-type telomere repeat (ATR) sequence probes and AT-rich banding. Reference karyotypes for loblolly and slash pines have been completed. Preliminary results for the rDNA genes show that both shortleaf and longleaf pines contain seven major intercalary 18S-28S rDNA sites. Shortleaf pine showed as many as three major and six medium-to-minor centromeric 18S-28S rDNA sites, while longleaf pine showed one major and six medium-to-minor centromeric 18S-28S rDNA sites. Both species showed one major and one minor site for 5S rDNA. There are as many as five ATR sites with different degrees of signal intensities located near the centromeres of each chromosome in both longleaf and shortleaf pine. In addition, strong AT-rich bands were found to flank the centromeres of most chromosomes in both species. Complete karyotypes for shortleaf and longleaf pines are being developed for comparison to each other and to the existing loblolly and slash pine karyotypes.

Keywords: cytogenetics, molecular cytology, ribosomal DNA, telomere repeat sequence

INTRODUCTION

The genus *Pinus* ($2n = 2x = 24$) includes many economically and ecologically important species worldwide. According to Sax and Sax (1933) all pines have eleven pairs of long metacentric chromosomes and one pair of short sub-metacentric chromosomes. The conventional cytology technique known as C-banding has been used extensively to characterize the pines, but found to be ineffective in discriminating the chromosomes except for the one pair of short sub-metacentric chromosomes (Mergen 1958; Borzan and Papes 1978; MacPherson and Filion 1981; Hizume and others 1990). Molecular cytology, *in situ* hybridization (ISH) coupled with conventional cytology, can provide information that greatly facilitates the individual identification of chromosomes (Heslop-Harrison 1991; Leitch and Heslop-Harrison 1992; Leitch and others 1992; Hizume and others 2002; Doudrick and others 1995). A reference karyotype

[1] Research Geneticist and Adjunct Professor, Southern Institute of Forest Genetics, USDA Forest Service, Southern Research Station and Department of Ecosystem Science and Management, Texas A&M University, College Station, TX; [2] Research Associate , [3] Graduate Research Assistant, Department of Ecosystem Science and Management, Texas A&M University, College Station, TX; [4] Research Geneticist and Project Leader, Southern Institute of Forest Genetics, USDA Forest Service, Southern Research Station, Saucier, MS.

† Paper presented at the Joint Meeting of the 29th Southern Forest Tree Improvement Conference and the Annual Meeting of the Western Forest Genetics Association, June 20-22, 2007, Galveston, Texas, USA.

(i.e., chromosome-specific description of a genome) is a pre-requisite for advanced genetic and genomic studies and such karyotypes for loblolly and slash pines have been completed (Islam-Faridi et al. 2007 and Doudrick et al. 1995, respectively). Here we report the preliminary FISH results for developing reference karyotypes for both longleaf pine and shortleaf pine using 18S-28S rDNA, 5S rDNA, and *Arabidopsis*-type telomere repeat (ATR) sequence probes and AT-rich banding.

MATERIALS AND METHODS

Plant Sample and Slide Preparation

Prior to germination, seeds from two shortleaf pine parental clones (WO-5 and WO-12) were treated with 1% H_2O_2 followed by cold, moist stratification (6 wk), while seeds from two open-pollinated longleaf pine parental clones (EM17 and EM24) were treated with 1% H_2O_2 only. Germinated seedlings were transferred to potting mix and allowed to grow in a greenhouse prior to root sampling for cytological analysis. Actively growing root tips, about 1.5 cm long, were excised and pretreated in 0.15% colchicine (Sigma, USA) for 7 hr at room temperature in the dark. Root tips were then fixed in 2:1:1 95% v/v ethanol, glacial acetic acid, and double distilled water. The fixed root tips were treated with 3% w/v cellulase RS and 1% w/v pectolyase Y23 as described by Jewell and Islam-Faridi (1994). The digested root tips were macerated on a 95% v/v ethanol-cleaned glass slide followed by light squashing under a clean cover glass as described by Islam-Faridi and Mujeeb-Kazi (1995).

