CYTOGENOMIC ANALYSES OF THE GENUS *SORGHUM*

A Dissertation

by

JASON CORRENTH ANDERSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2010

Major Subject: Plant Breeding

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Approved by:

Co-Chairs of Committee,	David M. Stelly
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ABSTRACT

Cytogenomic Analyses of the Genus *Sorghum.* (May 2010)

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Co-Chairs of Advisory Committee: Dr. David M. Stelly
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A phylogenetic tree based on ITS1, *Adh*1 and *ndh*F grouped the species of the genus *Sorghum* into one distinct monophyletic group, but including two sister lineages, one with *x*=5, the other with *x*=10 as basic chromosome numbers. The goal of this study was to elucidate major patterns in *Sorghum* genome evolution, particularly *n*=5 *vs. n*=10 genomes. A very recent molecular cytogenetic study in our laboratory revealed striking structural karyotypic rearrangements between *S. bicolor* (*x*=10) and an *x*=5 *Sorghum* species, *S. angustum*; so an immediate objective here was to determine if identical or similar rearrangements exist in other wild *Sorghum* species. Our approach was [1] to extend similar methods to additional species, i.e., fluorescent *in situ* hybridization (FISH) analyses of sorghum genomic bacterial artificial chromosome clones and multi-BAC cocktail probes to mitotic chromosomes of *S. angustum, S. versicolor, S. brachypodum* and *S. intrans*; and [2] to augment the BAC-FISH findings by comparing telomeric and ribosomal DNA FISH signal distributions to *x*=5 and *x*=10 *Sorghum* species. Signals from *in situ* hybridizations of BAC-based probes were insufficiently robust and insufficiently localized to delineate FISH signal patterns akin to those discovered previously in *S. angustum*. Southern blots of the same BACs to restricted

DNA of these species revealed relatively moderate affinity to smeared DNA, suggesting homology to non-tandemized sequences. FISH of the A-type TRS (*Arabidopsis*-like telomeric repeat sequence) revealed its presence is limited to terminal chromosomal regions of the *Sorghum* species tested, except *S. brachypodum*, which displayed intercalary signal on one chromosome and no detachable signal at its termini region. The hybridization of 45S and 5S rDNA revealed that the respective sites of tandemized clusters differ among species in terms of size, number and location, except *S. angustum* versus *S. versicolor*.

Well localized BAC-FISH signals normally occur when signals from low-copy sequences discernibly exceed background signal, including those from hybridization of dispersed repetitive elements. The low level of signal intensity from BAC low-copy sequences relative to the background signal "noise" seems most likely due to low homology and(or) technical constraints. Extensive dispersal of low-copy sequences that are syntenic in *S. bicolor* seems unlikely, but possible.

In conclusion, the result was a lack of clear experimental success with BAC-FISH and an inability to effectively screen for S. angustum-like rearrangements using BAC-FISH. The telomeric and rDNA FISH indicated that the *x*=5 genomes vary extensively. One can surmise that although the arrangements seen in *S. angustum* might extend to *S. versicolor*, they certainly do not extend to *S. versicolor*, they certainly do not extend to *S. intrans* or *S. brachypodum*. It is clear that *S. brachypodum* has telomeric repeats that are either very short or rely on some sequence other than the A-type TRS.

DEDICATION

This dissertation is dedicated to my Lord and Savior Jesus Christ for allowing me to do His will and His will be done, not mine.

ACKNOWLEDGMENTS

I would first like to acknowledge my late major professor, my mentor, Dr. H. James Price, for giving me the opportunity to pursue my Ph.D. Special thanks go to Dr. C. Wayne Smith for assisting me through my entire graduate program. I would also like to thank my advisor, Dr. David Stelly, for guiding me through my maturation process as a student. You saw my potential and allowed me to discover it at my own pace; for this I am grateful to you. I also would like to thank co-advisor, Dr. Patricia Klein, for challenging me and always being honest. I would also like to thank Dr. Hongbin Zhang and Dr. William L. Rooney for serving on my committee and for their collaborative efforts with my research. Special thanks go to the entire Klein and Zhang laboratories for opening their respective doors to me.

I would like to acknowledge Dr. George Hodnett, Dr. Nurul Faridi and present and past lab colleagues for their advice, opinion and friendship.

I would like to express my love to my family for supporting, encouraging and standing by me through my doctoral education. I would have never accomplished anything with you all. Lastly, I thank my Lord and Savior Jesus Christ for never abandoning me, for strengthening me when I was weak and comforting me when I was down.

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CHAPTER I

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.)Moench] is the fifth most important major cereal crop grown in the world (www.fao.org), and relative to the other cereals, it is especially well adapted to hot, arid areas. Grain sorghum has a capacity to tolerate conditions of limited moisture and reproduce during periods of extended drought, circumstances that would impede production in most other grains. Sorghum leaves roll along the midrib when moisture-stressed, making the plant more drought resistant than other grain plants. Therefore, it is extensively cultivated in marginal rainfall areas of the tropics and subtropics (Lazarides et al. 1991).

In the U.S., sorghum is a principal feed component for both cattle and poultry. However, sorghum is an integral part of the diets of millions of people around the world, where it is processed into unleavened breads, boiled porridge, malted beverages including beer, and specialty foods such as popped grain and syrup. With increased use of marginal farmland and global climatic and economical trends, sorghum will be an important crop of interest to feed the world's expanding populations.

In some cultures, the base of the sorghum plant is an important source of fuel for cooking and the stems of wild varieties are used to make baskets and fish traps. In West Africa a red dye is extracted from sorghum that is used to color leather. Increased

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This dissertation follows the style and format of *Genome.*

interests in alternative sources of renewable fuels in the United States have propelled sorghum as a biofuel crop. Currently, sorghum is the second source of grain-based ethanol in the US (after maize) (Paterson 2008). In fact, sorghum has many advantages over maize, such as having generally lower water demands, market price and equal per-bushel ethanol yields. "Sweet sorghums", already grown for forage and silage can serve as an excellent source of cellulosic biofuel, which can offer advantages over seed-based production (Farell et al. 2006). Sweet sorghums contain large, lignocellulose-rich stalks that are completely irrelevant to its use a food crop. With the advanced knowledge of the genetic control of perenniality (Paterson et al. 1995; Hu et al. 2003) and growing knowledge of functional genomics of perenniality (Kresovich et al. 2005), sorghum can be molded into an efficient cellulosic biofuel crop. Sorghum seems to offer nations around the world opportunities for increased energy independence and development, while reducing deleterious environmental impacts that have been historically associated with industrialization.

