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Cytogenetic Analysis of *Populus trichocarpa* – Ribosomal DNA, Telomere Repeat Sequence, and Marker-selected BACs

M.N. Islam-Faridi[a] C.D. Nelson[b] S.P. DiFazio[c] L.E. Gunter[d] G.A. Tuskan[d]

[a]Southern Institute of Forest Genetics, Southern Research Station, U.S. Forest Service, Forest Tree Molecular Cytogenetics Laboratory, Department of Ecosystem Science and Management, Texas A&M University, College Station, Tex., [b]Southern Institute of Forest Genetics, Southern Research Station, U.S. Forest Service, Harrison Experimental Forest, Saucier, Miss., [c]Department of Biology, West Virginia University, Morgantown, W.Va., [d]Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tenn., USA

Key Words
BAC · FISH · *Populus trichocarpa* · rDNA · Telomere

Abstract
The 18S-28S rDNA and 5S rDNA loci in *Populus trichocarpa* were localized using fluorescent in situ hybridization (FISH). Two 18S-28S rDNA sites and one 5S rDNA site were identified and located at the ends of 3 different chromosomes. FISH signals from the *Arabidopsis*-type telomere repeat sequence were observed at the distal ends of each chromosome. Six BAC clones selected from 2 linkage groups based on genome sequence assembly (LG-I and LG-VI) were localized on 2 chromosomes, as expected. BACs from LG-I hybridized to the longest chromosome in the complement. All BAC positions were found to be concordant with sequence assembly positions. BAC-FISH will be useful for delineating each of the *Populus trichocarpa* chromosomes and improving the sequence assembly of this model angiosperm tree species.

The genus *Populus* consists of many species that are ecologically and economically important across the Northern Hemisphere. Poplars (*Populus* spp.) are early successional, fast growing species that can be cultivated for high wood yields [Stettler et al., 1996]. The wood has many uses, most notably as fiber for paper products but more recently as biomass for bioenergy feedstocks [Rubin, 2008]. In addition, their fast growth rate and globally wide distribution makes poplars valuable in sequestering carbon. Therefore, wider cultivation of these species may benefit the carbon balance and assist in moderating global temperature change [Lemus and Lal, 2005]. The poplars are considered to be model forest tree species for genetics and genomics research in part because of their relatively small genome size (480 Mb/1C, 2n = 2x = 38), amenability to tissue culture and genetic transformation, relative ease of controlled-pollination and high seed set, and short generation time (<5 years). In particular this distinction has fallen on *Populus trichocarpa* (black cottonwood) as it was the 3rd plant species (after *Arabidopsis* and rice) and 1st tree species to have its genome sequenced [Tuskan et al., 2006].

Prior to the genome sequence, a number of genetic linkage (i.e., recombination-based) maps were developed

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Nurul Faridi
Forest Science Laboratory, Texas A&M University
2585-TAMU
College Station, TX, 77843-2585 (USA)
Tel. +1 979 862 3908, Fax +1 979 845 3272, E-Mail nfaridi@tamu.edu

and reported for poplars [e.g., Bradshaw et al., 1994; Grattapaglia and Sederoff, 1994; Bradshaw, 1996; Cervera et al., 1996, 2001; Yin et al., 2004]. However, little work has been completed at the cytogenetic level, especially when compared to other plant models such as *Arabidopsis* [Jackson et al., 1998; de Jong et al., 1999; Fransz et al., 2000, 2002], rice [Cheng et al., 2001, 2002; Zhao et al., 2002; Cheng et al., 2005; Tang et al., 2007], and sorghum [Islam-Faridi et al., 2002; Kim et al., 2005a, b]. Cytogenetic analysis in these plant species has been useful in aiding genome assembly by facilitating integration of linkage maps with physical maps and in determining the order of sequence scaffolds and their respective contigs [Zhao et al., 2002; Cheng et al., 2005; Kim et al., 2005a]. This has provided a more complete understanding of the structural and functional properties of these genomes.

