Cytogenomic Characterization of rDNA and the Chromatin Composition of the NOR-Associated Satellite in American and Chinese Chestnuts

Nurul Islam-Faridi[1], Tetyana Zhebentyayeva[2], George L Hodnett[3], Laura L Georgi[4], Paul H Sisco[5], Frederick V Hebard[4], C Dana Nelson[6,7]

[1]Research Mol. Cytogeneticist, USDA Forest Service, Southern Research Station, Southern Institute of Forest Genetics, Texas A&M University, College Station, TX 77843 USA; [2]Department of Forestry and Natural Resources, University of Kentucky, Lexington, KY 40546, USA; [3]Department of Soil and Crop Sciences, Texas A&M University, College Station, TX 77843 USA; [4]The American Chestnut Foundation, Meadowview, VA 24361 USA; [5]The American Chestnut Foundation, Asheville, NC 28804 USA; [6]USDA Forest Service, Southern Research Station, Forest Health Research and Education Center, Lexington, KY, USA 40546; [7]USDA Forest Service, Southern Institute of Forest Genetics, Saucier, MS 39574 USA;
*m.n.faridi@usda.gov; nfaridi@tamu.edu

Abstract: Several genetic linkage maps of American and Chinese chestnuts are being utilized to accelerate the American chestnut breeding program. Both chestnut genomes have recently been sequenced, facilitating gene discovery and assisting in molecular-based breeding and genetic engineering. To complement and extend this genomic work, we analyzed the distribution and organization of their ribosomal DNAs (35S and 5S rDNA), and the chromatin composition of nucleolus organizing region (NOR)-associated satellites. Two loci of 35S (one major and the other minor) and one 5S rRNA locus were identified using fluorescent *in situ* hybridization. An additional 5S locus was observed intermingled distally with the major 35S site in Chinese chestnut. The major 35S site is located terminally and sub-terminally in American and Chinese chestnut, respectively. The 35S site originates at the end of the short arm of its chromosome, extending through the secondary constriction (the central region of the NOR) and into the proximal region of the satellite in Chinese chestnut, while in American chestnut the 35S site covered the entire satellite. In Chinese chestnut, a distinct demarcation was observed separating the distal region of the satellite, which is highly heterochromatic (i.e., gene-poor), from the proximal region containing the rRNA gene(s). This region may be euchromatic (i.e., gene-rich). This heterochromatic DNA could be species and/or chromosome specific. A striking accumulation of high 35S rDNA gene copy number was noticed at one of the minor 35S sites in a Chinese chestnut line, suggesting an evolutionary strategy that may favor the emergence of a new variant within this species.

Introduction: The American chestnut tree, *Castanea dentata* [(Marsh.) Borkh., $2n = 2x = 24$], once revered as the 'Majestic Forest Tree' of eastern North America, covered an impressive expanse of over 800,000 sq. km., ranging from Maine to Mississippi. However, it was tragically decimated, first in the 19th century by Phytophthora root rot in the Southeast and then by the middle of the 20th century across its entire range by chestnut blight. These devastating diseases are respectively caused by invasive pathogens *Phytophthora cinnamomi* Rands., and *Cryphonectria*