Sorghum is one of many grass plants that belong to the family Poaceae, which contains about 700 genera and 11,000 species (Chen et al. 2006). Beyond their practical and aesthetic importance in modern society, these cereals also serve as model systems for comparative genetics (Bennetzen and Freeling 1993; Freeling 2001). Sorghum ($2n$=20) has a small genome (~818 Mb) (Price et al. 2005) and is considered to be an attractive model for functional, structural and evolution of cereal genomes (Paterson et al. 2008). Shotgun sequencing of US sorghum inbred line BTx623 has been achieved with approximately 8X coverage. The availability of the sorghum genome sequence

encourages scientists to correlate sorghum genes to its functions. One can expect that as the knowledge of functional genomics in sorghum increases, it will narrow the gap of knowledge that exists in comparative genomics of the cereal grasses. Bennett and Smith (1976; Bennett et al. 2000) have noted that one of the major hurdles to overcome for comparative genomics is the fact that each of these model systems, such as *A. thaliana* and *Oryza sativa*, has major differences in the nuclear DNA content of their genomes. For example, more than 60% of the maize (*Zea mays*) genome is composed of retrotransposons, often arranged as nested insertions within insertions (SanMiguel et al.1996; SanMiguel and Bennetzen 1998; Meyers et al. 2001). The smaller grass genomes, including those of rice and sorghum, have lower amounts of repetitive DNA and fewer retrotransposons inserted between genes (Chen et al. 1997, 1998; Tikhonov et al. 1999; Klein et al. 2000; Tarchini et al. 2000). Comparative linkage mapping studies have demonstrated that grass genomes strongly exhibit gross colinearity across large segments and even entire chromosomes (SanMiguel and Bennetzen 1998; Meyers et al. 2001). Such alignments have enabled comparisons of genomic content, organization, recombination and other features of orthologous regions. Detailed structure analyses of characteristics of local evolution of plant genomes have revealed extensive microdeletions of maize (Coghlan et al. 2005). The roles of such perturbations at the phylogenetic level remain to be delimited. For example, Kim et al. (2005*c*) were able to analyze collinear regions of sorghum and rice chromosomes, comparing genes and average rates of recombination.

Comparison of RFLP maps of sorghum and maize using maize-derived probes showed that many linkage groups are conserved between these two genomes (Whitkus et al. 1992). There are many models that try to explain the large-scale duplications of the maize genome and its relationship to sorghum. Gaut and Doebley (1997) proposed a segmental allotetraploid model and tentatively suggested that one of the maize subgenomes is more closely related to sorghum than to the other maize subgenome. Swigonova et al. (2004) extensively studied the evolutionary relationship of sorghum and maize. They sequenced large duplicated chromosomal fragments in five different loci that are located on seven different maize chromosomes. They reported microcollinearity between maize and rice is preserved in this region; however, only eleven genes were conserved between two duplicated regions of maize and the sorghum and rice genomes. Further phylogenetic analyses demonstrated a close relationship of the two maize progenitor genomes and the sorghum genome, which led them to hypothesize a trichotomous speciation of the three genomes. They hypothesized that the two progenitor genomes of maize and the sorghum genome diverged from each other ~11.9 million years ago (mya).

The genus *Sorghum* belongs to the Andropogoneae tribe, along with other important crops and potential crops, including maize, sugarcane, and miscanthus. The base chromosome number for the Poaceae has been considered to be either five or ten (Garber 1950; Celarier 1956). Garber (1950) concluded that the base number of the tribe could be ten based upon the predominance of genera with chromosome numbers of x=10 or 5 based on the presence of genera with x=5. Spangler et al. (1999) tried to resolve the

base chromosome number of the Andropogoneae by superimposing chromosome numbers onto their phylogenetic tree based upon *ndh*F sequence analysis. The wide variety of taxa near the base of the tree with *x*=10 led them to suggest that ten, not five, is the base number of the tribe (Spangler et al. 1999; Spangler 2003).

There are opposing views on the phylogenetic and ancestral origin of the genus *Sorghum.* According to Garber (1950) and Dillon et al. (2001; 2004; 2007), the genus *Sorghum* consists of twenty-five recognized species that have been traditionally subdivided into five subgenera or sections: *Eu-Sorghum* (including *S. bicolor*), *Chaetosorghum*, *Heterosorghum*, *Para-sorghum* and *Stiposorghum*. The occurrence of both *x*=5 and *x*=10 species of *Sorghum* has raised questions regarding the base number of this genus and thus the evolution of its genomes (Price et al. 2005). Spangler (1999; 2003) concluded there was little evidence that the genus *Sorghum* is monophyletic. Using many morphological traits, Spangler (2003) organized the genus Sorghum into three genera: *Sorghum*, *Sarga* and *Vacoparis*. However, Dillon et al. (2001; 2004; 2007) used the ribosomal ITS gene (ITS1), the chloroplast *ndh*F gene and *Adh*1 gene to create a molecular phylogeny that has resolved all *Sorghum* species into a distinct monophyletic group, within which two clear lineages are evident (Dillon et al. 2007). One lineage contains species that have a base chromosome number of five (*x*=5) and the other lineage contains species that have a base chromosome number of 10 (*x*=10). The ancestral origin of *S. bicolor* has always been viewed with ambiguity. There is genetic and molecular evidence that *S. bicolor* is may have a tetraploid origin. Whereas linkage maps and available sequence data indicate that redundancy within the *S. bicolor*

genome is very limited and thus now diploid, certain cytogenetic and molecular cytogenetic characteristics of the genome are more polyploid-like than diploid-like and thus strongly indicate a polyploid origin of sorghum, followed by very extensive diploidization.

Chromosome numbers are the best known cyto-taxonomic data for most plant genera (Guerra, 2008). Dillon et al. (2007) reported that *S. bicolor* cascaded into a separate lineage that has a base chromosome number of 10, with each species possessing relatively small genomes and small chromosomes. Studies conducted by Kim *et al*. (2002; 2005*a*) yielded a cytogenomic map of ten *S. bicolor* chromosomes. BAC clones containing molecular markers mapped to each linkage group were hybridized to *S. bicolor* chromosomes, producing a FISH-based karyotyping and nomenclature system for all ten sorghum chromosomes.

Brown (1943), Kidd (1952) and Endrizzi and Morgan (1955) reported bivalents in meiosis from haploid *S. bicolor*, indicating homology among chromosomes (Price et al. 2005). Celarier (1956) and Mehra and Sharma (1975) suggested that the haploid ancestral chromosome number for the entire tribe Andropogoneae is five (n=5). Using FISH, Gomez et al. (1998) and Zwick et al. (2000) respectively discovered a BAC clone of sorghum genomic DNA and a 280-bp tandem repeated DNA sequence contained therein, CEN38, which hybridized differentially to the peri-centromeric regions of half of the sorghum genome, i.e., to 10 of the 20 chromosomes. The strong bimodality of a repetitive sequence strongly suggested that the sorghum genome, while extensively diploidized, has an underlying allotetraploid architecture and arose as an allotetraploid.

According to this interpretation, pericentromeric regions of the 10 chromosomes displaying strong FISH signals represent remnants of one subgenome of the tetraploid, whereas the other 10 chromosomes represent the other subgenome.

The advent of multi-BAC FISH probe cocktails enabled cytogeneticists to develop chromosome- and segment-specific "paints" for analysis of chromosomal structure without the cumbersome difficulties of microdissection, flow-sorting and DOP-PCR (Fransz et al. 2000; Islam-Faridi et al. 2002). Kim et al. (2002; 2005*a*; *b*; *c*) extended the use of this powerful technique to study the entire *S. bicolor* genome and later to examine the relationship between the *x*=10 genome of cultivated *S. bicolor* and the *x*=5 genome of the wild Australian species *S. angustum* (Kim et al. unpublished). These results revealed a striking pattern of gross karyotypic differences between *S. angustum* and *S. bicolor*. BAC-probe cocktails from euchromatic regions of all ten *S. bicolor* linkage groups (SBI) were successfully FISHed to the *S. angustum* complement (*n*=5). These hallmark findings raise many questions about the evolution in *Sorghum* *x*=5 and *x*=10 genomes, the relationships between *S. angustum* (*x* =5) and *S. bicolor* (*x*=10) genomes, and the evolutionary and taxonomic relationships among other *Sorghum* species.

