GENOME ORGANIZATION AND CYTO-MOLECULAR MAP OF CHESTNUT

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INTRODUCTION

The American chestnut (*Castanea dentata*, 2*n* = 2*x* = 24), once a foundational forest species over 800,000 km^2 in eastern North America, was decimated by chestnut blight caused by an introduced fungal pathogen, *Cryphonectria parasitica*. The devastating disease was first reported in 1904 by Hermann Merkel, a forester at the New York Zoological Park (Murrill, 1906). Numerous measures were taken to control the pathogen, but these attempts failed as the disease spread rapidly, covering the entire species range by the late 1920s and killing nearly 4 billion trees by the 1950s (Hepting, 1974). Although now reduced to an understory species (Anagnostakis, 1987; Burnham, 1988), American chestnut persists through repeated, vigorous sprouting from its root collar, occasionally producing flowers and seeds before succumbing again to the blight (Paillet, 2002).

Chinese chestnut (*Castanea mollissima*), a species closely related to American chestnut, is relatively resistant to blight. Efforts are underway to transfer resistance from Chinese chestnut to American chestnut, including a backcross breeding program operated by the American Chestnut Foundation (TACF) (Hebard, 2006; *www.acf.org*) and a biotechnology-based program sponsored by the Forest Health Initiative (Thompson, 2012; Nelson et al., 2014; www.foresthealthinitiative.org). Recently an integrated genetic/physical map of Chinese chestnut was published (Kubisiak et al., 2013) and the species genome is being sequenced (Nelson et al., 2014); however, little cytogenetic data are available to confirm and correct these genomic resources. Fluorescence *in situ* hybridization (FISH) is an important cytogenetic technique for assigning and orienting genetic markers to specific chromosomes. Presently we are using as many as 16 genetically and physically mapped BAC clones, distributed across each linkage group, as FISH probes to develop a cyto-molecular map of chestnut.

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MATERIALS AND METHODS

Actively growing root tips were harvested from chestnut seedlings growing in potting soil and treated with an aqueous solution of α-monobromonaphthalene (0.8 % v/v) for 1.5 h at room temperature in the dark to accumulate metaphase stage cells, then fixed in 4:1 (95% ethanol : glacial acetic acid). Fixed root tips were treated with cell wall degrading enzymes in 0.01 M citrate buffer and the chromosome spreads were prepared as described previously (Sakaanokho and Islam-Faridi, 2013).

The 18S-28S rDNA of maize (Zimmer et al., 1988), 5S rDNA of sugar beet (Schmidt et al., 1994) and various BAC clones from Chinese chestnut (Fang et al., 2013) were used as probes. The BAC clones were selected from BAC contigs associated with genetically mapped markers near the ends of each linkage group (Kubisiak et al., 2013). In addition the BAC clones were selected to avoid repetitive regions of the genome, using information available from the physical mapping project (Fang et al., 2013). Probe DNAs were labeled with biotin-16-dUTP (Biotin Nick Translation Mix, Roche, USA) and/or digoxigenin-11-dUTP (Dig Nick Translation Mix, Roche, USA) following the manufacturer's instructions.

Fluorescent *in situ* hybridization (FISH) was performed as described previously (Islam-Faridi et al. 2009). Probe hybridization sites were detected with Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories, USA) for biotin labeled probes and FITC-conjugated anti-digoxygenin (Roche, USA) for digoxigenin labeled probes. The FISH preparations were mounted with Vectashield containing DAPI (Vector Laboratories, USA) to prevent photo-bleaching the fluorochromes. Digital images were recorded using an epi-fluorescence microscope (AxioImager M2, Carl Zeiss, Germany) with suitable filter sets (Chroma Technology, USA) and a Cool Cube high performance CCD camera, and processed with ISIS V5.1 (MetaSystem Inc., USA) and Adobe Photoshop CS v8 (Adobe System, USA).

RESULTS AND DISCUSSION

The chestnut genome size is 780 Mb/1C, composed of 12 pairs of metacentric and near meta-centric chromosomes. We were able to obtain chromosome spreads that were free of cell walls, nuclear membranes and cytoplasmic debris, allowing for efficient probe hybridization.

The 18S-28S and 5S rDNA probes showed two 18S-28S rDNA (one major and the other minor) and one 5S rDNA sites. These three rDNA loci are located on three different homologous pairs of chromosomes in both Chinese chestnut and American chestnut. The positioning of ribosomal genes in Chinese chestnut reported here is in agreement with that in American chestnut reported previously (Islam-Faridi et al., 2009).