Nick Translation and *In situ* Hybridization

Whole plasmids of 18S-28S rDNA and 5S rDNA were labeled with biotin-16-dUTP (Biotin-Nick Translation Mix, Roche, Germany) following instructions provided by the manufacturer. An ATR DNA sequence $(TTTAGGG)_n$ of about 300 base pairs (kindly provided by Dr. T. McKnight, Texas A&M University) was labeled with digoxigenin-11-dUTP (Dig-Nick-Translation Mix, Roche, Germany). Labeled DNA was dot-blotted to verify incorporation of labeled nucleotides. A standard hybridization technique was used as described elsewhere (Hanson et al. 1996; Islam-Faridi et al. 2002). The probe hybridization sites were detected with Cy3-cojugated streptavidin (Jackson Immuno Research Laboratories, USA; for biotin labeled probe), and fluorescein conjugated anti-digoxigenin (Roche, Germany; for dig labeled probe) – followed by fluorescein conjugated anti-sheep (Roche, Germany). The preparation was counter stained with DAPI (4 ug/ml) and mounted using Vectashield (Vector Laboratories, USA).

Microscopy

Digital images were recorded from an AxioImager Z-1 Epi-fluorescence microscope with suitable filter sets (Chroma Technology, USA), using a COHU High Performance CCD Camera and the Metafer v4 MetaSystems Finder digital image system (MetaSystem Inc., USA). Images were processed with Ikaros and ISIS v5.1 and then further processed with Adobe Photoshop CS v8 (Adobe Systems, USA). The Metafer software allows scanning of the whole slide in less than

5 min and records the coordinates of each cell, while the "photo gallery" aspect of Metafer ensures the re-capture of the same cell in repeated experiments.

RESULTS AND DISCUSSIONS

We have modified a technique for preparing pine somatic chromosome spreads that consistently provides a high number of metaphase cells in root tip samples of the southern pine species. As many as 731 mitotic divisions, mostly in metaphase, have been observed from a single root tip preparation. In addition, chromosome morphology generally appears to be sharp and clear after *in situ* hybridization. This feature is critical for obtaining accurate measurements of total chromosome lengths, centromere indices, and distances from centromere to FISH signal positions. Strong DAPI positive (AT-rich) bands occurred in various patterns near or around the centromeres of 22 chromosomes each of longleaf pine and shortleaf pine. Numerous light or weaker DAPI bands appeared interstitially (i.e., area between a centromere and telomere) in some chromosomes of both species.

FISH experiments were carried out in two phases. First, we used 18S-28S and 5S rDNA clones (both labeled with biotin) on longleaf pine and shortleaf pine chromosome spreads. The hybridization sites were detected with Cy3 streptavidin. The 5S rDNA site was identified in an earlier experiment, so there would no confusion of its location with the 18S-28S rDNA sites (Figs. 1 and 2). FISH images were captured as described above, then the slides were incubated in 0.5X SSC for 2 hr to remove the probes and then re-probed with ATR. In our earlier experiments we observed that the first probe hybridization sites could not be eliminated completely. To obtain a better image we re-used the rDNA probes in the second FISH experiment along with the ATR probe (Figs. 1 and 2).

One major and one minor 5S rDNA sites were detected in both longleaf pine and shortleaf pine. Similar results were reported in loblolly pine (Islam-Faridi et al. 2007). Seven major interstitial 18S-28S rDNA sites were observed, similar to other pine species (e.g., loblolly pine, Jacobs et al. 2000 and Islam-Faridi et al. 2007, and slash pine, Doudrick et al. 1995). One major and six medium-to-minor 18S-28S rDNA sites were identified at or around the centromeres in longleaf pine, and three major and six medium-to-minor 18S-28S rDNA sites were identified in shortleaf pine. One of the interstitial sites of shortleaf pine is heterologous, i.e., one homologue showed a major 18S-28S rDNA FISH signal and the other showed a medium-to-minor signal. These results indicate that the medium-to-minor 18S site contains fewer repeat units of 18S-28S rDNA sequence than the other homologue. A similar result was reported in slash pine (Doudrick et al. 1995), suggesting that at least for this cytological feature shortleaf pine appears more closely related to slash pine than to loblolly pine or longleaf pine. However other genetic evidence suggests that shortleaf pine is more closely related to loblolly pine or longleaf pine than to the other southern pines (Dorman 1976; Wagner et al. 1991; Krupkin et al. 1996; Dvorak et al. 2000).