The goal of this research endeavor is to clarify long-standing ambiguity of karyotypic evolution and relationships in the genus *Sorghum*. Cytogenomic analyses, like those of Kim et al. (unpublished), could explicate the convoluted evolutionary state of the *Sorghum* genus. The knowledge gained from comparative cytogenomics of *S. bicolor* (*x*=10) and numerous *x*=5 wild species would expectedly elucidate the

evolutionary relationships that exist in the genus and complement the various phylogenetic studies that have been undertaken. Thus, specific objectives of this study are to test the hypothesis that the types of karyotypic rearrangements which distinguish the genomes of *S. bicolor* and *S. angustum* are prevalent in other *x*=5 *Sorghum* species and to compare and analyze patterns of *in situ* hybridization and localization of telomeric and ribosomal DNA to *x*=5 and *x*=10 *Sorghum* species to determine if the resulting data augment, refute or refine the hypothesized patterns in *Sorghum* genome evolution. My approach to elucidating *x*=5 versus *x*=10 *Sorghum* evolution is to rely mostly on *in situ* hybridization of BACs, ribosomal DNA (rDNA) and telomoric DNA. The BAC-FISH is expected to reveal major chromosomal and segmental relationships among the tested species, while the rDNA will expectedly provide additional supportive data, and the telomeric may pinpoint sites of some of the rearrangements that have shaped each of the genomes. At the very least, the resulting comparative maps will reveal karyomopholgical data for species that have never been investigated. This information will help determine the extent of genomic changes during evolution of the genus.

CHAPTER II

INTERSPECIFIC CHROMOSOMAL AND GENOMIC RELATIONSHIPS IN THE GENUS *SORGHUM*

Introduction

Sorghum bicolor genomics research ranges from applications in plant breeding to functional and structural genomics. There are many factors that make sorghum an attractive model for genomic studies and agricultural biology, such as its small genome (~818 Mb) and C4 photosynthesis. The advancements made in genetic and physical mapping of the sorghum genome have allowed scientists to cross-utilize results and simultaneously advance knowledge of many important crops (Paterson et al. 2008). Intraspecific and interspecific high-density linkage maps from *S. bicolor* (Xu et al. 1994; Bhattramakki et al. 2000; Menz et al. 2002) and *S. bicolor* x *S. propinquum* (Chittenden et al. 1994; Bowers et al. 2003) have made available a plethora of molecular advances. Sorghum was also the first angiosperm for which a BAC library was published (Woo et al. 1994). Now high-coverage BAC libraries are available for sorghum inbred line BTx623. Approximately 456 *S. propinquum* (closest relative of sorghum) and 303 *S. bicolor* BAC contigs (41% of BACs, 80% of single-copy loci) are well-anchored to euchromatic regions, with the percentage of the genome attributable to euchromatin likely to rise with additional anchoring (Paterson et al. 2008). Kim et al. (2005*c*) were able to delimit the boundaries of euchromatic and heterochromatic regions of *S. bicolor*

and estimate the amount of DNA located in each type of chromatin, for each chromosome and overall.

The genome sequencing for *S. bicolor* is creating more facile approaches to functional and comparative genomics. Although many genomic studies have focused on sorghum and other cereal grasses, they have yet to reveal the origin of *Sorghum bicolor* and related species. Thus, genome evolution in Sorghum remains a scientific conundrum. A more thorough description of genomic evolution in the genus will almost certainly enhance our understanding of the origin of *Sorghum bicolor* and very possibly, other grasses, such as maize, sugarcane and miscanthus.

The genus *Sorghum* is a distinct monophyletic group, within which two lineages are clearly evident (Dillon et al. 2007). The *Eu-sorghum* section (*S. bicolor* included) and *Chaetosorghum*/*Heterosorghum* sections form one lineage (B), whereas the *Para-sorghum* and *Stiposorghum* sections form a well-supported second lineage (F). Each lineage can be delineated by their differences in base chromosome number, chromosome morphology and gene content. The x=5 lineage (F) ($2n$=10, 20, 30 and 40) is characterized by species with large genomes and large chromosome morphology whilst the x=10 lineage (B) ($2n$=20 and 40) is characterized by small genomes and small chromosome morphology (Price et al. 2005).

In general, the evolution of chromosome components can be resolved by genome sequencing, comparative genetic (linkage) mapping and comparative chromosome painting (Lysak et al. 2005). In the *Sorghum* genus, each species features a basic chromosome number or either x=5 or 10, with the chromosomes differing vastly in size.

Gene sequence data enabled synthesis of a genome-wide phylogenetic interpretation (Dillon et al. 2007). Genetic maps are available for *S. bicolor* (Chittenden et al. 1994; Bhattramakki et al. 2000; Menz et al. 2002) and species of the *x*=10 *Sorghum* lineage, but lack of such maps for species of the *x*=5 lineage precludes broad application of comparative genetic linkage maps for map-based evolutionary interpretations of *x*=5 versus *x*=10 lineages. Using an alternative approach, comparative chromosome painting (CCP), Kim et al. (unpublished) compared *S. bicolor* (*n*=10) and *S. angustum* (*n*=5) discovered dramatic cytogenomic rearrangements between them, which allowed them to detect multi-chromosomal synteny shared between *S. bicolor* (*x*=10) and *S. angustum* (*x*=5).

Kim et al. (unpublished) developed a CCP strategy with probes made from BACs landed to genetic map of sorghum. They observed extensive intra-chromosomal rearrangements in *S. angustum* relative to *S. bicolor*. Because the experiments of Kim et al. (unpublished) were confined to just a couple of taxa, it is not known if the most dramatic results were species-specific, lineage-specific or general. To deduce prevalence of phenomena discovered by Kim et al., similar experiments must be applied to other *Sorghum* species.

The objective of this study is to determine if extensive karyotypic rearrangements observed in *S. angustum* are prevalent in other *x*=5 *Sorghum* species. By extending the approach of Kim et al. (unpublished) to other *x*=5 species, it should be possible to determine if these karyotypic rearrangements can be generalized for the entire genus or classified as an evolutionary phenomenon exclusive to *S. angustum*.

Materials and Methods

Plant Materials

The *Sorghum* species used in this research were propagated from seeds and grown in a glasshouse. Accession numbers, herbarium voucher numbers, life forms and origins are listed in Table 1.