Detailed cytogenetic analysis of poplar chromosomes should help improve the genome assembly, which currently consists of 2,447 major scaffolds >5 kb [Tuskan et al., 2006]. In this paper, we utilized fluorescent in situ hybridization (FISH) to study and localize the 18S-28S and 5S rDNA and the *Arabidopsis*-type telomere repeat sequence (ATRS) sites in *P. trichocarpa*. In addition, we used FISH to localize 6 marker-selected BAC clones that represent 2 linkage groups of *P. trichocarpa*. Our specific objectives were to 1) determine the number and location of the rDNA sites, 2) determine the distribution of the ATRS sites, and 3) study the feasibility of utilizing BAC-FISH in *Populus* for chromosome localization. The 3rd objective is important for furthering our work in establishing a cytomolecular map for each poplar chromosome and to better understand the structural details of the *Populus* genome.

Materials and Methods

Chromosome Preparation

Chromosome spreads were prepared for *P. trichocarpa* clone Nisqually-1 (383–2499), the same genotype that was used for whole genome sequencing [Tuskan et al., 2006]. Actively growing root tips about 1.5 cm long were harvested from rooted cuttings growing in potting soil in a greenhouse. Harvested root tips were immediately pre-treated with an aqueous solution of α-monobromonaphthalene (0.8%, Sigma) for 1.5 to 1.75 h at room temperature (RT) in the dark and then fixed in 4:1 ethanol:glacial acetic acid to arrest cell division at metaphase. Fixed root tips were processed enzymatically (5% cellulase Onozuka R-10, Yakult Honsha Co. Ltd., and 1.25% pectolyase Y-23, Kyowa Chemical Products Co. Ltd.) in 0.01 M citrate buffer, and the chromosome spreads were prepared as described elsewhere [Jewell and Islam-Faridi, 1994]. The chromosome spreads were checked with a phase contrast microscope (Axioskop, Carl Zeiss, Inc.), and slides containing good chromosome spreads were selected and stored at −80°C for use in FISH.

Probe DNA

The following probes were used in the current experiments: 18S-28S rDNA of maize [Zimmer et al., 1988], 5S rDNA including a spacer region of sugar beet [Schmidt et al., 1994], *Arabidopsis*-type telomere repeat sequence $(TTTAGGG)_n$ (kindly provided by Dr. T. McKnight, Department of Biology, Texas A&M University), and BAC clones from 2 *P. trichocarpa* linkage groups LG-I and LG-VI.

The BAC clones were derived from a library prepared from Nisqually-1 [Stirling et al., 2001]. Positions of the BACs were determined by alignment of end sequences to the genetic map-anchored whole genome sequence assembly. The repeat content of each BAC clone was inferred based on the frequency of constituent 16mers in the full set of 7.5 million sequence reads from the *Populus* genome sequencing project and the abundance of protein coding sequences contained in the BAC. The selected BACs were 66B19, 75P22, and 87F21 from LG-I and 78O18, 88A10, and 93N12 from LG-VI (http://www.bcgsc.ca/platform/mapping/data/?searchterm=poplar).

BAC DNA was isolated from overnight liquid cultures in selective media by alkaline lysis, digested with *Eco*RI, and followed by further purification using Plant DNeasy spin columns (QIAGEN, Valencia, CA) as described elsewhere [Childs et al., 2001]. Probe DNAs with and without whole plasmids were labeled with biotin-16-dUTP (Biotin-Nick Translation Mix, Roche Diagnostics) and/or digoxigenein-11-dUTP (dig) (Dig-Nick Translation Mix, Roche Diagnostics) by nick translation as recommended by the manufacturer. Labeled probes were dot-blotted to verify incorporation of label.

Fluorescent in situ Hybridization (FISH)

The hybridization mixture consisted of deionized formamide (50%, Fisher Chemical), dextran sulfate (10%, Fisher Chemical), 2× SSC, labeled probe DNA (30 ng/slide), carrier DNA (*E. coli* DNA, 7.5 μg/slide), and blocking DNA (poplar Nisqually-1 Cot-1 DNA, 300 to 600 ng/slide, depending on single- or dual-color FISH, respectively). The poplar Cot-1 DNA was prepared as described by Zwick et al. [1997] and was used only for FISH with BAC probes (BAC-FISH). The BAC-FISH hybridization mixture was denatured in a boiling water bath for 10 min, immediately placed on ice for 5 to 10 min, and then incubated at 37°C for 30 min to allow the Cot-1 DNA to hybridize with the repetitive DNA sequences of the BAC probes. Chromosome spreads were denatured in 70% deionized formamide at 72°C for 1.5 min in an oven on a metal block followed by dehydration through a series of ethanol (70, 85, 95, and 100%) washes at −20°C for 3 min each. The slides were dried with forced-air and on the bench at RT for 25 to 30 min prior to hybridization with probe DNA.