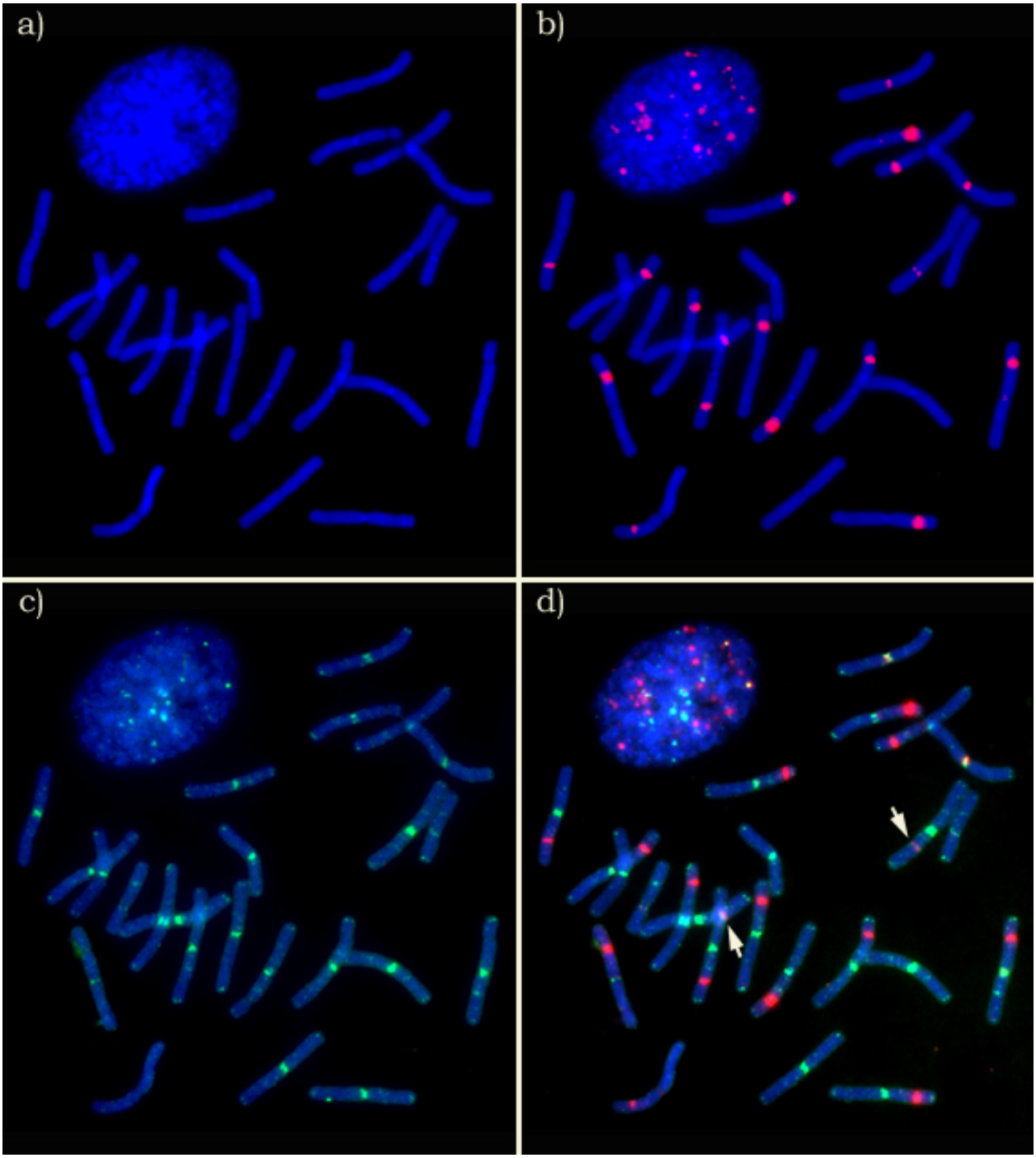

Figure 1. Longleaf pine chromosomes probed with 18S-28S rDNA and 5S rDNA clones (b and d, red signals) and *Arabidopsis*-type telomere repeat DNA sequence (c and d, green signals). Images "a" and "b" are from the first FISH experiment and Images "c" and "d" are from the second FISH experiment. Image "a" was taken under a DAPI filter and shows AT-rich bands; Image "b" was taken under DAPI and Cy3 filters, and then super-imposed; Image "c" was taken under FITC (green) and DAPI filters, and then super-imposed; Image "d" was taken under DAPI, Cy3 and FITC filters, and then super-imposed. Arrows point at the major 5S rDNA signals (d).

