Reference karyotype and cytomolecular map for loblolly pine (*Pinus taeda* L.)

M. Nurul Islam-Faridi, C. Dana Nelson, and Thomas L. Kubisiak

Abstract: A reference karyotype is presented for loblolly pine (*Pinus taeda* L., subgenus *Pinus*, section *Pinus*, subsection *Australes*), based on fluorescent in situ hybridization (FISH), using 18S-28S rDNA, 5S rDNA, and an *Arabidopsis*-type telomere repeat sequence (*A*-type TRS). Well separated somatic chromosomes were prepared from colchicine-treated root meristems, using an enzymatic digestion technique. Statistical analyses performed on chromosome-arm lengths, centromeric indices, and interstitial rDNA and telomeric positions were based on observations from 6 well-separated metaphase cells from each of 3 unrelated trees. Statistically, 7 of the 12 loblolly pine chromosomes could be distinguished by their relative lengths. Centromeric indices were unable to distinguish additional chromosomes. However, the position and relative strength of the rDNA and telomeric sites made it possible to uniquely identify all of the chromosomes, providing a reference karyotype for use in comparative genome analyses. A dichotomous key was developed to aid in the identification of loblolly pine chromosomes and their comparison to chromosomes of other *Pinus* spp. A cytomolecular map was developed using the interstitial 18S-28S rDNA and *A*-type TRS signals. A total of 54 bins were assigned, ranging from 3 to 5 bins per chromosome. This is the first report of a chromosome-anchored physical map for a conifer that includes a dichotomous key for accurate and consistent identification of the *P. taeda* chromosomes.

Key words: FISH, loblolly pine, 18S-28S rDNA, *A*-type TRS, reference karyotype, cytomolecular map, dichotomous key, TRS/18-28S bins.

Résumé : Un caryotype de référence est présenté pour le pin à encens (*Pinus taeda* L., sous-genre *Pinus*, section *Pinus*, sous-section *Australes*). Celui-ci est basé sur les motifs d'hybridation in situ en fluorescence (FISH) à l'aide des ADNr 18S-28S et 5S ainsi que la séquence télomérique (TRS de type A) de l'arabidopsis. Des chromosomes somatiques bien séparés ont été préparés à partir de méristèmes radiculaires traités à la colchicine et une technique de digestion enzymatique. Des analyses statistiques ont été effectuées sur la longueur des bras chromosomiques, les indices centromériques, la localisation des ADNr internes et la position des centromères suite à des observations faites sur six cellules en métaphase bien préparées, celles-ci provenant des trois arbres non-apparentés. Statistiquement, sept des chromosomes du pin à encens se différenciaient sur la base de leurs longueurs relatives. Les indices centromériques n'ont pas permis de distinguer d'autres chromosomes. Cependant, la position et l'intensité relatives des sites d'ADNr et d'ADN télomérique ont rendu possible d'identifier tous les chromosomes, procurant ainsi un caryotype de référence pour fins d'analyse comparée des génomes. Une clé dichotomique a été développée pour faciliter l'identification des chromosomes du pin à encens et la comparaison avec les chromosomes d'autres espèces du genre *Pinus*. Une carte cytomoléculaire est présentée, celle-ci faisant appel aux signaux de l'ADNr 18S-28S et des séquences TRS de type *A*. Au total, 54 segments (« bins ») ont été définis à raison de 3 à 5 segments par chromosome. Il s'agit de la première description d'une carte physique à chromosomes ancrés chez les conifères incluant une clé dichotomique pour l'identification précise et reproductible des chromosomes chez le *Pinus taeda*.

Mots-clés : FISH, pin à encens, ADNr 18S-28S, TRS de type *A*, caryotype de référence, carte cytomoléculaire, clé dichotomique, segments chromosomiques TRS/18S-28S.

[Traduit par la Rédaction]

Received 13 September 2006. Accepted 7 December 2006. Published on the NRC Research Press Web site at http://genome.nrc.ca on 13 April 2007.

Corresponding Editor: J.P. Gustafson.

M.N. Islam-Faridi.[1] Forest Tree Molecular Cytogenetics Laboratory, Southern Institute of Forest Genetics, Southern Research Station, USDA Forest Service, Texas A&M University, 2585-TAMU, College Station, TX 77843-2585, USA.
C.D. Nelson and T.L. Kubisiak. Southern Institute of Forest Genetics, Southern Research Station, USDA Forest Service, Harrison Experimental Forest, 23332 Mississippi 67, Saucier, MS 39574, USA.

[1]Corresponding author (e-mail: nfaridi@tamu.edu).

Introduction

The genus *Pinus* ($2n = 2x = 24$), originally confined almost entirely to the northern hemisphere, includes many economically and ecologically important species. Several species, such as loblolly pine (*Pinus taeda* L.), slash pine (*P. elliottii* Engelm. var. *elliottii*), Monterey pine (*P. radiata* D. Don), and Mediterranean pine (*P. pinaster* Ait.), with desirable growth and wood quality characteristics are being extensively planted on a worldwide basis. A number of tree improvement programs for these species use molecular genetic markers to construct linkage maps (Remington et al. 1999; Sewell et al. 1999; Costa et al. 2000; Kondo et al. 2000; Kubisiak et al. 2000; Li and Yeh 2001) for the purpose of mapping quantitative trait loci to accelerate breed-

 doi:10.1139/G06-153

ing efforts through marker-assisted selection. Despite the many linkage maps, little attention has been focused on developing a reference karyotype to unambiguously identify all 12 homologous chromosomes of pine. A reference karyotype based on robust cytological landmarks would greatly facilitate cross-referencing among these many recombination-based maps. Such information has the potential to significantly increase our understanding of genome evolution in this genus and lead to improved marker-based applications in tree breeding and species conservation programs.

Pinus genomes are large (1C = 20–30 Gb) (Wakamiya et al. 1993; Bogunic et al. 2003), and most of the chromosomes are cytologically indistinguishable because they are similar in length and centromeric index. Several attempts have been made to construct a karyotype for pine based on traditional cytological tools, such as Giemsa, C, and chromamycin A_3 (CMA) banding. However, insufficient levels of variation in banding patterns have made it difficult to unambiguously discriminate among most chromosomes (Borzan and Papes 1978; MacPherson and Filion 1981; Drewry 1982; Saylor 1983; Hizume et al. 1989, 1990). Molecular cytological techniques, such as in situ hybridization (ISH), coupled with conventional cytology can provide more accurate information about genomes (Heslop-Harrison 1991; Leitch and Heslop-Harrison 1992; Leitch et al. 1992); in fact, karyotypes have been developed for several pine species using rDNA, CG-rich repeat sequence, and (or) *Arabidopsis*-type telomere repeat sequence (*A*-type TRS) probes and CMA banding (*P. elliottii* var. *elliotti* (Doudrick et al. 1995); *P. sylvestris* L. (Lubaretz et al. 1996); *P. radiata* and *P. taeda* (Jacobs et al. 2000); *P. densiflora* Sieb & Zucc., *P. thunbergii* Franco, *P. sylvestris*, and *P. nigra* Arnold (Hizume et al. 2002)). However, none of these karyotypes were based on rigorous quantitative data and statistical analysis, and therefore each is fraught with enough uncertainty to make its use contraindicated in most gene mapping studies.