The hybridizations were accomplished by loading 25 μl of hybridization mixture on the chromosome spread and placing and sealing a 22 × 30 mm glass coverslip over the mixture. The coverslips were sealed with rubber cement, and the slides were placed in a humidity chamber and incubated over night at 37°C. Following hybridization, the coverslips were washed off with 2×

SSC, 37°C, using a squeeze bottle. The slides were then washed twice in 2× SSC, 30% deionized formamide, and 2× SSC for 5 min each at 40°C, followed by washing twice in 2× SSC and once in 4× SSC, 0.2% Tween 20 for 5 min each at RT. Finally, the slides were incubated in 200 μl blocking solution consisting of 5% bovine serum albumin (BSA, IgG-free, protease-free; Jackson ImmunoResearch Laboratories, Inc.) in 4× SSC, 0.2% Tween 20.

The hybridization sites were detected with 5 μg/ml of fluorescein (FITC)-conjugated anti-digoxigenin (Roche Diagnostics), or 0.75 μg/ml Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc.), or both depending on the labeled probe DNA used in the hybridization mixture. All detection reactions were completed at 37°C for 25 to 30 min using the blocking solution. The slides were then washed 4 times in 4× SSC/0.2% Tween 20 for 5 min each at 37°C to remove the unbound antibodies, counterstained with 4 μg/ml 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) in McIlvaine buffer, pH 7.0 (9 mM citric acid, 80 mM Na_2HPO_4, 2.5 mM $MgCl_2$) for 10 to 12 min, and briefly washed in 4× SSC, 0.2% Tween 20 followed by washing in 2× SSC. The slides were dried with forced-air, and a small drop (~6 μl) of anti-fade solution (Vectashield, Vector Laboratories) was added under a glass coverslip (22 × 50 mm). Slides were incubated overnight at 4°C to stabilize the fluorochromes before observation under an epifluorescence microscope. All detection reactions and treatments were conducted in subdued light.

The FISH experiments using 18S-28S and 5S rDNA and *Arabidopsis*-type telomere repeat sequence (ATRS) probes were carried out in 3 phases. First, we used a dig-labeled 18S-28S rDNA probe and detected the hybridization sites with FITC-conjugated anti-dig to provide green signals. Second, we used biotin-labeled 18S-28S rDNA and dig-labeled 5S rDNA probes simultaneously. The 18S-28S rDNA probe was detected with Cy3-conjugated streptavidin to provide red signals, and the 5S rDNA probe was detected with FITC-conjugated anti-dig to provide green signals. Third, we used dig-labeled 18S-28S rDNA and biotin-labeled ATRS probes, respectively, with signal detection as described above.

The BAC-FISH experiments for LG-I and LG-VI were also carried out in 3 phases. First, we used 2-color FISH with 2 BACs, where BAC 1 (to simplify here we code the BACs 1 to 3) was labeled with biotin and detected with Cy3-conjugated streptavidin (red) and BAC 2 was labeled with dig and detected with FITC-conjugated anti-dig (green). Second, we used 2-color FISH with BACs 2 and 3 (labeling and detection as before for BACs 1 and 2). Third, we hybridized all 6 BACs pooled together, 3 from each LG, followed by simultaneous detection of both colors.

Digital Image Capture and Process

Digital images were recorded from an Olympus AX-70 epifluorescence microscope with suitable filter sets (Chroma Technology) using a 1.3 MP Sensys (Roper Scientific) camera and a MacProbe v4.2.3 digital image system (Applied Imaging International) and then further processed with Adobe Photoshop CS v8 (Adobe Systems).

Results

The chromosome spread technique of Jewell and Islam-Faridi [1994] with air-drying routinely provided unifocal views of the *Populus* chromosomes. These chromosome spreads were mostly free of cell walls, nuclear membranes, and cytoplasmic debris providing good accessibility for the probes and low background hybridization.