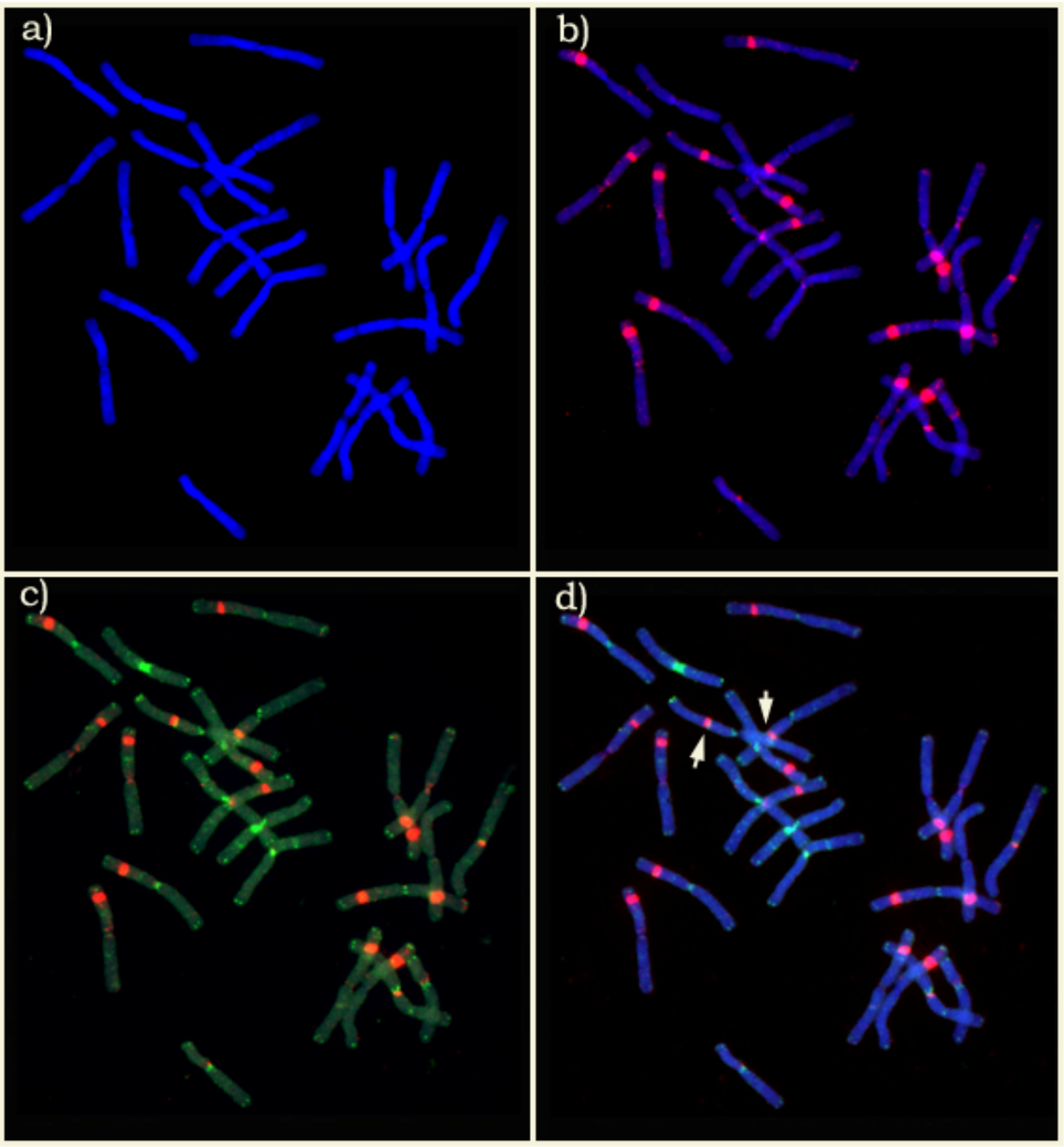

Figure 2. Shortleaf pine chromosomes probed with 18S-28S rDNA and 5S rDNA clones (b, c and d, red signals) and *Arabidopsis*-type telomere repeat DNA sequence (c and d, green signals). Images "a" and "b" are from the first FISH experiment and Images "c" and "d" are from the second FISH experiment. Image "a" was taken under a DAPI filter and shows AT-rich bands; Image "b" was taken under DAPI and Cy3 filters, and then super-imposed; Image "c" was taken under FITC (green) and Cy3 filters, and then super-imposed; Image "d" was taken under DAPI, Cy3 and FITC filters, and then super-imposed. Arrows point at the major 5S rDNA signals (d).

Strong to weak ATR signals were observed at or around the centromeric positions of all but one pair of chromosomes in both longleaf pine and shortleaf pine (Figs. 1c, 1d, 2c and 2d). Interstitial ATR signals were also observed on both chromosomal arms in longleaf pine and shortleaf pine. Similar results were reported in various pine species (Doudrick et al. 1995; Hizume et al. 2002; Islam-Faridi et al 2007), in contrast to other plant species where interstitial and centromeric telomere sites are rare (Fuchs et al. 1995). Finally, a pair of "snake-eyed" ATR signals was observed at the end of each chromosomal arm clearly revealing the telomere sequences at their usual terminal position.