Somatic Chromosome Preparation

Somatic metaphase chromosome spreads were prepared following a modified technique described in Kim et al. (2002) and Andras et al. (1999). Excised young root tips were treated with saturated aqueous α-bromonapthalene solution at room-temperature in the dark for 1.5 hr, and then promptly fixed in 3:1 ethanol: acetic acid. One to two mm of root meristimatic tissue was dissected from numerous similar-sized root tips into 0.5 ml micro-centrifuge tube containing H_20. The water was decanted and replaced with 0.2M HCL and incubated at room temperature for 10 min. After ten minutes, the HCL was decanted and root tips were washed 3X with H_20. After last washing, the H_20 was decanted and 400µl of enzyme solution (5% cellulase: 2% pectolyase) was added to the tube and incubated in a 37°C water bath for 2-3 hrs (depends on root tip size and experience). After incubation, the root tips are washed with H_20 and centrifuged ~2500 rpm 3X for 10 min each time. After the last washing, the H_20 is decanted and replaced with 4:1 methanol: acetic acid fixative. A pipetter was used to remove 2-8µl suspension and drop the solution onto clean glass slide.

Table 1. Accession number, life form and origin of five *Sorghum* species used in this study (Modified from Price et al. (2005).

Species	Herbarium voucher	Accession number[†]	Life form	Collection date and site, or source of seeds
S. angustum S. T. Blake	BRI AQ585981[§]	302605[§]	Annual	19-May –95, Windmill Ck crossing, 18.8 km S. of Musgrave Station on Peninsula. Development Road, QLD, Australia
S. bicolor Tx623 (L.) Moench			Annual	Seeds obtained from W. Rooney, Texas A&M University
S. brachypodum Lazarides	DNA D133019	302670	Annual	Oenpelli Rd (road to Jabiru), approx 2km E of Magela Creek, Kakadu National Park, NT
S. intrans F. Muell. Ex Benth	DNA D133021	302668	Annual	Rod to Howard river floodplain, SE of Darwin
S. versicolor Anderss.			Annual	East Africa, seeds obtained from G. Liang, Kansas State University

[§]BRI = Queensland Herbarium, Mt Coot-tha, QLD Australia

Metaphase cells were visually observed using phase contrast microscopy. Slides that contained metaphase cells were preserved in -20°C freezer until needed.

Selection of BACs for FISH

The BACs used in this study came from a genomic BAC library that derived from sorghum cultivar BTx623 (Tables 2, 3) (Woo et al. 1994). To obtain multiple BAC-FISH signals, twenty BACs from SBI-02 were selected based on their euchromatic origins (Kim et al. 2005b). Eight of these BACs were exclusively used by Kim et al. (unpublished) in BAC-FISH study of *S. bicolor* (*x*=10) and *S. angustum* (*x*=5) (Table 3).

BAC DNA Extraction and Purification

BAC DNA was isolated by alkaline lysis and then further purified using Plasmid Mini kit (Qiagen, Valencia, CA).

Probe Labeling and *In situ* Hybridization

Purified BAC DNA was labeled with biotin-dUTP and digoxygenin-11-dUTP by the BioNick Labeling system (Roche Molecular Biochemicals, Indianapolis, Indiana, USA). *In situ* hybridization techniques were performed following modifications of the protocol described in Jewell and Islam-Faridi (1994). The chromosomal DNA on the glass slide was denatured at 70°C in 70% formamide in 2X SSC for 1.5 min followed by dehydration in 70 (pre-chilled at -20°C), 85, 95 and 100% ethanol for 2-3 min each. Subsequently, the slides were allowed to air-dry for ten minutes. The hybridization

Table 2. List of BAC clones from SBI-02 used for comparative BAC-FISH.

BAC clone [a]	**Chromosome arm location**
112a4	p
56h12	p
120e10	p
200g12	p
48d12	p
86b12	p
109b1	p
86g4	p
119b9	q
245a8	q
182b4	q
105b9	q
44h5	q
123f1	q
99d7	q
226b3	q
39d3	q
193b6	q
121a6	q
19h7	q

[a] BAC clones from the BTx623 BAC libraries arranged in 96-well format

Table 3. List of BAC clones from SBI-02 used as probes for comparative Southern Hybridization.

BAC clone [a]	**Chromosome arm location**
112a4	p
120e10	p
48d12	p
109b1	p
105b9	q
39d3	q
99d7	q
245a8	q

[a] BAC clones from the BTx623 BAC libraries arranged in 96-well format

mixture (25 µl for each slide) contained 25 ng of labeled probe DNA (plus 40 µl of sheared S. bicolor DNA that served as blocking DNA for *S. bicolor* BAC-FISH), 50% formamide, 10% dextran sulfate and 2X SSC. The mixture was denatured at 90°C for 10 min (30 min for *S. bicolor* BAC-FISH), subsequently chilled on ice for 5 min and added to the slide. Following overnight incubation at 37°C, slides were rinsed at 40°C in 2X SSC for 5 min (3 times), then in 2X SSC for 5 min RT and finally in 4X SSC plus 0.2% Tween-20 for 5 min. Slides were blocked 5 min at RT with 5% (w/v) BSA in 4X SSC plus 0.2% Tween-20. Biotin-labeled probes were detected with 1% Cy3-conjugated streptavidin and digoxygenin- labeled probes with 1% fluorescein isothiocyanate (FITC)-conjugated anti-digoxygenin antibody and incubated at 37°C humidity chamber for 30 min. Afterwards, slides were washed 3 times in 4X SSC plus 0.2% Tween-20 at 40°C for 1-2 min each. The chromosomes are detected by adding 100-200 µL of 3 µg/mL DAPI with Vectashield®.

Southern Hybridization

The presence and organization of specific BAC DNA (Table 3) in five species of the genus *Sorghum* were detected by Southern blotting. Procedures for Southern blotting and hybridization followed the protocol by Zhang (2005). Genomic DNA from the five *Sorghum* species was digested with the restriction enzyme *Hind*III at 37ºC for exactly 2 h. While the DNA was being digested, a 0.8% agarose gel (250 ml of 1x NEB and 2.0 g of agarose) was prepared. Once the gel solidified, it was submerged with the gel mold tray in 1x NEB in the buffer chamber. After digestion, 5 µL of 10x loading dye

Table 4. Lane assignments for species analyzed by Southern blotting, and restriction enzymes used to digest their DNA.

Lane #†	Species/Marker	Restriction enzyme
1	*λDNA* marker	*Hind*III
2	*S. bicolor*	*Hind*III
3	*S. angustum*	*HindIII*
4	*S. versicolor*	*Hind*III
5	*S. brachypodum*	*Hind*III
6	*S. intrans*	*Hind*III
7	Empty	None
8	*λDNA* marker	*Hind*III
9	*S. bicolor*	*Hind*III
10	*S. angustum*	*HindIII*
11	*S. versicolor*	*Hind*III
12	*S. brachypodum*	*Hind*III
13	*S. intrans*	*Hind*III
14	Empty	None
15	*λDNA* marker	*Hind*III
16	*S. bicolor*	*Hind*III
17	*S. angustum*	*HindIII*
18	*S. versicolor*	*Hind*III
19	*S. brachypodum*	*Hind*III
20	*S. intrans*	*Hind*III
21	Empty	None
22	*λDNA* marker	*Hind*III
23	*S. bicolor*	*Hind*III
24	*S. angustum*	*HindIII*
25	*S. versicolor*	*Hind*III
26	*S. brachypodum*	*Hind*III
27	*S. intrans*	*Hind*III
28	Empty	None
29	Empty	None
30	Empty	None
1a	*λDNA* marker	*Hind*III
2a	*S. bicolor*	*Hind*III
3a	*S. angustum*	*HindIII*
4a	*S. versicolor*	*Hind*III
5a	*S. brachypodum*	*Hind*III

