Cytogenetic Analysis of American Chestnut (*Castanea dentata*) Using Fluorescent In Situ Hybridization

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Abstract

The American chestnut (*Castanea dentata*), once known as 'The King of the Forest' in the Appalachian Mountains of the eastern United States and southeastern Canada, has been all but extirpated by chestnut blight disease caused by an Asiatic bark fungus, *Cryphonectria parasitica*. A group of scientists at The American Chestnut Foundation has been working since 1983 to develop blight resistant American chestnut by inter-species backcross breeding with the relatively resistant Chinese chestnut (*Castanea mollissima*). In an effort to facilitate this breeding program we have initiated cytogenetic studies of these two species and their F_1 and backcross hybrids. Here we report our successes in preparing somatic chromosomes using enzymatic digestion of American chestnut seedling root tips and demonstrate the quality of the chromosome spreads using fluorescent in situ hybridization with ribosomal DNA probes. Our results show two 18S-28S rDNA sites, one major and one minor, and one 5S rDNA site in American chestnut. This is the first such report for American chestnut and our preliminary results appear similar for Chinese chestnut.

INTRODUCTION

Up until the early part of the 20th century, American chestnut (*Castanea dentata*, 2*n*=2*x*=24) was a dominant forest tree species in the hardwood forests of much of the eastern United States and adjoining areas of southeastern Canada. The species was extremely valuable to local communities providing straight-grained, durable wood for many purposes and abundant, nutritious nuts for human and wildlife consumption (Buttrick, 1915; Detwiler, 1915). However, by about 1950 nearly all American chestnut trees had been killed by an exotic bark and stem fungus (*Cryphonectria parasitica*, causing chestnut blight disease) that was accidentally introduced in the late 1800s from Asia (Hepting, 1974).

In an effort to restore the American chestnut to its former silvical status, The American Chestnut Foundation (TACF) initiated a backcross breeding program designed to transfer the blight resistance genes from Chinese chestnut (*Castanea mollissima*) to American chestnut (Burnham et al., 1986; Kubisiak et al., 1997; Hebard, 2006). Since the efficiency of inter-species backcross breeding is partially dependent on the similarity of the species' chromosomes, we initiated a cytogenetic research program aimed at

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characterizing the chromosomal complements of the two species. The objective of the current study was to evaluate an enzymatic chromosomal preparation technique using fluorescent in situ hybridization (FISH) with ribosomal DNA probes.

MATERIALS AND METHODS

Chromosome Preparation

Root tips were harvested from healthy chestnut seedlings growing in potting soil in a greenhouse and immediately pre-treated with an aqueous solution of α-bromo-naphthalene (0.8%) for 1.75 to 2 h in the dark at room temperature followed by fixing in a 4:1 solution of 95% ethanol to glacial acetic acid. Fixed root tips were processed enzymatically and chromosomes were spread on to ethanol (95%) washed glass slides as described by Jewell and Islam-Faridi (1994).

Probe DNA Preparation

Whole plasmid DNA with 18S-28S rDNA insert of maize (Zimmer et al., 1988) or 5S rDNA of sugar beet including the spacer region (Schmidt et al., 1994) were labeled by nick translation using either biotin-16-dUTP (Biotin-Nick Translation Mix, Roche Diagnostics, Indianapolis, IN, USA) or digoxigenin-11-dUTP (Dig-Nick Translation Mix, Roche Diagnostics, Indianapolis, IN, USA). Probe fragment sizes were checked by agarose gel electrophoresis and labeled nucleotide incorporation was verified by dot-blotting.

Fluorescent In Situ Hybridization (FISH)

Standard FISH technique was utilized as previously reported (Islam-Faridi et al., 2002, 2009). Probe hybridization sites were detected with Cy3-conjugated streptavidin (Jackson Immuno Research Laboratories, West Grove, PA, USA) for biotin labeled probe and FITC-conjugated anti-digoxigenin (Roche Applied Science, Indianapolis, IN, USA) for digoxigenin labeled probe. The FISH preparations were counterstained with DAPI (4 ug/ml) in McIlvaine buffer (pH 7.0) and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) for microscopy.

Microscopy and Image Analysis

Digital images were recorded from an Olympus AX-70 epifluorescence microscope with suitable filter sets (Chroma Technology, Rockingham, VT, USA) using a 1.3 MP Sensys camera and the MacProbe v42.3 digital image system (Applied Imaging International, San Jose, CA, USA). Selected images were further processed with Adobe Photoshop CSv8 (Adobe Systems, San Jose, CA, USA).