parasitica (Murr.) Burr. In contrast, the Chinese chestnut (*Castanea mollissima* Blume, $2n = 2x = 24$) exhibits resistance to both pathogens, having co-evolved with them and consequently suffers little damage (Anagnostakis 1992; 2012). Efforts spearheaded by the American Chestnut Foundation and the State University of New York are currently underway to identify and transfer the resistance genes from the Chinese chestnut to the American species, through backcross breeding and from wheat by genetic engineering (Powell 2020). Several genetic maps of Chinese chestnut have been developed (Kubisiak et al. 1997, 2013; Zhebentyayeva et al. 2019), integrated with a physical map (Fang et al. 2013) and more recently, the genomes of both chestnut species have been sequenced (Staton et al. 2020; Westbrook et al. 2022). These valuable resources are expected to significantly bolster genomic research and breeding efforts aimed at the development of resilient American chestnut varieties. Despite these advancements, knowledge of chestnut cytogenetics is limited, especially when compared to other plant species, such as Arabidopsis, rice, sorghum, and maize, and others (see review by Garcia et al. 2017; Jiang 2019). Knowledge of structural organization of the chestnut genome is important for inter-species breeding, particularly when target genes are located on rearranged chromosomes. Fluorescent *in situ* hybridization (FISH) can precisely locate specific DNA sequences on individual chromosomes and shed light on the structural and compositional organization of a genome and its molecular evolution (Cerbah et al. 1998; Heslop-Harrison 2000; Islam-Faridi et al. 2002; Jiang 2019).

In this study, we report:

i) compositional details of the major 35S rDNA at the NOR region in both American and Chinese chestnuts; and
ii) repositioning of the major 35SrDNA locus from a major site to a minor site in the Chinese chestnut.

Materials and Methods: Root tip collection and pre-treatment to accumulate chestnut metaphase chromosome spreads were carried out as described by Islam-Faridi et al. 2023. Fluorescent *in situ* hybridization coupled with capture of digital images by epi-fluorescence microscopy and subsequent processing were performed as described previously (Islam-Faridi et al. 2023). Two experiments were performed; in the first experiment, American and Chinese chestnut chromosome spreads were probed with 35S, 5S and telomere repeat Oligo probes (Islam-Faridi et al. 2020). In the second, a 35S rDNA probe was used in a Chinese chestnut x American chestnut F_1 hybrid spread as described elsewhere (Islam-Faridi et al. 2023).

Results and Discussion: In plant cells, ribosomes play a crucial role in protein synthesis, and their production is driven by ribosomal genes, namely 35S and 5S rRNAs (Rosselló et al. 2022). We observed that both American (AC) and Chinese chestnuts (CC) contained two 35S rDNA loci (one major and one minor) and a single 5S rDNA locus, each independently located on three different chromosomes. Additionally, a second 5S locus was identified in Chinese chestnut located on the satellite one (SAT-1). The major 35S (mj-35S) locus was positioned at the terminal end in AC, while it was sub-terminal in CC. The minor 35S (mn-35S) and the 5S loci were proximally located in both species [Fig. 1 (AC) and Fig. 2 (CC)]. Interestingly, our findings regarding the Chinese chestnut contrast with those reported by Ribeiro et al. 2011, in which the mj-35S and the second 5S loci in *C. mollissima* were identified as pericentromeric on the short arm. They proposed that these rDNA loci underwent restructuring during the evolutionary history of *Castanea* species. A

detailed cytomorphological analysis of the rDNA loci in AC and CC, along with numerous evolutionary insights, was recently reported in Islam-Faridi et al. (2023).

From an analysis of their length, we identified two types of satellites (SAT) in Chinese chestnut, viz., SAT-1 and SAT-2 (Fig 2; for details see Islam-Faridi et al. 2023). We observed that the satellites in both AC and CC appear detached from their respective mother-chromosome [mo-ch, see Fig. 1 (AC) and Fig. 2 (CC)] until they reach full condensation (Fig. 3a; the chromosome spread was from a CC x AC F_1 hybrid). A second 5S locus was identified on the SAT-1 of CC (see enlarged image in lower-right box, Fig. 2) (for additional details see Islam-Faridi et al. 2023).

Telomere length in plants, which ranges from 1 to 50 kb, plays a crucial role in safeguarding the chromosome ends and maintaining chromosomal integrity (Fuchs et al. 1994). Using a telomere-specific probe in FISH, we confirmed their presence at the chromosomal termini (Figs. 1 and 2). The telomere signals on AC satellites and SAT-2 of CC are initially overshadowed by the mj-35S signal (green). Notably, no telomere signals were detected at the end of the short arm of the mj-35S mother-chromosome [mo-ch, arrowheads, Fig. 1 (AC) and Fig. 2 (CC)]. However, they were visible on the satellites that extend beyond the short arm when the green signals were dimmed and the red signals enhanced through image analysis [see inserts, enlarged images of the satellites, Fig. 1 (top-left) and Fig. 2 (lower middle-right)].