Table 4. Continued

Lane #†	**Species/Marker**	**Restriction enzyme**
6a	*S. intrans*	*Hind*III
7a	Empty	None
8a	*λDNA* marker	*Hind*III
9a	*S. bicolor*	*Hind*III
10a	*S. angustum*	*HindIII*
11a	*S. versicolor*	*Hind*III
12a	*S. brachypodum*	*Hind*III
13a	*S. intrans*	*Hind*III
14a	Empty	None
15a	*λDNA* marker	*Hind*III
16a	*S. bicolor*	*Hind*III
17a	*S. angustum*	*HindIII*
18a	*S. versicolor*	*Hind*III
19a	*S. brachypodum*	*Hind*III
20a	*S. intrans*	*Hind*III
21a	Empty	None
22a	*λDNA* marker	*Hind*III
23a	*S. bicolor*	*Hind*III
24a	*S. angustum*	*HindIII*
25a	*S. versicolor*	*Hind*III
26a	*S. brachypodum*	*Hind*III
27a	*S. intrans*	*Hind*III
28a	Empty	None
29a	Empty	None
30a	Empty	None

† letter “a” designates the lanes on the bottom-half of the gel

was added to each sample and the samples were inserted into the gel. One lane of marker DNA (λ DNA digested with *Hind*III) was loaded per gel. The gel was run at 24V for approximately 16 h. The gel tray was carefully removed and transferred into a staining tray containing ethidium bromide. The tray was agitated on an orbital shaker with gentle shaking and allowed to stain for 30 min. The gel was carefully transferred to another tray containing water and destained on the orbital shaker for 20 min. The gel was carefully removed from the tray and view on a UV light box. The gel was photographed and prepared for blotting (Fig. 1). A blotting tray was filled with 1 L of 0.4 N NaOH. A glass plate was placed over the tray (used as a bridge) and a wick was constructed using two layers of blotting paper soaked in 0.4 N NaOH. Bubbles were removed between the plate and the blotting paper with a glass pipette. The corner of the gel without samples was cut with a razor blade for identification purposes and placed upside down on the blotting paper wick. A piece of Hybond-N+® membrane the same size as the gel was placed over the gel. Again the bubbles were removed from the gel with a glass pipette. Two sheets of blotting paper with sizes slightly larger than the gel were cut, soaked in the reservoir buffer (0.4 N NaOH) and placed on the membrane. A large stack of paper towels (2.5 – 5.0 cm) was placed over the blotting paper. A glass plate was placed over the paper towels and large weight (500 - 1000 g) was placed on top of the plate. The DNA from the gel was allowed to diffuse to the membrane overnight. The next day, the membrane was transferred into a tray containing 500 mL of 2x SSC with a forceps and soaked on an orbital shaker for 10 min. Then the membrane was wrapped with SaranWrap® and stored at 4ºC until hybridization. The membrane

was incubated in hybridization solution (250 mL of 20x SSC, 25mL of 0.5% SDS, 25 mM 0.5 M KPB,

pH6.5, 100x Denhardt's and 625 mL of dd H_2O) at 65ºC for >2 h. The probes were radioactively-labeled by incubating at 37ºC for 30 min in LS (labeling solution), 0.5U/ul Klenow, ^{32}P-dCTP, and dd H_20. The labeled probes DNA were denatured by adding one volume of 0.4 N NaOH and incubating the reaction at 95ºC for 10 min. The labeled probes were carefully transferred into the hybridization solution (without touching the membrane) and incubated overnight at 65ºC with gentle shaking. On the next day, the membrane was washed several times at 65ºC with a washing buffer (20x SSC [0.2x final concentration], 20% SDS [0.1% final concentration] and dd H_20). After the third washing, the membrane was blotted with paper towels to remove excess fluid and wrapped with SaranWrap®. The membrane was placed in an autoradiograph cassette, covered with x-ray film. The cassette containing the film was exposed at room temperature for 2 d. The x-ray film was then developed and further analyzed.

Microscopy

Chromosomes and FISH signals were viewed through an Olympus AX-70 epifluorescence microscope equipped with filter cubes appropriate for DAPI, Cy3 and fluorescein. Images from a Peltier-cooled 1.3M pixel Sensys camera (Roper Scientific) were captured with the MacProbe v.4.2.3 digital image system (Applied Imaging Corp., Santa Clara, California, USA).

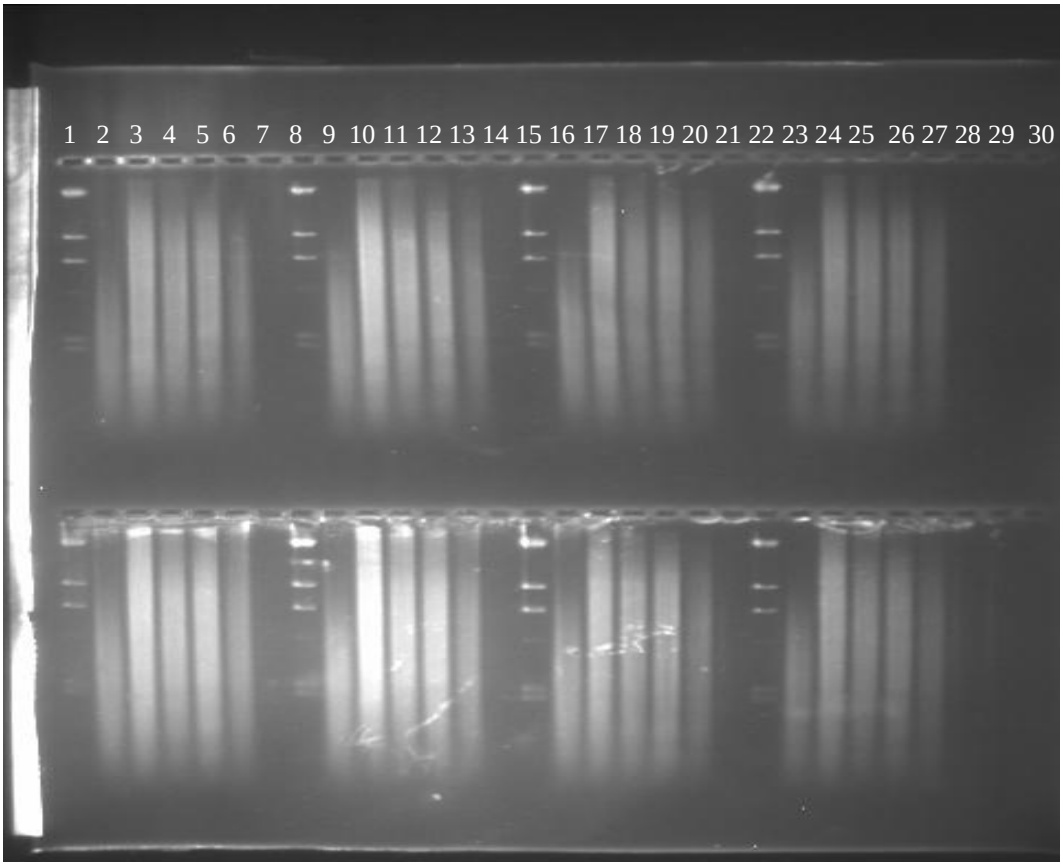


Fig. 1. Agarose gel of *Hind*III-digested genomic DNA of five select *Sorghum* species.