Well-spread metaphase chromosomes that are free of cell walls and cytoplasmic debris are a prerequisite for ISH. Because the chromosomes of pine are extremely large, well-spread metaphases are difficult to obtain (Doudrick et al. 1995; Jacobs et al. 2000; Schmidt et al. 2000). Well-separated chromosome spreads with minimal distortion are essential for accurate length and distance measurements. For the current pine cytogenetics work, a modified somatic chromosome preparation technique was used to improve chromosome spreading and morphology. Fluorescent ISH (FISH) was then used to quantitatively locate 18S-28S ribosomal, 5S ribosomal DNA, and *A*-type TRS sites in 6 cells from each of 3 unrelated trees to develop a robust reference karyotype for loblolly pine. The data were statistically analyzed to determine the significance of differences between means observed for chromosome length, arm ratio, and FISH signal positions. A dichotomous key was developed to aid in the identification of loblolly pine chromosomes and to compare these results with previous results for slash pine (Doudrick et al. 1995) and several species of *Pinus* section *Pinus* subsection *Sylvestris* (Hizume et al. 2002). Finally, a cytomolecular map comprising 54 bins, ranging from 3 to 5 bins per chromosome, was developed to serve as a framework map for FISH-based physical mapping in loblolly pine.

Materials and methods

Plant materials

Open-pollinated seeds from 3 loblolly pine clones (7-56, SIFG-J, and LSG-62) were stratified for 5 d in 1% hydrogen peroxide (H_2O_2, changed daily) after soaking in 30% H_2O_2 for 20 min and rinsing in running water overnight. Stratified seeds were germinated on moist filter paper in Petri dishes at 24 °C in the dark. The clones were selected to represent different geographic areas of the species native range: 7-56 is from coastal South Carolina, SIFG-J is from southeast Louisiana, and LSG-62 is from east Texas.

Slide preparation

Healthy root tips shorter than 1.5 cm were excised, pretreated in 0.15% colchicine (Sigma, P-9754) for 7.5 h at room temperature in the dark, and then fixed in 4:1 ethanol (95%)/glacial acetic acid. The roots were digested with cell-wall degrading enzymes, as described by Jewell and Islam-Faridi (1994). The digested root tips were macerated on clean slides (1 root tip per slide) in 3:1 ethanol/glacial acetic acid. After complete evaporation, a small drop of 45% glacial acetic acid was added to the macerated tissue, which was then gently squashed under a glass cover slip (following Islam-Faridi and Mujeeb-Kazi 1995).

Probe DNA nick translation and fluorescent in situ hybridization

Either whole plasmid (18S-28S rDNA and 5S rDNA) or isolated insert (*A*-type TRS, $(TTTAGGG)_n$) DNA was labeled by nick translation, using either biotin-14-dATP (BioNick Labeling System, Invitrogen, Carlsbad, Calif.) or digoxigenin-11-dUTP (Dig-Nick Translation Mix, Roche, Indianapolis, Ind.), in accordance with the manufacturers' instructions.

Microscopy

Digital images were recorded using an Olympus AX-70 epifluorescence microscope with suitable monochrome filter sets (Chroma Technology, Rockingham, Vt.), a 1.3 megapixel Sensys (Roper Scientific, Tucson, Ariz.) camera, and the MacProbe v. 4.2.3 digital image system (Applied Imaging, San Jose, Calif.). Final magnification was 1000×. Images were processed with Adobe Photoshop CS v. 8.0 (Adobe Systems, San Jose, Calif.).

Karyotype analysis

For each of the 3 open-pollinated families, 6 complete undistorted chromosome spreads were selected for karyotype analysis. In each spread, chromosomes were numbered arbitrarily from 1 to 24, and paired (12 pairs) on the basis of 18S-28S rDNA, 5S rDNA, *A*-type TRS FISH signals and centromeric DAPI bands (AT-rich regions). Distances (relative units, where total chromosome length for the set was 100) from the centromere to each signal and to the chromosome end were measured 3 times, and averaged before statistical analysis. In addition, the 18S-28S rDNA and *A*-type TRS signals at the centromere were classified by signal strength on a scale from 0 to 5, where 0 indicates no signal and 5 indicates a signal of the highest intensity observed for the set. All data were collected using Optimas v. 6 (Pixera,

Table 1. Means and standard errors (SE) for the 12 chromosomes in loblolly pine.

Chromosome	Mean RL (Duncan group), SE	Mean CI (Duncan group), SE (centricity class[b])	Number and type of diagnostic features (assigned arm)[a]	
			Arm 1	Arm 2
1	9.55 (A), 0.048	48.5 (AB),[c] 0.40 (M)	1 i5S (S)	0 i5S (L)
2	9.16 (B), 0.049	48.7 (AB), 0.28 (M)	1 i18S (L)	0 i18S (S)
3	8.81 (C), 0.051	48.2 (AB), 0.26 (M)	1 i18S (S)	0 i18S (L)
4	8.75 (C), 0.035	47.7 (B), 0.33 (M)	1 i18S (S)	0 i18S (L)
5	8.58 (D), 0.037	49.1 (A),[c] 0.31 (M)	1 c18S (L)	0 c18S (S)
6	8.58 (D), 0.044	49.1 (A),[c] 0.16 (M)	2 iTelo (L)	1 iTelo (S)
7	8.55 (D), 0.031	48.4 (AB), 0.20 (M)	1 i18S (L)	0 i18S (S)
8	8.39 (E), 0.041	49.2 (A), 0.29 (M)	1 i18S (S)	0 i18S (L)
9	8.17 (F), 0.036	48.9 (A), 0.30 (M)	1 i18S (S)	0 i18S (L)
10	7.69 (G), 0.043	46.1 (C), 0.20 (NSM)	1 i18S (S)	0 i18S (L)
11	7.48 (H), 0.030	48.7 (AB),[c] 0.61 (M)	1 c18S (S)	0 c18S (L)
12	6.27 (I), 0.034	39.9 (D), 0.37 (SM)	1 iTelo (L)	0 iTelo (S)

Note: All estimates are based on 36 chromosomes except where noted in footnote 'c'. CI, centromeric index; RL, relative length.

[a]Arms 1 and 2 were recognized by the features listed (i18S, c18S, iTelo or i5S), and arm-length differences were tested for significance ($p \leq 0.05$). Short (S) and long (L) chromosome arms were assigned only when results were significant.

[b]Centricity class: M, metacentric (CI ≥ 47); NSM, near submetacentric (42 ≥ CI < 47); SM, submetacentric (CI < 42).

[c]Estimates based on fewer than 36 chromosomes, because the arms were not always distinguishable. Numbers of chromosomes were 10, 34, 20, and 22 for chromosomes 1, 5, 6 and 11, respectively.

Los Gatos, Calif.) after zooming 400% to improve resolution and minimize measurement error. Homologous pairs of chromosomes were numbered (1 to 12) according to mean relative length (RL; the sum of 2 arm lengths), starting with the longest RL.