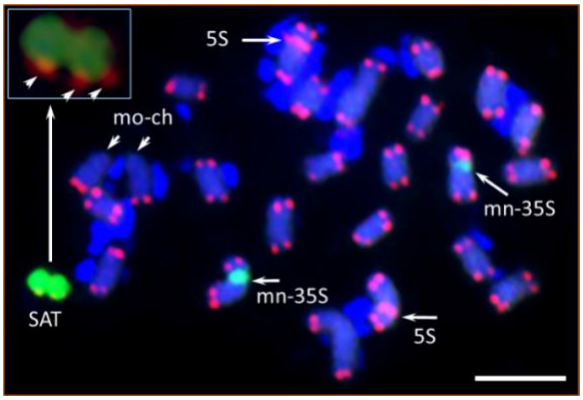


Fig. 1. Somatic chromosome spreads of American chestnut, showing two 35S rDNA loci [one major (mj-35S), one minor (mn-35S), green signals] and one 5S rDNA locus (red signals) and telomere repeat sites at each chromosomal end (red signals). Both satellites appeared detached from their respective mother chromosomes (mo-ch, arrows). The image was captured under a 100x objective. Scale bar = 5 µm.

Based on DAPI staining, we have recently reported that the AC satellite is likely euchromatic, while the proximal region of the CC satellite's might be euchromatic and its distal region heterochromatic. In CC, the mj-35S originates at the end of the short arm, passes through the secondary constriction (SC) and extends into the proximal region of the satellite [CC mj-35S, Fig. 3a and Fig. 3a1 (an enlarged image)]. The same path is observed for AC, but it covers the entire satellite (AC mj-35S, Fig. 3a). The SAT-2 of CC appeared the same as that in AC, suggesting it might have been contributed by a chestnut species other than Chinese chestnut (for details see Islam-Faridi et al. 2023).

Expressed DNAs are predominantly or preferentially euchromatic and stained weakly or less intensely with DAPI. In contrast, heterochromatic regions, which are AT-rich, stain strongly or more intensely with DAPI and appear bright blue under a UV-filter (Schweizer 1976; de Jong et al. 1999). Surprisingly, we observed a robust 35S signal on one of the mn-35S site homologues (an enlarged image shown in top-right box, Fig. 2). This accumulation of additional rDNA copies, potentially an evolutionary strategy, might result from jumping events and/or unequal