Further analysis on measurements of chromosome lengths and distances between FISH signals are being carried out to develop a reference karyotype for both longleaf pine and shortleaf pine, which will then be compared to our loblolly pine reference karyotype (Islam-Faridi et al 2007) and two slash pine karyotypes (Doudrick et al. 1995; Islam-Faridi et al., unpublished data). It is our hope that cytogenetic analyses including karyotype comparisons between species will be useful in identifying structural rearrangements within and between species that may be used to infer evolutionary relationships, to inform gene conservation efforts, and to guide interspecies hybrid breeding projects.

Acknowledgements: We thank Daniel Peterson and George Hodnett for providing thoughtful reviews of this manuscript; Floyd Bridgewater (retired, U. S. Forest Service, Southern Research Station) for supporting this research through Cooperative Agreement, SRS 03-CA-11330126-040; National Science Foundation for partial funding (award DBI-0421717, NIF and CDN); and Tom Byram (Texas Forest Service and Texas A&M University) for administrative leadership, scientific discussion, and greenhouse space.

LITERATURE CITED

Borzan, Z. and D. Papes. 1978. Karyotype analysis in *Pinus*: A contribution to the standardization of the karyotype analysis and review of some applied techniques. Silvae Genetica 27:144-150.

Dorman, K.W. 1976. The Genetics and Breeding of Southern Pines. U. S. Department of Agriculture, Forest Service, Agricultural Handbook No. 471, 407 p.