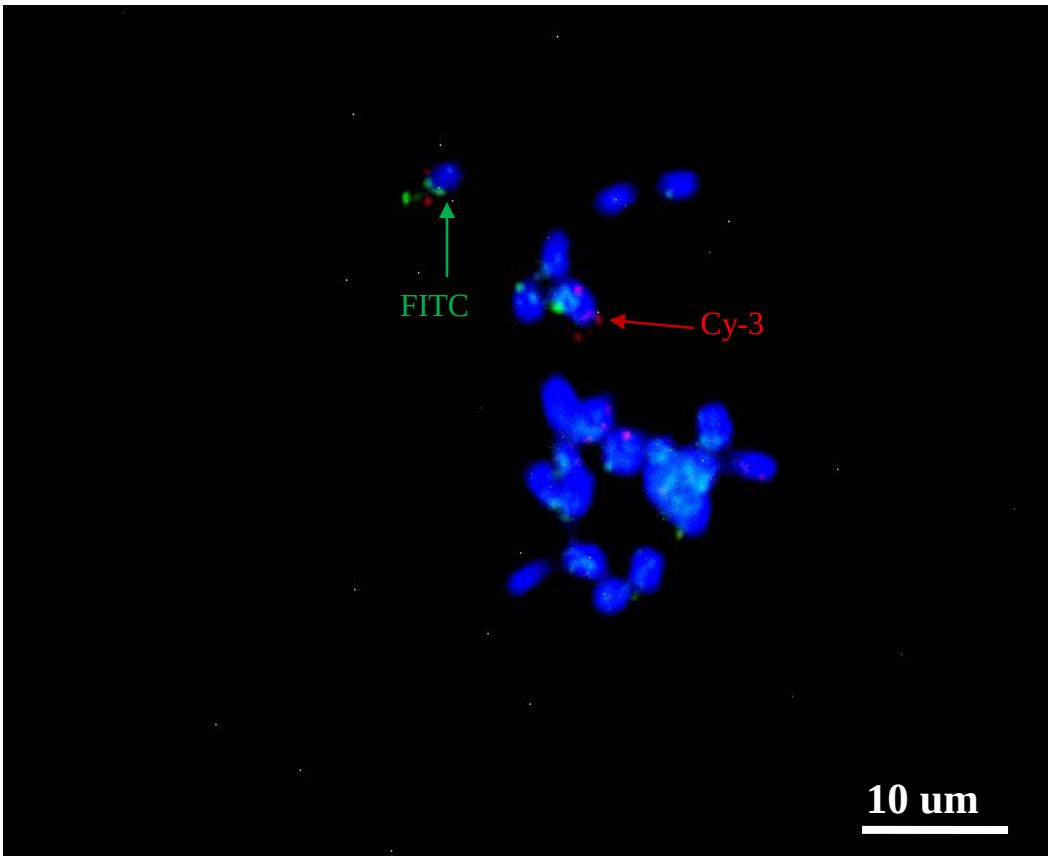


Fig. 2. FISH signals hybridized on somatic metaphase *S. bicolor* chromosomes using a 20-probe BAC cocktail of SBI-02 and 10X blocking DNA. Pooled BACs from the p-arm (short) were labeled with digoxygenin (DIG) and detected with anti-digoxygenin-Cy3 conjugate system (red signal). Pooled BACs from the q-arm (long) were labeled with biotin and detected with streptavidin-DTAF conjugate signal (green signal).

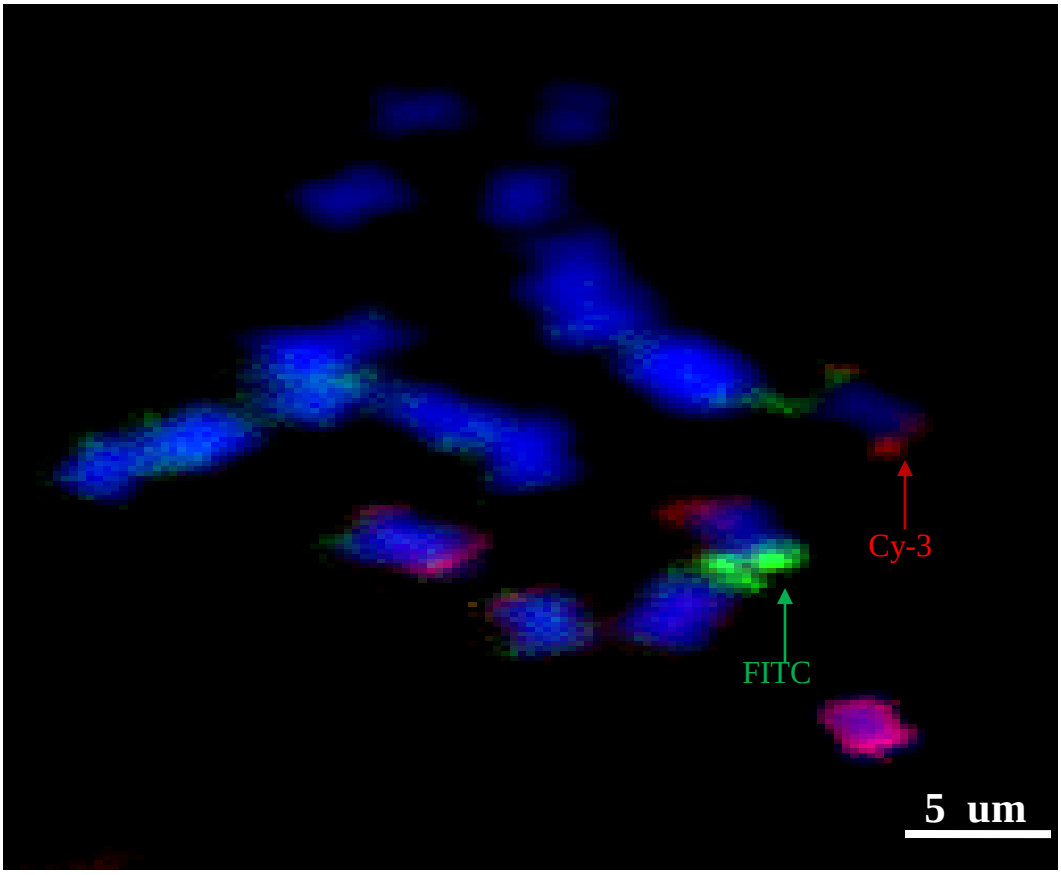


Fig. 3. FISH signals hybridized on somatic metaphase *S. bicolor* chromosomes using a 20-probe BAC cocktail of SBI-02 and 10X blocking DNA. Pooled BACs from the p-arm (short) were labeled with digoxygenin (DIG) and detected with anti-digoxygenin-Cy3 conjugate system (red signal). Pooled BACs from the q-arm (long) were labeled with biotin and detected with streptavidin-DTAF conjugate signal (green signal).