For FISH with BAC clones as probes, we used two BACs (one labeled with biotin and the other with digoxigenin) from either end of each linkage group to identify the corresponding chromosome and their physical positions and orientations within the chromosome. We followed those experiments by using four BAC clones (two from either end of a linkage group) to confirm that all four BACs hybridize to a specific chromosome. To date we have not observed any discrepancies between integrated physical/genetic map and cytogenetic data, i.e., BAC clones from an individual linkage group hybridized to a specific chromosome, and the physical positions of the BACs in chromosome spreads were relatively similar to their respective genetic map positions.

In a FISH experiment with four BAC clones (BB134N22, 1.3 cM; BB171MO4, 6.3 cM; BD176N08, 50.2 cM; and BB055C18, 57.9 cM) from linkage group H, hybridization signals identified a homologous pair of satellited chromosomes, suggesting that this linkage group contains the major 18S-28S rDNA locus. In another FISH experiment, two BAC clones from opposite ends of linkage group H (BB171M04, 6.3 cM and BB055C18, 57.9 cM) and 18S-28S rDNA were used as probes (Fig. 1). The results showed that the 18S-28S rDNA probe hybridized to the nucleolus organizer region (NOR) of the satellited chromosome, confirming that the major rDNA locus is located on linkage group H.

We are now in the process of locating as many as 16 genetically and physically mapped BAC clones to each linkage group to form the basis of a cyto-molecular map for chestnut. Such a map should help improve the understanding of the chestnut genome by serving as a reference karyotype for comparative genomic studies that will facilitate gene discovery and mapping, interspecies breeding and genetic engineering.

CONCLUSION

The major 18S-28S rDNA locus in chestnut is located on linkage group H. Chestnut BAC clones can be selected based on genetic and physical map positions and physical mapping characteristics that facilitate FISH. Using mapped BACs as FISH probes allows the integration of the genetic and physical maps with the karyotype and the production of a cyto-molecular map.

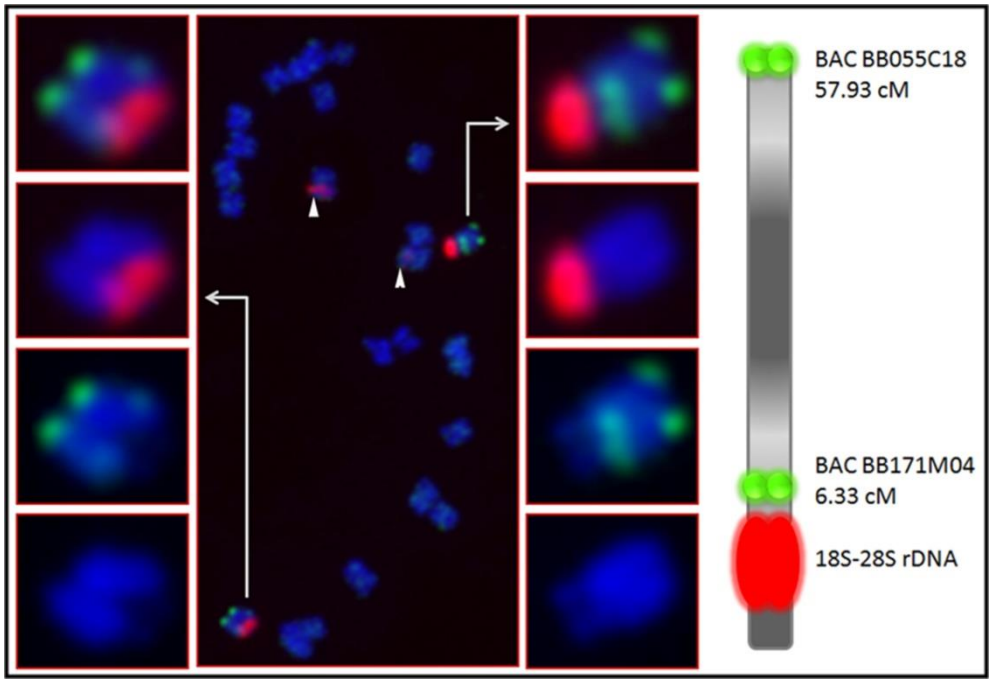

Figure 1. Fluorescent in situ hybridization of Chinese chestnut using BAC BB171M04 (green signal), BAC BB055C18 (green signal) and 18S-28S rDNA (red signals) as probes. The arrow heads show the minor 18S-28S rDNA signals. A schematic diagram of the satellited chromosome (i.e., linkage group H) is presented on the right hand side.

Acknowledgements: Funding for this research was provided by TACF and the Southern Research Station (USDA Forest Service). We thank Dr. Tom Byram (Texas A&M Forest Service) for greenhouse facilities.

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