Doudrick, R.L., J.S. Heslop-Harrison, C.D. Nelson, T. Schmidt, W.L. Nance and T. Schwarzacher. 1995. Karyotype of slash pine (*Pinus elliottii* var. *elliottii*) using patterns of fluorescence *in situ* hybridization and fluorochrome banding. Journal of Heredity 86:289-296.

Dvorak, W.S., A.P. Jordan, G.P. Hodge and J.L. Romero. 2000. Assessing evolutionary relationships of pines in the *Oocarpae* and *Australes* subsections using RAPD markers. New Forests 20:163-192.

Fuchs, J., A. Brandes and I. Schubert. 1995. Telomere sequence localization and karyotype evolution in higher plants. Plant Systematics and Evolution 196:227-241.

Hanson, R.E., M.N. Islam-Faridi, E.A. Percival, C.F. Crane, Y. Ji, T.D. McKnight, D.M. Stelly and H.J. Price. 1996. Distribution of 5S and 18S-28S rDNA loci in a tetraploid cotton (*Gossypium hirsutum* L.) and its putative diploid ancestors. Chromosoma 105:55-61.

Heslop-Harrison, J.S. 1991. The molecular cytogenetics of plants. Journal of Cell Science 100: 15-21.

Hizume, M., M. Arai and A. Tanaka. 1990. Chromosome banding in the genus *Pinus*. III. Fluorescent banding pattern of *P. luchuensis* and its relationships among the Japanese diploxylon pines. Botanical Magazine, Tokoyo 103: 103-111.

Hizume, M., F. Shibata, Y. Matsusaki and Z. Garajova. 2002. Chromosome identification and comparative karyotypic analyses of four *Pinus* species. Theoretical and Applied Genetics 105: 491-497.

Islam-Faridi, M.N. and A. Mujeeb-Kazi. 1995. Visualization of *Secale cereale* DNA in wheat germplasm by fluorescent *in situ* hybridization. Theoretical and Applied Genetics 90: 595-600.

Islam-Faridi, M.N., C.D. Nelson and T.L. Kubisiak. 2007. Reference karyotype and cytomolecular map for loblolly pine (*Pius taeda* L.). Genome 50:241-251.

Jacobs, M.D., R.C. Gardner and B.G. Murray. 2000. Cytological characterization of heterochromatin and rDNA in *Pinus radiata* and *P. taeda*. Plant Systematics and Evolution 223: 71-79.

Jewell, D.C. and M.N. Islam-Faridi. 1994. Details of a technique for somatic chromosome preparation and C-banding of maize. In: M. Freeling and V. Walbot, (eds) The Maize Handbook. Springer-Verlag, New York, pp 484-493.

Krupkin, A.B, A. Liston and S.H. Strauss. 1996. Phylogenetic analysis of the hard pines (*Pinus* Subgenus *Pinus*, *Pinaceae*) from chloroplast DNA restriction site analysis. American Journal of Botany 83: 489–498.

Leitch, I.J. and J.S. Heslop-Harrison. 1992. Physical mapping of the 18S-5.8S-25S rRNA genes in barley by *in situ* hybridization. Genome 35: 1013-1018.

Leitch, I.J., W. Mosgoller, M. Shi and J.S. Heslop-Harrison. 1992. Different patterns of rDNA organization at interphase in nuclei of wheat and rye. Journal of Cell Science 101: 751-757.

MacPherson, P. and W.G. Filion. 1981. Karyotype analysis and the distribution of constitutive heterochromatin in five species of *Pinus*. Journal of Heredity 72: 193-198.

Mergen, F. 1958. Natural ploidy in slash pine. Forest Science 4:283-295.

Sax, K and H.J. Sax. 1933. Chromosome number and morphology in the conifers. Journal of the Arnold Arboretum 15:356-375.

Wagner, D.B., W.L. Nance, C.D. Nelson, T. Li, R.N. Patel and D.R. Govindaraju. 1991. Taxonomic patterns and inheritance of chloroplast variation in a survey of *Pinus echinata, Pinus elliottii, Pinus palustris,* and *Pinus taeda.* Canadian Journal of Forest Research 22: 683–689.