Results

Comparative Chromosome Painting (CCP) among *Sorghum* Species

On the basis of their map positions and euchromatic origins, twenty sorghum BACs from SBI-02 were selected for CCP (Kim et al. 2005*b*). Since Kim et al. (2005*b*) evaluated these BACs via single-color FISH and selected them for multiple BAC-FISH, there was no question of their efficacy and the BACs were immediately labeled and used for BAC-FISH. Two multi-BAC FISH cocktails were used to locate the distribution of individual BAC-FISH pools, which were differentially-labeled by chromosome arms (Table 2).

In situ hybridization of pooled BAC probes yielded underwhelming results. As expected, hybridization of BAC pools formed from SBI-02 was detected in *S. bicolor* (Figs. 2, 3). The localization of primary hybridization to a single chromosome conformed to expectations and established credibility of the *in situ* hybridization. Hybridization of one BAC pool (p-arm) was clearly detected without the aid of blocking DNA in to *S. angustum* (Fig. 4), even though all chromosomes were not full condensed as evident by the bright-stained DAPI regions (Fig. 5). In contrast, the patterns of hybridization signal were sparse and weak after FISH of the combined BAC cocktail (p and q arms) used in *S. bicolor* sans sheared *S. bicolor* DNA (blocking DNA) to *S.*

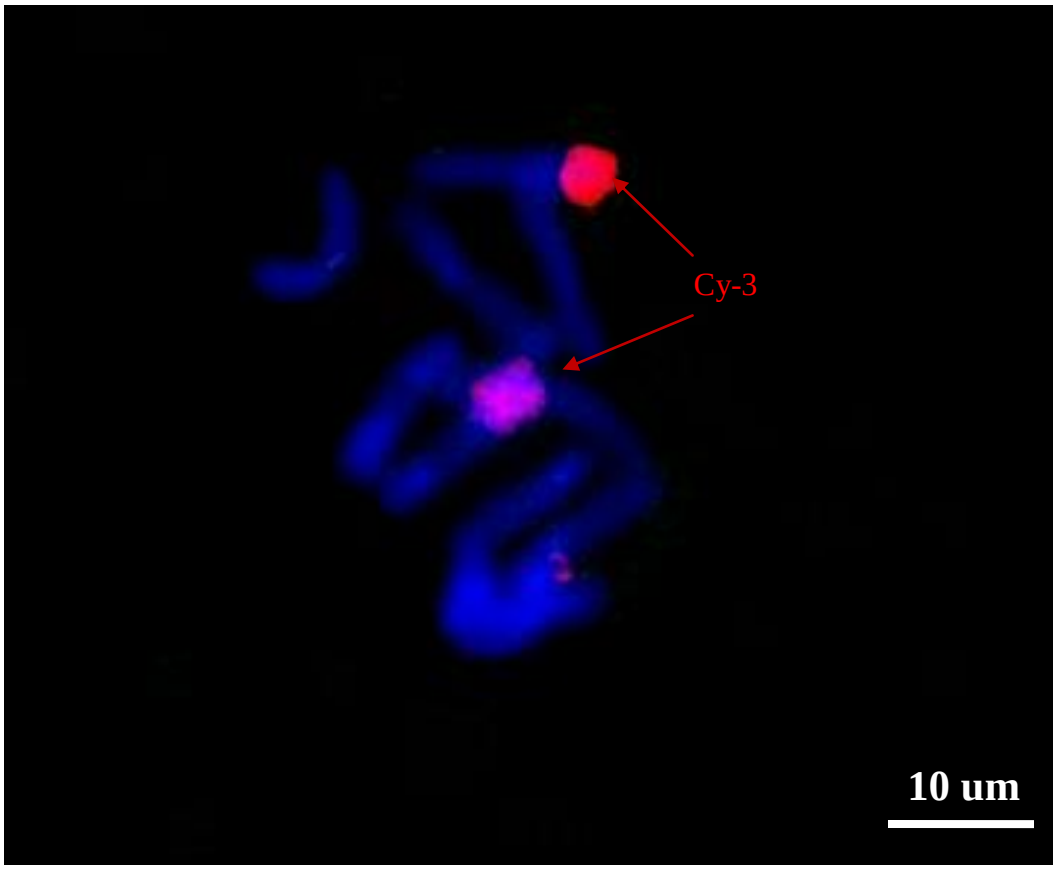


Fig. 4. FISH signals hybridized on somatic metaphase *S. angustum* chromosomes using pooled BACs from the p-arm (short) of SBI-02. BACs were labeled with digoxygenin (DIG) and detected with anti-digoxygenin-Cy3 conjugate system (red signal).

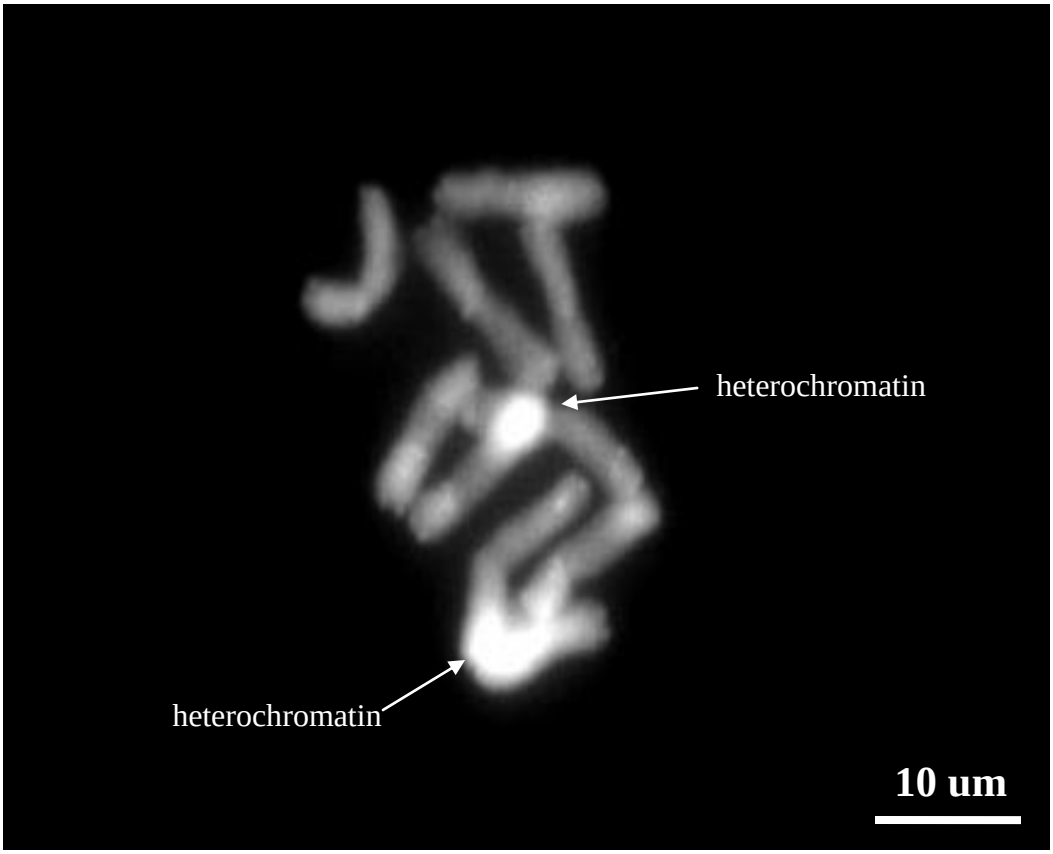


Fig. 5. Inverted DAPI image of somatic metaphase *S. angustum* chromosomes. Brightly stained DAPI regions are characterized as overlapped chromosomes or possibly heterochromatic DNA.