Statistical analysis

For each set, homologous chromosomes were identified and aligned on the basis of their DAPI stain and FISH patterns (see Table 1). Within each homologous pair, *t* tests were used to test the significance of the difference between chromosome-arm lengths. In instances where significance was established ($p \leq 0.05$), chromosome arms were then assigned as either long or short. The centromeric index (CI) was computed as short-arm length / (short-arm length + long-arm length). RL and CI were subjected to analyses of variance and Duncan's multiple-range tests, using homologous pair as the dependent variable (Proc GLM, SAS Institute Inc.). Distances to the interstitial (i.e., between centromere and telomere) *A*-type TRS (iTelo) and 18S-28S rDNA (i18S) signals were analyzed in the same way, after converting the relative-distance values to arm-index (AI) values (distance from centromere / arm length). For all position and signal intensity variables, means and standard errors (SE) were computed (Tables 1 and 2).

Homoeologous groups, subsection *Australes*

We attempted to align our loblolly pine reference karyotype with a slash pine karyotype published by Doudrick et al. (1995). Both of these species are in subgenera *Pinus*, section *Pinus*, and subsection *Australes*, and both studies tested and detected similar genomic features, although with some notable variations. For each loblolly pine (our study) and slash pine (Doudrick et al. 1995) chromosome, we coded data for 6 features, as shown in Table 3. A difference matrix was then computed for all 144 combinations of the 12 loblolly and 12 slash pine chromosomes for each feature. A single distance measure was calculated with a linear combination of the individual feature squared differences. Both equal weightings of features and various unequal weightings were evaluated. For a given weighting, the differences were ranked (smallest difference, 1; largest difference, 12) by loblolly and by slash pine chromosomes, and the 2 rankings were superimposed on each other and summed. For each chromosome (using the loblolly pine chromosome numbers), the smallest sum was taken as a homoeologous match, providing that both rankings were among the best 4. Where more than 1 minimum sum occurred in a row (loblolly chromosomes) or column (slash chromosomes), the next best choice was made, again providing that there was a choice where both rankings were among the best 4. If no choice could be found using these criteria, then the homoeologous grouping for that chromosome was considered unknown.

Results

We developed a modified pine chromosome spread technique that consistently provides a large number of high-quality metaphase cells. On average, a single pine root preparation yielded 50 or more dividing cells (prophase to metaphase) per slide, with a maximum count of 731. For a number of cells on each slide, all chromosomes were well separated without distortion, and their morphology was clearly discernible after DAPI staining or FISH. Figures 1 and 2 show photomicrographs of FISH images of loblolly pine metaphase chromosomes probed with 18S-28S rDNA, 5S rDNA and *A*-type TRS.

Relative length and centromere index

In many instances, differences among RLs and CIs of the loblolly pine chromosomes were significant (Table 1). We

Table 2. Means and SE for distinguishing features of the 12 chromosomes in loblolly pine.

Chromosome	cTelo[a,b] (RL units)	c18S[a,b] (RL units)	iTelo1[b,c] (AI units)	iTelo2, 3[b,c,d] (AI units)	i18S[b,c] (AI units)	i5S[b,c] (AI units)
1	–0.017, 0.009 0.89 0.89, 0.05	0.067, 0.006 1.00 1.22, 0.07	36.7, 0.63 0.89	–35.9, 0.47 1.00		**40.0**[e], **1.43** **1.00**
2	0.004, 0.006 1.00 2.28, 0.16	0.006, 0.008 0.78 0.78, 0.07	36.3, 0.73 0.83	–34.3, 0.89 0.86	**–62.1, 0.60** **0.81**	93.7, 3.48 0.21
3	0.034, 0.014 0.39 0.39, 0.08	**0.0, 0** **1.00** **4.83, 0.06**	34.4, 0.54 0.83	–33.9, 1.22 0.25	**61.6, 0.72** **0.94**	[f]
4	0.012, 0.010 0.06 0.69, 0.08	0.051, 0.006 1.00 1.11, 0.08	35.0, 0.60 0.81	–35.1, 1.30 0.36	**62.2, 0.55** **0.94**	
5	0.014, 0.008 1.00 3.06, 0.04	–0.045, 0.010 1.00 2.56, 0.14	37.3, 0.55 0.92	–37.8, 0.64 0.89		
6	**0.0, 0** **1.00** **4.75, 0.08**	0.007, 0.008 0.72 0.72, 0.08	37.4, 0.75 0.94	–32.0, 1.20 0.94 –41.1, 1.09 0.56		
7	–0.002, 0.010 0.06 0.42,[g] 0.08	**0.0, 0** **0.94** **4.94, 0.04**	39.6, 1.17 0.47	–41.5, 1.12 0.67	**–56.6, 0.48** **1.00**	
8	0.008, 0.005 1.00 2.78, 0.09	0.025, 0.008 0.94 0.89, 0.05	34.5, 0.95 0.69	–33.4, 1.22 0.61	**64.5, 0.99** **0.69**	
9	**0.0, 0** **1.00** **4.72, 0.08**	–0.019, 0.013 0.06 0.25, 0.07	37.5, 0.77 0.86	–35.8, 0.62 0.53	**60.5, 0.93** **0.86**	
10	0.0, 0 0.22 0.22, 0.07	**0.0, 0** **1.00** **5.00, 0.0**	37.9, 0.51 0.89	–35.5, 0.97 0.61	**71.3, 0.80** **1.00**	
11	**0.0, 0** **1.00** **5.00, 0.0**	0.024, 0.007 1.00 1.00, 0.0	41.8, 0.86 0.81	–40.1, 0.88 0.61		
12	–0.074, 0.003 1.00 1.11, 0.08	0.083, 0.005 1.00 1.00, 0.0		–47.6, 0.62 1.00		

Note: AI, arm index; RL, relative length.

[a]Values given in each cell are as follows: first line, AI mean and SE; second line, proportion of cells scored; third line, signal strength mean and SE.

[b]cTelo and c18S are centromeric *A*-type TRS and 18S-28S rDNA signals, respectively; iTelo1 to iTelo3 are interstitial *A*-type TRS signals; and i18S and i5S are interstitial 18S-28S rDNA and 5S rDNA signals, respectively.

[c]Values given in each cell are as follows: first line, AI mean and SE; second line, proportion of cells scored. Where a third and fourth line appear, these indicate the same as the first 2 lines for a second signal.

[d]A third iTelo site appeared on chromosome 6 only. In rare cases (<10% of the cells), a third iTelo site was observed on chromosomes 2, 5, or 10.

[e]Signal strength classes were determined from mean signal strength, where trace <0.50; 0.50 ≤ minor < 2.0; 2.0 ≤ intermediate < 4; and major ≥ 4. Data for all major signals are shown in bold.

[f]The third i5S site is trace-to-minor and appears to be on chromosome 3, but this assignment remains tentative.

[g]cTelo site on chromosome 7 was observed in 1 of 3 families.

found that RL could be used to statistically differentiate 7 of the chromosomes using Duncan's multiple-range test ($p \leq 0.05$). In this test, mean values for a particular variable (e.g., RL) are categorized into groups (each designated by an uppercase letter) based on the standard errors of the means and the number of means between the 2 chromosomes being compared (Steel and Torrie 1980). Two ranges contained multiple chromosomes: range C with chromosomes 3 (mean RL, 8.81; SE, 0.05) and 4 (RL, 8.75; SE, 0.04); and range D with chromosomes 5 (RL, 8.58; SE, 0.04), 6 (RL, 8.58; SE, 0.04), and 7 (RL, 8.55; SE, 0.03). CIs were less variable among the chromosomes. Duncan's

Table 3. Dataset attributes for comparing loblolly and slash pine karyotypes.