18S-28S rDNA and 5S rDNA Sites

Two major 18S-28S rDNA sites were clearly identified in *P. trichocarpa* and they are located at the ends of 2 different chromosomes (figs. 1, 2). This is in contrast to results inferred from interphase to prophase stages where numerous 18S-28S rDNA FISH signals were observed (figs. 1c–e, 2c, d). A single 5S rDNA site was found in *P. trichocarpa* and it is likewise located at the end of a 3rd chromosome (green signals, arrows, fig. 2a, b).

Arabidopsis-*type Telomere Repeat Sequence (ATRS)*

The ends of each chromosome of *P. trichocarpa* showed ATRS signals (fig. 2c), while interphase nuclei suggested numerous ATRS signals scattered throughout the chromosomal complement (fig. 2d).

LG-I and LG-VI BACs

Two-color FISH with 2 BAC clones of both LG-I and LG-VI allowed an evaluation of the relative copy number of the probes, i.e., whether either combination of the BAC clones contains unique/low copy or high copy DNA sequences. BAC 87F21 (red signal, fig. 3a) and BAC 66B19 (green signal, fig. 3a) were observed to be located on the same chromosome as expected, and it appeared to be the longest chromosome in the complement. Pairs of red and green signals were also observed in interphase nuclei (fig. 3b). BAC 87F21 was located in a low DAPI stained (euchromatic) region at the end of the long arm while BAC 66B19 was proximally located in a high DAPI stained (heterochromatic) region. All but one BAC (66B19) showed unique hybridization signals at their respective chromosomal locations. Interestingly, BAC 66B19 showed a strong hybridization signal near the putative centromere (i.e., apparent primary constriction as observed under the microscope) of the longest chromosome along with some scattered FISH signals on all of the other chromosomes, which indicates that 66B19 contains some degree of repetitive DNA. For the LG-I chromosome, BACs 87F21 and 66B19 appear to be located on the long arm, and BAC 75P22 appears to be located on the short arm