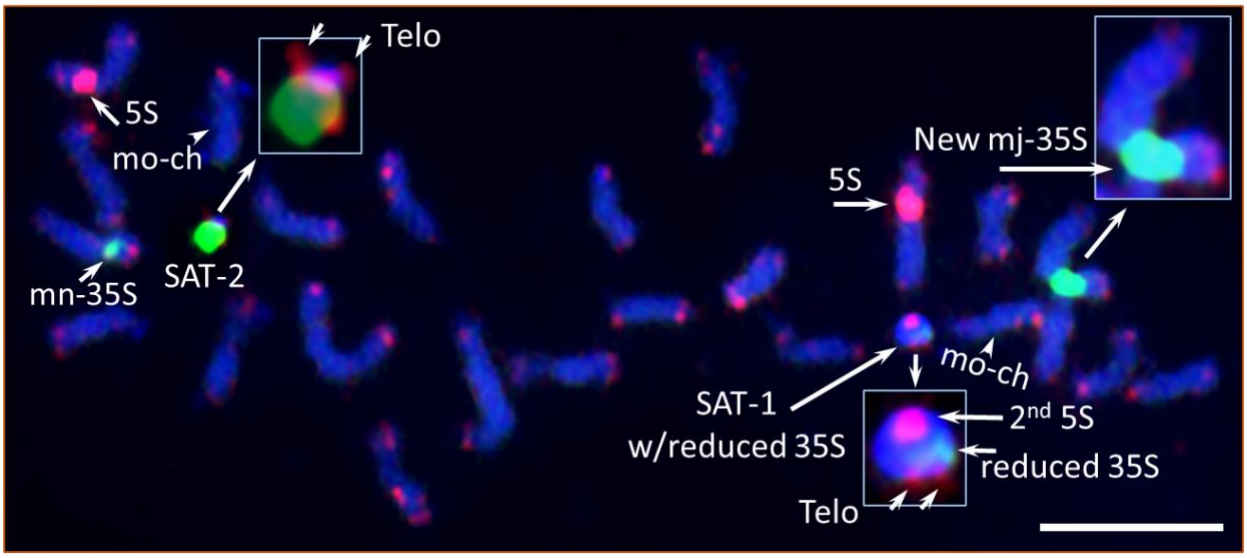
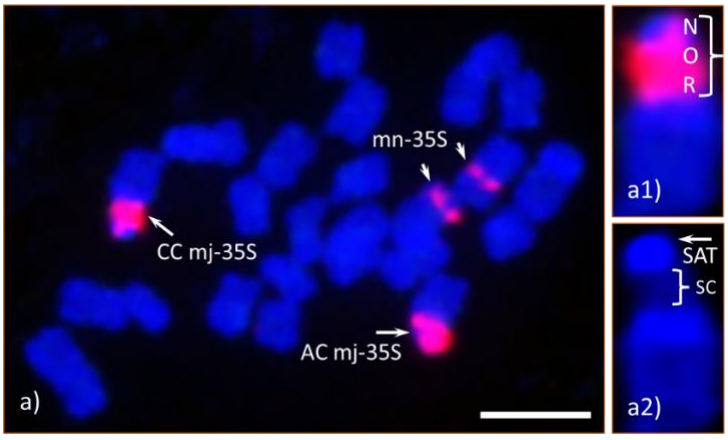


Fig. 2. Somatic chromosome spreads of Chinese chestnut, showing two 35S rDNA loci (green signals) and one 5S rDNA locus (red signals) and telomere repeat sites at each chromosomal end (red signals). Both satellites appeared detached from their respective mother chromosomes (mo-ch, arrowheads). An enlarged image of the new mj-35S locus at minor 35S site shown in upper-right box. The image was captured under a 63x objective. Scale bar = 5 µm.



a1): Enlarged image (RGB) of Chinese chestnut mj-35S rDNA bearing chromosome.
NOR = nucleolus organizing region

a2): DAPI image, same as "c1" under UV-filter
SAT = satellite
SC= secondary constriction

Fig. 3a. Somatic chromosome spread of Chinese x American chestnut F_1 hybrid hybridized with 35S rDNA probe, illustrates the expected two 35S sites. Notably, one of the mj-35S signals shows a prominent satellite, contributed by the CC parent, while the other was from AC. The image was captured under a 100x objective. Scale bar = 5 µm.

crossing-over between the mj-35S mo-ch and the mn-35S rDNA chromosome [Schubert 1984; Mohannath et al. 2019; observed in maize and ash, Islam-Faridi (unpublished)]. Consequently, this event might lead to the emergence of a new Chinese chestnut variant with an interstitial major 35S locus, while the original satellite (SAT-1), possibly carrying fewer 35S gene copies at the NOR (an enlarged image shown in lower-right box, Fig. 2), may remain unchanged. Alternatively, the satellite might eventually be lost over time. In Arabidopsis, significant variation in the copy number of the rDNA genes was associated with changes in gene expression profiles of several hundred genes (Lopez et al. 2021). Whether variation in the cytogenetic organization of the 35S locus affects the chestnut transcriptome remains to be elucidated.

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