Signal	Probe or detection method and species used	Region	Sequence(s) detected	Scale[a]
i18S intensity	FISH, both species	Interstitial	18S-28S rDNA	0–5
c18S intensity	FISH, loblolly CMA, Slash	Centromeric	18S-28S rDNA	0–5
cTelo intensity	FISH, loblolly DAPI, Slash	Centromeric	*A*-type telomere repeat	0–5
5S intensity	FISH, both species	Interstitial	5S rDNA	0–3
5S location	FISH, both species	Interstitial	5S rDNA	0–2
RL class	RL, both species	Overall	None	0–2

Note: CMA, chromamycin A_3. FISH, fluorescent in situ hybridization; RL, relative length.

[a]Range of values used. For intensity values, the first number, 0 in all cases, indicates a complete lack of signal, and the second number represents the most intense signal observed. For 5S location, 0 indicates no signal, 1 medial interstitial location, and 2 distal location. For RL class, 0 indicates the shortest chromosome, 1 indicates the next 2 shortest chromosomes, and 2 indicates the longest 9 chromosomes.

test suggests 3 overlapping ranges, containing 10 chromosomes with the highest (i.e., most metacentric) CIs. These ranged from 47.7 (SE, 0.33) for chromosome 4 to 49.1 (SE, 0.31) for chromosome 5. Only chromosomes 10 and 12 were distinct, with CIs of 46.1 (SE, 0.20) and 39.9 (SE, 0.37), respectively.

18S-28S rDNA location

All 12 chromosomes showed 1 or 2 18S-28S rDNA signals (Table 2). There were 10 major and as many as 9 intermediate-to-trace 18S-28S rDNA sites identified in each of the 3 loblolly pine families (Figs. 1*a*, 1*d*, and 2). This is consistent with a previous finding (Hizume et al. 2002) and represents the largest number of 18S-28S rDNA sites reported for a diploid plant species. Seven of the 10 major 18S-28S rDNA sites were located at interstitial positions (i18S), and 3 were at centromeric positions (c18S), which flanked the primary constriction. The remaining 9 intermediate-to-trace sites were located at or around the centromere (c18S).

5S rDNA location

Only 1 major 5S rDNA site was observed in loblolly pine, and it was located at a medial interstitial position (mean AI, 40.0; SE, 1.43) on the short arm of chromosome 1 (Table 2, Figs. 1*f* and 1*g*). Two other minor 5S rDNA sites were observed, but these were located toward the ends of 2 different chromosomes (minor signal on chromosome 2 (Figs. 1*h* and 1*i*) and trace signal possibly on chromosome 3).

A-type TRS location

A-type TRS was found at as many as 6 different sites along a chromosome; 1 at each end (i.e., the telomeres), 1 to 3 at interstitial positions (iTelo1–3), and 1 at or around the primary constriction (cTelo) (Table 2, Figs. 1*b*, 1*d*, and 2). Most of the chromosomes showed an interstitial *A*-type TRS signal as a streak or band on both chromosome arms (the exceptions being chromosomes 6 and 10, which had 2 bands on their long arms; and chromosome 12, which had no band on its short arm). All chromosomes showed major-to-trace *A*-type TRS signals at or around the primary constriction, which is infrequent in plant species (Fuchs et al. 1995).

AT-rich regions

Eleven (and in rare cases 10) of 12 chromosomes showed strong AT-rich (i.e., DAPI bands) heterochromatic sites at or around the centromere (Figs. 1*c*, 1*e*, and 2). Only chromosome 4 varied in this respect; 1 member (and in rare cases both) of the homologous pair consistently failed to show an AT-rich site in the centromeric region (Figs. 1*e*, 1*j*, and 1*k*). Interstitial AT-rich regions were observed in most but not all of the chromosome arms, typically associated with the *A*-type TRS sites.

Loblolly pine karyotype and dichotomous key of chromosomes

Using the FISH probe data, we constructed a reference karyotype (Fig. 2) and dichotomous key (Table 4) to assist in identifying the chromosomes of loblolly pine. Additional distinguishing characteristics for each of the 12 chromosomes are given in Supplemental Table S1[2]. We attempted to align our karyotype data and chromosomal designations with those for slash pine, a related species occurring in the same subsection as loblolly pine for which the first standardized numbering system for conifer chromosomes was proposed (Doudrick et al. 1995). Various weighting of the genomic features produced essentially identical results, so equal weightings were used to obtain the designations provided in Supplemental Table S1[2]. Finally, we subdivided each of the chromosomal arms into bins, using the interstitial 18S-28S rDNA and interstitial *A*-type TRS sites as bin boundaries. The bins were numbered consecutively from 1–54 across the 12 chromosomes, in order of RL, starting with the short arms and proximal locations. This is the first report of its kind in a conifer species; we have developed a chromosome-anchored cytomolecular map and a dichotomous key for accurate and consistent chromosome identification. The resulting loblolly pine ideogram is presented in Fig. 3.

Discussion

We have presented the first comprehensive karyotype for loblolly pine, using cytologically discriminatory features to differentiate each of the chromosomes ($n = x = 12$) and

[2] Supplementary data for this article are available on the journal Web site (http://genome.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 5144. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/irm/unpub_e.shtml.

Fig. 1. In situ hybridization (ISH) of somatic metaphase chromosomes of loblolly pine (family LSG-62): (*a*) major and minor 18S-28S rDNA sites (red signals, Cy3 filter); (*b*) terminal, interstitial, and centromeric *Arabidopsis*-type telomere repeat sequence (*A*-type TRS) signals (green, FITC filter); (*c*) centromeric and interstitial AT-rich bands (blue, DAPI stained, UV filter); (*d*) superimposed image of UV, Cy3, and FITC filters; (*e*) gray-scale inverted image of Fig. 1*c* showing improved resolution of AT-rich bands; (*f*) major 5S rDNA site located on chromosome 1 (arrowhead, superimposed image of UV, Cy3, and FITC filters); (*g*) same as Fig. 1*f* except with Cy3 filter only; (*h*) second 5S rDNA site located on chromosome 2 (arrowhead, UV, Cy3, and FITC filters); (*i*) same as Fig. 1*h* except with Cy3 filter only; (*j*) chromosome 4 lacking A-T rich band (arrowhead, DAPI stained, UV filter), and (*k*) gray-scale inverted image of Fig. 1*j*. Scale bar = 10 μm.