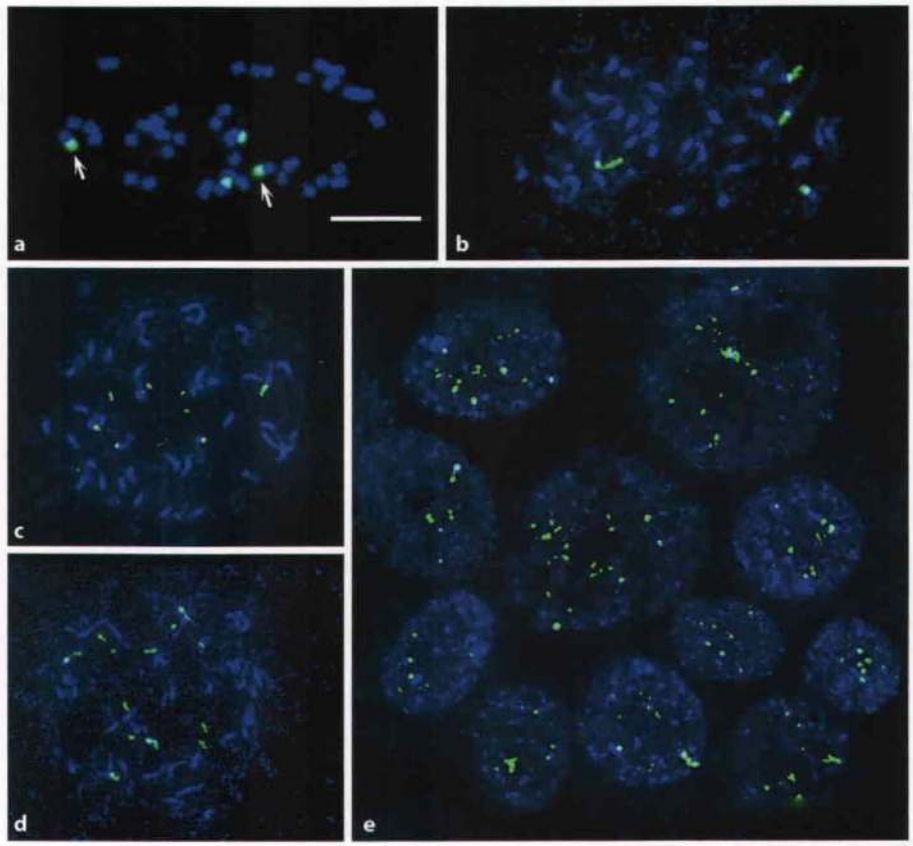

Fig. 1. FISH results using 18S-28S rDNA probe on mitotic chromosome spreads and intact nuclei in *Populus trichocarpa*. **a** Metaphase chromosome spread, arrows point to signals that are slightly stronger than the others. **b** Late prophase chromosome spread. **c** Mid-prophase chromosome spread. **d** Early prophase nuclei. **e** Interphase nuclei. Bar = 10 μm.

(fig. 3a, c, e). For the LG-VI chromosome, BACs 78O18 and 93N12 are located on one arm, and BAC 88A10 is located interstitially on the other arm (fig. 3d, f).

Discussion

Our 18S-28S rDNA FISH results support the findings of Faivre-Rampant et al. [1992] based on Southern hybridization, where they inferred the presence of two 18S-25S rDNA loci in *P. trichocarpa*. Using FISH, we observed 2 major 18S-28S rDNA sites in *P. trichocarpa*, located on 2 different chromosomes. In addition, we found one site to be slightly stronger in FISH signal intensity than the other site (arrows, fig. 1a). This difference is most likely due to variation in copy number of rDNA repeat units located at the 2 sites as suggested by Faivre-Rampant et al. [1992]. They analyzed restriction fragments of the 18S-25S rDNA loci in *P. trichocarpa* and found the 11.7-kb *Eco*RV fragment to be present in higher frequency than the 11.4-kb fragment, suggesting the presence of some minor loci. In contrast, our FISH results showed no evidence of minor 18S-28S rDNA loci in *P. trichocarpa*.

The 18S-28S rDNA loci were located at the end of each of 2 homologous pairs of chromosomes (figs. 1a, b, 2). In addition, a red Cy3 ATRS signal was observed directly distal of each of the 18S-28S rDNA signals. As expected, the telomeric end of each chromosome arm showed a pair of ATRS signals [Fuchs et al., 1995]. Furthermore, the 18S-28S rDNA loci are located in satellited regions of both chromosomes, as shown by FISH with the ATRS probe and DAPI staining (fig. 2c, d). In contrast, only one satellited chromosome was reported in *P. alba*, *P. balsamifera*, *P. deltoides*, *P. euroamericana*, and *P. nigra*, while one to three 18S-28S rDNA sites were reported for these species [Prado et al., 1996].

Numerous 18S-28S rDNA FISH signals (sometimes more than 20) were observed in interphase nuclei, and more than 4 signals were observed in early-prophase to mid-prophase. These variations of signal numbers are most likely due to the highly decondensed nature of DNA in interphase nuclei and to a lesser degree in prophase. Collecting data at these decondensed chromatin stages