RESULTS AND DISCUSSION

High quality mitotic metaphase (somatic) chromosome spreads of American and Chinese chestnuts were obtained using the described technique. The spreads were mostly free of cell walls, nuclear membranes and cytoplasmic debris, and the chromosome morphology was excellent, allowing for clear visualization of non-distorted chromosomes (Fig. 1).

We identified two 18S-28S rDNA sites in American chestnut, one major and one minor, located on two different homologous pairs of chromosomes (Fig. 1a). The major site is clearly higher in signal intensity (FITC, green color) than the minor site. For 5S rDNA, we observed one site in American chestnut (Fig. 1c). To our knowledge, this is the first report of FISH using ribosomal DNA probes in chestnut trees. A simultaneous two-color FISH (dual FISH) with the two rDNA probes is needed to determine whether the 5S rDNA site is syntenic to either of the 18S-28S rDNA sites.

Our preliminary FISH results on Chinese chestnut somatic chromosome spreads show a similar result-- two 18S-28S rDNA sites and one 5S rDNA site. In addition, one of the 18S-28S rDNA bearing chromosomes of Chinese chestnut appears to be

structurally different from that of American chestnut. Further FISH analysis using F_1 hybrid somatic chromosome spreads should confirm the synteny of the 18S-28S and 5S rDNA sites in American and Chinese chestnuts and clarify the structural differences noted for one of the 18S-28S rDNA sites. An enhanced understanding of the major structural differences between these two species' chromosomes will facilitate the transfer of blight resistance genes and improve the accuracy of our expectations of the backcross breeding program.

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Figures

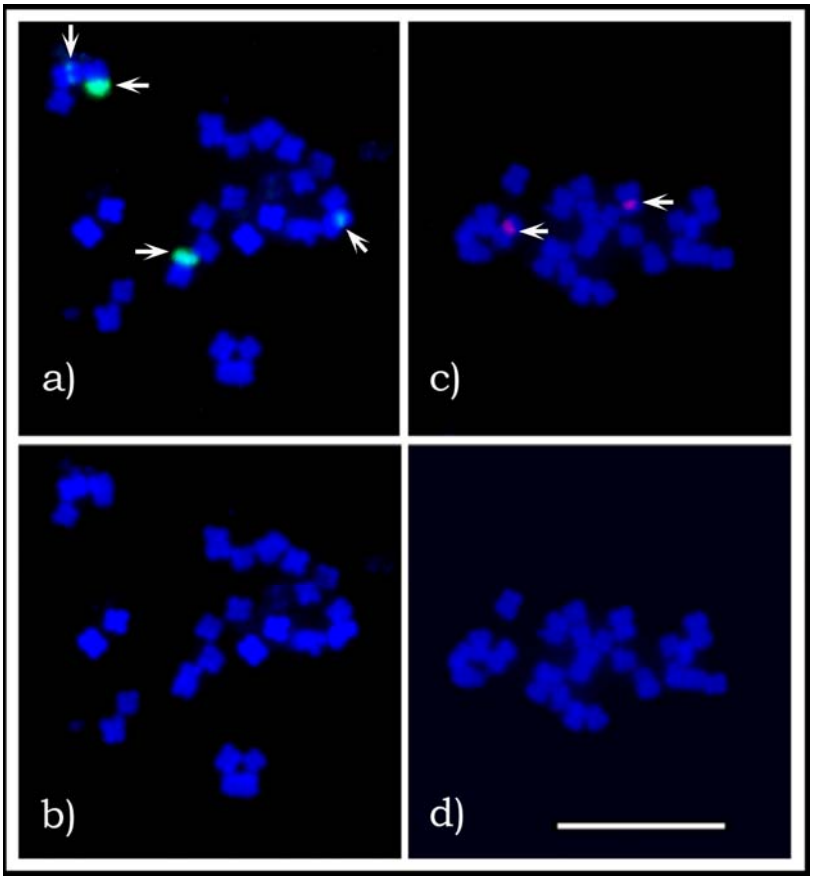

Fig. 1. Ribosomal DNA (18S-28S and 5S) sites detected with fluorescent in situ hybridization (FISH) on somatic chromosome spreads of American chestnut. a) Metaphase chromosomes with 18S-28S rDNA probe detected (arrows, green signal); b) DAPI image of the same chromosomes as shown in "a"; c) Metaphase chromosomes with 5S rDNA probe detected (arrows, red signal); and d) DAPI image of the same chromosome spread as shown in "c". Bar is 10 µm.