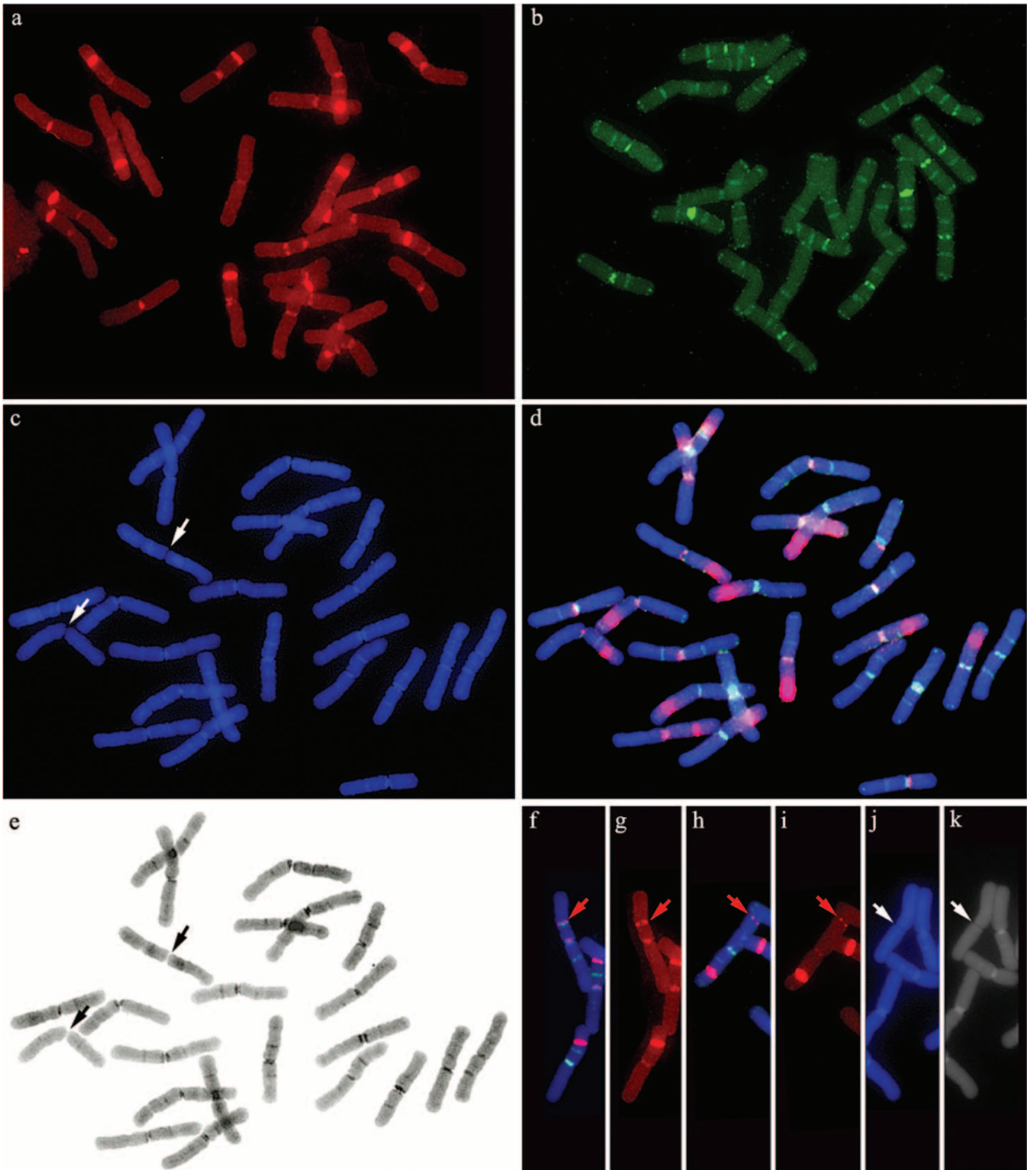
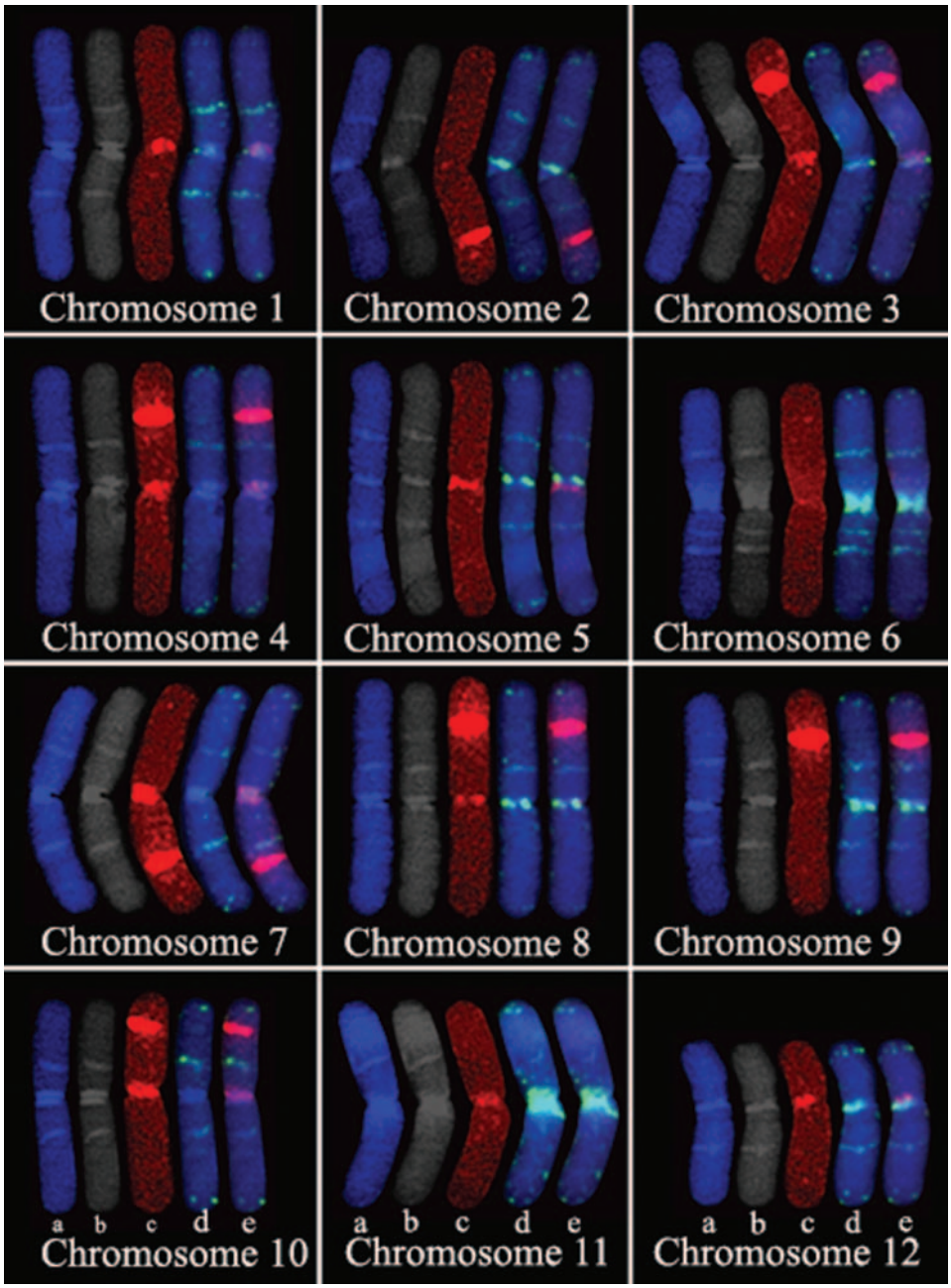


Fig. 2. A reference karyotype of loblolly pine (family SIFG-J). Homologous chromosomes, numbered 1 to 12, are arranged in descending order of mean relative length: (*a*) DAPI stained, (*b*) gray-scale inverted image of Fig. 2*a*, (*c*) 18S-28S rDNA sites (red signal), (*d*) *A*-type TRS sites (green signal), and (*e*) superimposed image consisting of Figs. 2*a*, 2*c*, and 2*d*.