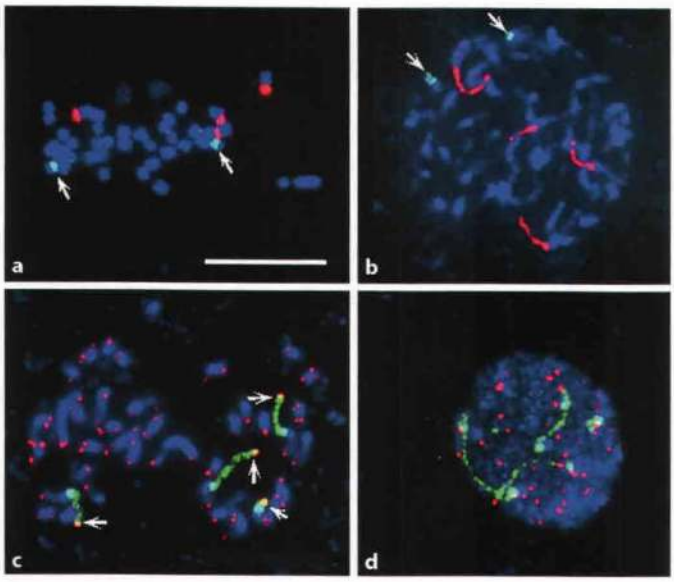

Fig. 2. FISH results using 18S-28S rDNA, 5S rDNA, and ATRS probes on somatic chromosome spreads and an interphase nucleus in *Populus trichocarpa*. **a** Metaphase chromosome spread with 18S-28S rDNA signals (red) and 5S rDNA signals (green, arrows). **b** Mid-prophase chromosome spread with 18S-28S rDNA signals (red) and 5S rDNA signals (green, arrows). **c** Prometaphase chromosome spread with 18S-28S rDNA signals (green), ATRS signals (red), and overlapping probes (arrow pointing to yellowish color). **d** Interphase nucleus with 18S-28S rDNA signals (green) and telomere repeat DNA sequence signals (red). Bar = 10 μm.

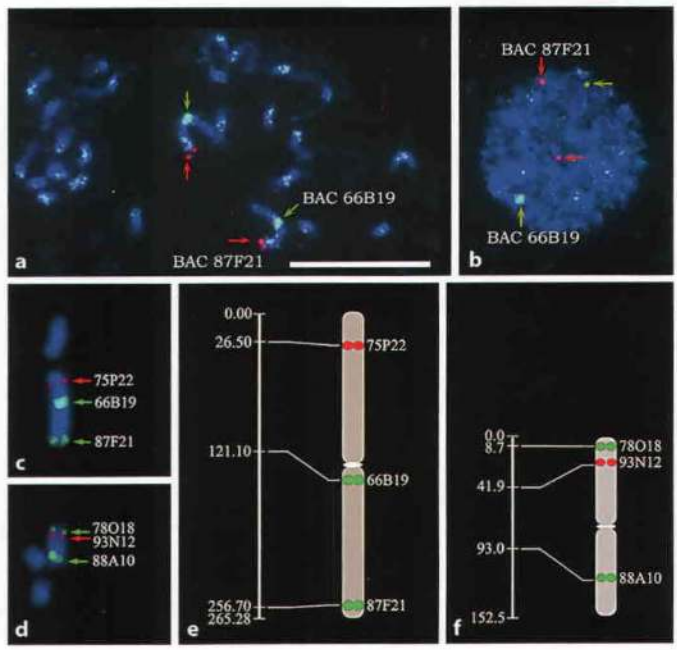

Fig. 3. Linkage group specific BAC FISH and diagrammatic representations of BAC map positions on LG-I and LG-VI in *Populus trichocarpa*. **a** LG-I BAC FISH signals (BAC 87F21 in red and BAC 66B19 in green) on chromosome complement. **b** Interphase nucleus showing red and green signals from the same BACs as in **a**. **c** LG-I BAC FISH signals (red and green) on a specific chromosome. **d** Three LG-VI BAC FISH signals (red and green) on a specific chromosome. **e** Diagrammatic representation of LG-I BAC positions on chromosomes along with linkage map. **f** Diagrammatic representation of LG-VI BAC positions along with linkage map. Primary constrictions (i.e., putative centromeres) are represented by white ellipses towards the center of the chromosomes (**e** and **f**). Bar = 10 μm.

provides an upwardly biased count of the number of tandemly repeated DNA loci such as 18S-28S rDNA. Because of this, interphase nuclei should not be used to determine the number of repetitive DNA sites such as 18S-28S rDNA. When chromatin is not fully condensed, these FISH signals, depending on the degree of condensation, can be observed like a series of dots along a filament over these loci (green signals, fig. 2d). These signals become increasingly dense when the nuclei advance from interphase to prophase (red signals, fig. 2b; green signals, fig. 2c) as the chromatin condensation process reaches a maximum level at metaphase, showing one signal (figs. 1a, 2a). Similar patterns of FISH signals also have been reported for *Brassica* [Maluszynska and Heslop-Harrison, 1993], alfalfa [Calderini et al., 1996], lentil, and peanut [Islam-Faridi, unpublished data].