chromosome arms (i.e., distinguishing long from short arms, independent of length measurements). Technical improvements, which provided a high number of metaphase spreads and nondistorted well-separated chromosomes, and statistical analyses of well-replicated experiments were combined to provide the best estimates to date of *Pinus* chromosome RLs and CIs. Given this and the importance of loblolly pine as a model conifer species, we propose the adoption of the loblolly chromosome numbers and chromosome arm designations (Tables 1 and 2) as the standard for *Pinus*, especially the pines of the *Australes* subsection. Ten of the 12 chromosomes can be homoeologously paired with slash pine, as provided by Doudrick et al. 1995: loblolly 1 with slash 9, loblolly 2 with slash 2, loblolly 4 with slash 5, loblolly 5 with slash 7, loblolly 6 with slash 1, loblolly 7 with slash 4, loblolly 8 with slash 3, loblolly 9 with slash 8, loblolly 11 with slash 10, and loblolly 12 with slash 12. The remaining 2 loblolly pine chromosomes (3 and 10) cannot be decidedly paired with the unassigned slash pine chromosomes (6 and 11).

Our study identified 12 centromeric 18S-28S rDNA sites, which is more than previously suggested by CMA banding or CMA band specific repeat (Doudrick et al. 1995; Lubaretz et al. 1996; Jacobs et al. 2000; Hizume et al. 2002). We chose not to use CMA banding, because it has been shown to provide limited information beyond the presence of 18S-28S

Table 4. Dichotomous key for the 12 chromosomes of loblolly pine.

Step	To	Chromosomes
A. i18S major (50<AI<80)	B	2, 3, 4, 7, 8, 9, 10
A. i18S not present	C	1, 5, 6, 11, 12
C. i5S major (AI<50)		1
C. i5S not present	D	5, 6, 11, 12
D. submetacentric (CI<43)		12
D. not submetacentric (CI≥43)	E	5, 6, 11
E. cTelo intermediate, c18S intermediate		5
E. cTelo major, c18S minor to trace	F	6, 11
F. cTelo exceptionally robust, typically 2 iTelo sites (1 on each arm)		11
F. cTelo major but not exceptionally robust, typically 3 iTelo sites (2 on long arm)		6
B. i5S minor (AI>80)		2
B. i5S not present	G	3, 4, 7, 8, 9, 10
G. c18S major	H	3, 7, 10
G. c18S minor to trace	I	4, 8, 9
H. iTelo close to i18S site (AI<20)		7
H. iTelo not close to i18S site (25<AI<40)	J	3, 10
J. i18S distal (AI>65)		10
J. i18S not distal (AI<65)		3
I. cTelo major		9
I. cTelo intermediate to trace	K	4, 8
K. cTelo intermediate		8
K. cTelo trace		4

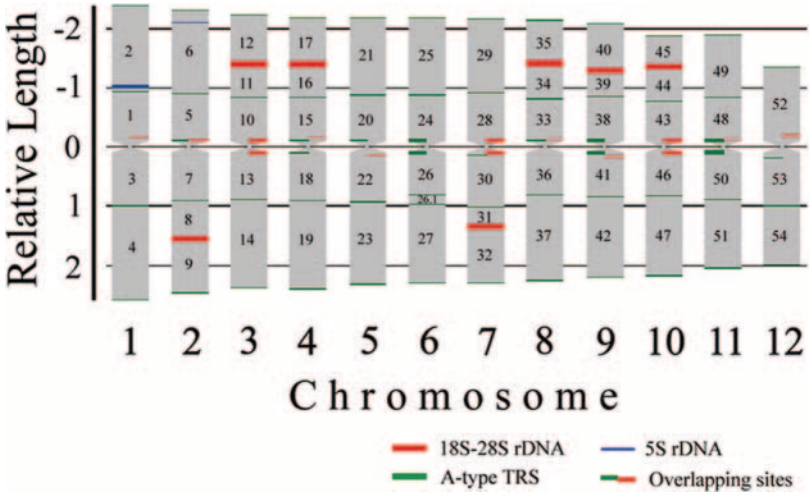
rDNA sites and primary constrictions (Doudrick et al. 1995, Jacobs et al. 2000; Schweizer 1980). Three of the 12 centromeric 18S-28S rDNA sites in loblolly pine are major sites (chromosomes 3, 7, and 10) (Table 2; Figs. 2 and 3). Weak 45S rDNA signals near the centromeric regions were observed in subsection 4 *Sylvestris* species and were found to be associated with PCSR (centromeric CMA band-specific repeat) signals (Hizume et al. 2002). No major 45S rDNA sites were observed in the centromeric regions in any of the *Sylvestris* species (Hizume et al. 2002).

Several chromosomes showed strong interstitial *A*-type TRS signals in all 4 *Sylvestris* species (Hizume et al. 2002). Strong centromeric *A*-type TRS signals were also reported. Somewhat to the contrary, in loblolly pine we observed interstitial *A*-type TRS signals as streaks or bands across the chromosome arms. All 12 loblolly pine chromosomes showed centromeric *A*-type TRS varying from trace-to-major signals. We also observed a small pair of dot signals (i.e., snake-eyes) of *A*-type TRS at the distal ends of each chromosome. *A*-type TRS signals were always found to be associated with DAPI bands, but not all DAPI (i.e., AT-rich) bands were associated with *A*-type TRS signals, suggesting that some AT-rich regions do not contain *A*-type TRS.

Major centromeric DAPI bands have been observed in almost all chromosomes studied in *Pinus* (Hizume et al. 1989, 1990; Doudrick et al. 1995; Hizume et al. 2002). We observed similar results in loblolly pine. Centromeric DAPI bands were consistently observed in 23 of the 24 homologues (the exception being chromosome 4), and they tended to flank the centromere. Interstitial DAPI bands have been reported in various *Pinus* species, including *P. sylvestris*, *P. densiflora*, *P. thunbergii*, *P. nigra* (Hizume et al. 2002), and *P. elliottii* var. *elliottii* (Doudrick et al. 1995), and many other plant taxa (Andras et al. 2000). For loblolly pine, both consistent and inconsistent interstitial DAPI banding patterns have been reported (Islam-Faridi et al. 2003; Jacobs et al. 2000), making them less than optimal for physical mapping, although they have been used in animal species (Breen et al. 1999). Given the inconsistent DAPI-banding results in *Pinus* and the consistent banding provided by *A*-type TRS and 18S-28S rDNA, we propose using the latter as markers for delimiting cytomolecular bins (Fig. 3) in physical mapping. For example, using Fig. 3, we note that the 2 5S rDNA sites in loblolly pine are located in bins 2 (short arm of chromosome 1) and 6 (short arm of chromosome 2). We propose referring to these delimited locations as TRS/18-28S bins.

Although several loblolly pine chromosomes were found to be quite similar, we were able to establish a quantitatively descriptive karyotype and dichotomous key using intrachromosomal position and intensity measurements of the FISH signals (Figs. 2 and 3). Major centromeric 18S-28S rDNA signals were helpful in differentiating loblolly pine chromosomes 3, 7, and 10 from chromosomes 2, 4, 8, and 9 (Table 4). Within these 2 groups, chromosomes exhibited similar major interstitial 18S-28S rDNA signals and can be distinguished only with careful observation and measurement. Within the former group, chromosome 7 can be identified by the interstitial *A*-type TRS signal position, which is more distal and closer to the interstitial 18S-28S rDNA signal than it is on chromosomes 3 and 10 (Tables 2 and 4). For chromosomes 3 and 10, the interstitial 18S-28S rDNA site is more distal on chromosome 10 than it is on chromosome 3. Within the latter group, chromosome 2 can be separated from chromosomes 4, 8, and 9 by its 5S rDNA site. For chromosome 9, the centromeric *A*-type TRS signal is

Fig. 3. Ideogram of the reference karyotype of loblolly pine. Chromosomes 1 to 12, are arranged in descending order of mean relative length (RL). Vertical height of bars is proportional to the mean signal intensity or mean proportion of times observed. Vertical position of bars is scaled to the mean position on the chromosomal arm. Chromosome-arm lengths are proportional to the mean RL of the respective chromosome's arms. In the centromeric region, *A*-type TRS sites are drawn on the left side and 18S-28S on the right side to reveal areas of signal overlap. TRS/18S-28S bins, numbered 1–54, are located at the mid-point of their respective chromosomal arms. One sub-bin is shown as 26.1 on chromosome 6. The subbin occurs between 2 interstitial *A*-type TRS sites located on the long arm.



major, whereas it is intermediate on chromosome 8 and trace on chromosome 4. In addition, the interstitial *A*-type TRS signal on chromosome 9 is closer to the interstitial 18S-28S rDNA site than it is on chromosomes 4 and 8 (Tables 2 and 4). Finally, for chromosome 4, at least 1 homologue consistently lacked centromeric DAPI bands in all 3 families. Future work should refine this key by identifying an additional probe (e.g., Brown et al. 1998; Vischi et al. 2003; Navrátilová et al. 2003) that will more clearly resolve differences among the more similar chromosomes.

Homoeologous groupings of chromosomes among 4 species in subgenus *Pinus* subsection *Sylvestris* have been proposed (Hizume et al. 2002), using similar chromosomal landmarks. The basic similarities between the karyotypes of these species and that of loblolly pine include 7 interstitial 18S-28S rDNA sites (5 on short arms and 2 on long arms) and 2 5S rDNA sites (1 major and 1 minor). In all cases, the 7 interstitial 18S-28S rDNA sites are on different chromosomes, whereas the 2 5S rDNA sites are on chromosomes containing interstitial 18S-28S rDNA for the *Sylvestris* species. In loblolly pine, the major 5S rDNA site is on a chromosome lacking an interstitial 18S-28S rDNA site. In addition, the major 5S rDNA site is interstitially located (mean AI, 40; SE, 1.43), and the minor site, located on a different chromosome, is relatively distal (AI, 93.7; SE, 3.48); in the *Sylvestris* species, the opposite is the case. For slash pine (Doudrick et al. 1995), the same number of 5S rDNA sites as for loblolly pine were observed, except that 2 minor sites were consistently found in relatively distal positions, 1 located on the same chromosome (opposite arm) as an 18S-28S rDNA site. We have some evidence of a second minor 5S rDNA site in loblolly pine also at a distal location, but it was not consistent enough in signal to include in the reference karyotype.

The differences noted above between members of *Australes* and *Sylvestris* make it very difficult to form homoeologous groups across subsections. A homoeologous group including *Australes* chromosome 2 (here and Doudrick et al. 1995) and *Sylvestris* chromosome 8 has been proposed (Hizume et al. 2002). Our data suggest the same, noting that, in loblolly and slash pines, the distal 5S rDNA site is much weaker than in *P. densiflora* and the other *Sylvestris* species. In loblolly and slash pines, the strong 5S rDNA site occurs at a median interstitial position on the short arm of chromosome 1 (chromosome 9 in Doudrick et al. 1995). Beyond these differences, the loblolly and slash pine karyotypes are very similar with respect to numbers and locations of the interstitial rDNA sites.

In each species, individual chromosome identification depends on interstitial rDNA locations, as well as rDNA characteristics near the centromere, and *A*-type TRS signals at both interstitial and centromeric positions. Careful measurement of the locations and the strength of these signals from nondistorted chromosome spreads are important for proper chromosome identification. We suggest that combining these characteristics systematically will allow for the chromosomes to be identified using a dichotomous key (Table 4). Ideally, such a key would provide for automated data collection and analysis, leading to rapid chromosome identification for use in physical mapping genes or gene-rich regions. In principle, the key we developed does not require comparison to other chromosomes in the karyotype; therefore, complete metaphase cells are not required. Instead, any chromosome in the microscopic field can be evaluated and identified. However, some distinguishing characteristics depend on length measurements (e.g., step D to distinguishing chromosome 12 from chromosomes 5, 6, and 11), and these lengths are not significant at $p \leq 0.05$. Furthermore, when signal intensity is important (e.g., step E to distinguish chromosome 5 from chromosomes 6 and 11), a within-cell intensity calibration will be necessary, but again, the whole chromosomal complement will not be required. Ultimately,

data collected in such a manner can be combined across cells using statistical approaches until all chromosomes have been identified many times for a given sample. This will save time and resources; slide preparation can take time when all 24 homologues in a cell have to be nondistorted and well-separated before analysis.

In future studies within *Pinus*, we suggest that a reference karyotype be developed for a "model" species in each subsection, using an objective comparative analysis, such as the one presented here, as a method for naming the chromosomes of the remaining subsection members. The reference karyotype should be built on data derived across diverse genotypes, using high-quality chromosome preparations and statistical analysis to provide support for numbering chromosomes by RL and assigning long and short arms on the basis of unique characteristics, not observed length measurements. In addition, we argue that chromosome karyotyping procedures and dichotomous keys should be developed that do not require the whole chromosomal complement to be available for measurement and analysis. A chromosome of *Pinus* should be identifiable on the basis of its own characteristics, not on comparative criteria with other chromosomes. Our technical improvements and dichotomous key are steps in this direction and provide a framework to meet these criteria. In addition, the definition of specific bins delimited by discrete FISH signals (*A*-type TRS and 18S-28S rDNA) will provide robust physical markers for characterizing the loblolly pine genome, including the integration of genetic and physical maps.

Acknowledgements

We thank T. Mullin (North Carolina State University) and T. Byram (Texas A&M University) and their respective Tree Improvement Cooperatives' members, especially International Paper and Weyerhaeuser for supplying seeds for this study. We thank F. Bridgwater (retired, USDA 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, MNIF and CDN); and D. Stelly and J. Price (Texas A&M University) for sharing lab space. Special thanks go to D. Stelly for providing technical assistance with Optimas v. 6 and to T. McKnight (Texas A&M University) for preparing the *A*-type TRS probe. We thank S. Ramakrishnan (graduate research assistant), V. Gullirmo and V. McNamara (undergrad research assistants, Texas A&M University) for excellent technical assistance. Finally, we note with respect the recent passing of our friend and colleague Dr. H. James Price.

References

Andras, S.C., Hartman, T.P.V., Alexander, J., Mcbride, R., Marshall, J.A., Power, J.B., et al. 2000. Combined PI-DAPI staining (CPD) reveals NOR asymmetry and facilitates karyotyping of plant chromosomes. Chromosome Res. **8**: 387–391. doi:10.1023/A:1009258719052. PMID:10997779.