Only one 5S rDNA locus was identified in *P. trichocarpa*, and it was located using two-color FISH on a chromosome different to either of the two 18S-28S rDNA loci bearing chromosomes (arrows, fig. 2a, b). In contrast, Prado et al. [1996] reported that there were two 5S rDNA loci in each of 5 diploid species of *Populus* and that they were located on 2 different chromosomes. However, they could not confirm whether the 5S rDNA and 18S-28S rDNA loci were on the same or different chromosomes. We observed that the 5S rDNA FISH signal in *P. trichocarpa* is much reduced compared to either of the 18S-28S rDNA signals, apparently because the 5S rDNA locus contains fewer repetitive units than either of the 18S-28S rDNA loci. Similar results were also reported for *P. nigra* [Ribeiro et al., 2008].

The BACs were selected from LG-I and LG-VI based on low or single copy BLAST hits against the whole-genome sequence assembly. We tested these BACs as a pilot

study to determine whether or not BAC-FISH could be useful in developing a BAC-based cytomolecular map in *Populus* as it has been for *Arabidopsis* [Fransz et al., 2000, 2002], sorghum [Islam-Fraidi et al., 2002; Kim et al., 2005a, b], and rice [Cheng et al., 2001, 2002; Zhao et al., 2002; Cheng et al., 2005; Tang et al., 2007]. All but one of the BAC clones (66B19) were observed to be located in euchromatic regions of the 2 chromosomes, and each showed almost no or very low background FISH signal. These results demonstrated that 5 of the 6 tested BAC clones (75P22, 87F21, 78O18, 93N12, and 88A10) contained low repetitive DNA compared with BAC clone 66B19. On the other hand, BAC clone 66B19 was found to be located in a heterochromatic region as determined by its co-location with a highly DAPI stained region. Since BAC clone 66B19 apparently originated from a heterochromatic region, it seems likely that it may contain some repetitive DNA, and the observed, dispersed FISH signals across most if not all chromosomes support this assertion. It is unclear why the repetitive nature of this BAC was not apparent from the frequency of 16mers in the assembled genome sequence. However, repetitive regions are notoriously difficult to assemble using a whole genome shotgun approach [Green, 2001], so some highly repetitive regions are probably not included in the assembled sequence, including the region spanned by BAC clone 66B19.

FISH with the LG-I BAC clones revealed the order of the BAC clones as telomere (of one chromosome arm) → BAC 75P22 → BAC 66B19 → BAC 87F21 → telomere of the other arm. For the BAC clones selected from LG-VI, the order observed was: telomere of one arm → BAC 78O18 → BAC 93N12 → BAC 88A10 → telomere of the other arm. The chromosomal positions of the 6 BACs clones appear to be syntenic to their inferred positions in the sequence-based assembly, but the relative positions (i.e., physical vs. genetic) vary, especially for the 3 BAC clones 66B19 (LG-I, fig. 3e), 93N12, and 88A10 (LG-VI, fig. 3f). It is not surprising that there is a discrepancy between BAC positions inferred by FISH and those inferred from the sequence assembly. The genome sequence contains many gaps, and the genetic map has relatively low resolution, being based on genotypes of only 44 progeny in many cases [Tuskan et al., 2006]. This highlights the importance of FISH for determining physical distances between markers in the *Populus* genome.

Using linkage group specific BAC clones as FISH probes, we found that the longest chromosome is associated with BAC clones from LG-I. Given these results, we propose using LG-specific BACs to identify and enumerate the poplar chromosomes. Considering this standard, we have studied the location of 3 BAC clones from each of chromosomes 1 and 6. We also found that 3 chromosomes contain rDNA loci, two 18S-28S loci and one 5S locus, and that none of these chromosomes are either chromosomes 1 or 6. Additional BAC-FISH will be needed to determine the chromosomes that contain the rDNA loci. In addition, a centromere-specific FISH probe will be needed to define the basic karyotype of *P. trichocarpa* and the other poplars. These experiments should lead to the development of a comprehensive cytomolecular map that is anchored to the genetic linkage map, the BAC-based physical map, and the whole-genome sequence. Furthermore the development and availability of a comprehensive cytomolecular map should help in closing and resolving the remaining gaps and issues in the whole-genome sequence assembly.

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