Bogunic, F., Muratovic, E., Brown, S.C., and Siljak-Yakovlev, S. 2003. Genome size and base composition of five Pinus species from the Balkan region. Plant Cell Rep. **22**: 59–63. doi:10.1007/s00299-003-0653-2. PMID:12835994.

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

Breen, M., Bullerdiek, J., and Langford, C.F. 1999. The DAPI Banded Karyotype of the Domestic Dog (*Canis familiariz*) Generated Using Chromosome-Specific Paint Probes. Chromosome Res. **7**: 401–406. doi:10.1023/A:1009224232134. PMID:10515215.

Brown, G.R., Newton, C.H., and Carlson, J.E. 1998. Organization and distribution of a *Sau*3A tandem repeated DNA sequence in *Picea* (Pinaceae) species. Genome, **41**: 560–565. doi:10.1139/gen-41-4-560. PMID:9796105.

Costa, P., Pot, D., Dubos, C., Frigerio, J.M., Pionneau, C., Bodenes, C., et al. 2000. A genetic map of Maritime pine based on AFLP, RAPD and protein markers. Theor. Appl. Genet. **100**: 39–48. doi:10.1007/s001220050006.

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

Drewry, A. 1982. G-banded chromosomes in *Pinus resinosa.* J. Hered. **73**: 305–306.

Fuchs, J., Brandes, A., and Schubert, I. 1995. Telomere sequence localization and karyotype evolution in higher plants. Plant Syst. Evol. **196**: 227–241. doi:10.1007/BF00982962.

Heslop-Harrison, J.S. 1991. The molecular cytogenetics of plants. J. Cell Sci. **100**: 15–21.

Hizume, M., Ohgiku, A., and Tanaka, A. 1989. Chromosome banding in the genus *Pinus*. II. Interspecific variation of fluorescent banding patterns in *P. densiflora* and *P. thunbergii.* Bot. Mag. Tokyo, **102**: 25–36. doi:10.1007/BF02488110.

Hizume, M., Arai, M., and Tanaka, A. 1990. Chromosome banding in the genus *Pinus*. III. Fluorescent banding pattern of *P. luchuensis* and its relationships among the Japanese diploxylon pines. Bot. Mag. Tokyo, **103**: 103–111. doi:10.1007/BF02489619.

Hizume, M., Shibata, F., Matsusaki, Y., and Garajova, Z. 2002. Chromosome identification and comparative karyotypic analyses of four *Pinus* species. Theor. Appl. Genet. **105**: 491–497. PMID:12582496.

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

Islam-Faridi, M.N., Nelson, C.D., Kubisiak, T.L., Gullirmo, M.V., McNamara, V.H., Ramakrishnan, S., et al. 2003. Developing standard karyotypes for loblolly and slash pines with fluorescent *in situ* hybridization and AT-rich banding. *In* Proceedings 27th Southern Forest Tree Improvement Conference. Oklahoma State University, Stillwater, Oklahoma, USA, 24–27 June 2003. *Edited by* C.R. McKinley. National Technical Information Service, Springfield, Virginia. pp. 184–188.

Jacobs, M.D., Gardner, R.C., and Murray, B.G. 2000. Cytological characterization of heterochromatin and rDNA in *Pinus radiata* and *P. taeda.* Plant Syst. Evol. **223**: 71–79. doi:10.1007/BF00985327.

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

Kondo, T., Terada, K., Hayashi, E., Kuramoto, N., Okamura, M., and Kawasaki, H. 2000. RAPD markers linked to a gene for resistance to pine needle gall midge in Japanese black pine (*Pinus thunbergii*). Theor. Appl. Genet. **100**: 391–395. doi:10.1007/s001220050051.

Kubisiak, T.L., Nelson, C.D., Nowak, J., and Friend, A.L. 2000. Genetic linkage mapping of genomic regions conferring tolerance to high aluminum in slash pine. J. Sustain. For. **10**: 69–78.

Leitch, I.J., and Heslop-Harrison, J.S. 1992. Physical mapping of the 18S–5.8S–26S rRNA genes in barley by *in situ* hybridization. Genome, **35**: 1013–1018.

Leitch, A.R., Mosgoller, W., Shi, M., and Heslop-Harrison, J.S. 1992. Different patterns of rDNA organization at interphase in nuclei of wheat and rye. J. Cell Sci. **101**: 751–757. PMID:1527177.

Li, C., and Yeh, F.C. 2001. Construction of a framework map in *Pinus contorta* subsp. *Latifolia* using random amplified polymorphic DNA markers. Genome, **44**: 147–153. doi:10.1139/gen-44-2-147. PMID:11341723.

Lubaretz, O., Fuchs, J., Ahne, R., Meister, A., and Schubert, I. 1996. Karyotype of three Pinaceae species via fluorescent *in situ* hybridization and computer-aided chromosome analysis. Theor. Appl. Genet. **92**: 411–416. doi:10.1007/s001220050143.

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

Navrátilová, A., Neumann, P., and Macas, J. 2003. Karyotype analysis of four *Vicia* species using *in situ* hybridization with repetitive sequences. Ann. Bot. (London), **91**: 921–926. doi:10.1093/aob/mcg099. PMID:12770847.

Remington, D.L., Whetten, R.W., Liu, B.H., and O'malley, D.M. 1999. Construction of AFLP genetic map with nearly complete genome coverage in Pinus taeda. Theor. Appl. Genet. **98**: 1279–1292. doi:10.1007/s001220051194. PMID:12238515.

Saylor, L.C. 1983. Karyotype analysis of the genus *Pinus*-subgenus *Strobus*. Silvae Genet. **32**: 119–121.

Schmidt, A., Doudrick, R.L., Heslop-Harrison, J.S., and Schmidt, T. 2000. The contribution of short repeats of low sequence complexity to large conifer genomes. Theor. Appl. Genet. **101**: 7–14. doi:10.1007/s001220051442.

Schweizer, D. 1980. Fluorescent chromosome banding in plants: applications, mechanisms, and implications for chromosome structure. *In* The Plant Genome. Proceedings of the 4th John Innes Symposium and Second International Haploid Conference. Norwich, England, September 1979. *Edited by* D.R. Davies and D.A. Hopwood. The John Innes Charity, Norwich, England. pp. 61–72.

Sewell, M.M., Sherman, B.K., and Neale, D.B. 1999. A consensus map for loblolly pine (*Pinus taeda* L.). I. Construction and integration of individual linkage maps from two out bred three-generation pedigrees. Genetics, **151**: 321–330. PMID:9872970.

Steel, R.G.D., and Torrie, J.H. 1980. Principles and procedures of Statistics – A biometrical approach. 2nd ed., McGraw-Hill, Inc., New York.

Vischi, M., Jurman, I., Bianchi, G., and Morgante, M. 2003. Karyotype of Norway spruce by multicolor FISH. Theor. Appl. Genet. **107**: 591–597. doi:10.1007/s00122-003-1306-0. PMID:12827248.

Wakamiya, I., Newton, R.J., Johnston, J.S., and Price, H.J. 1993. Genome size and environmental factors in the genus *Pinus*. Am. J. Bot. **80**: 1235–1241. doi:10.2307/2445706.