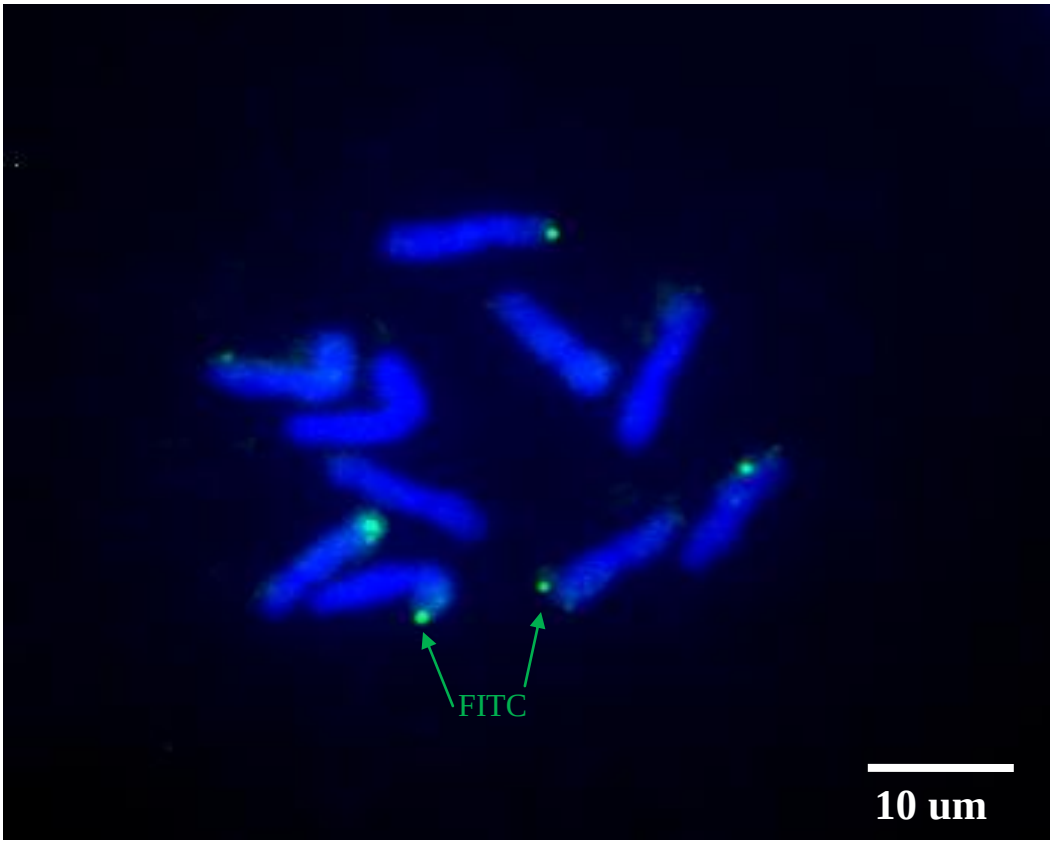


Fig. 6. FISH signals hybridized on somatic metaphase *S. brachypodum* chromosomes using pooled BACs from the q-arm (long) of SBI-02. Pooled BACs from the q-arm (long) were labeled with biotin and detected with streptavidin-DTAF conjugate signal (green signal).

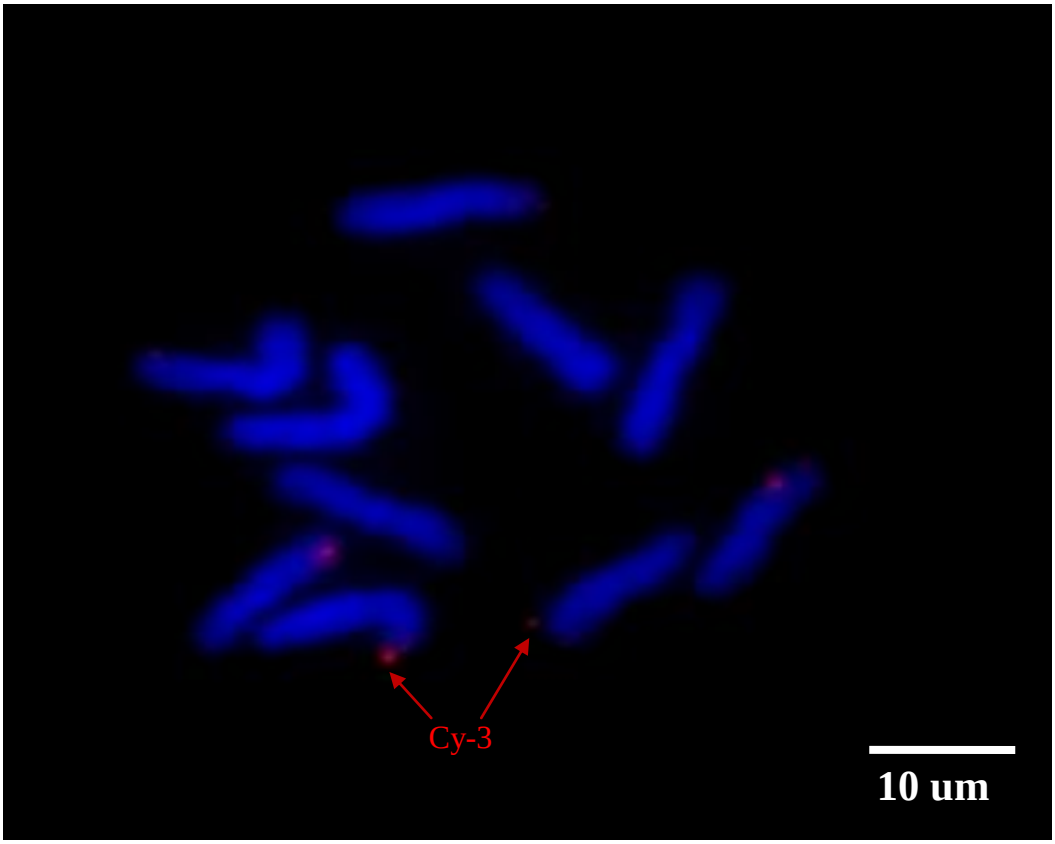


Fig. 7. FISH signals hybridized on somatic metaphase *S. brachypodum* chromosomes using pooled BACs from the p-arm (short) of SBI-02. BACs were labeled with digoxygenin (DIG) and detected with anti-digoxygenin-Cy3 conjugate system (red signal).

brachypodum and *S. versicolor*. Signals occurred on multiple pairs of chromosomes, not just one pair, and did not manifest any segmental chromosomal rearrangement. The FISH patterns for the short and long arms of SBI-02 were largely co-localized, which suggested that they share one or more repeats with each other and these two wild species, and that in the latter, they may be tandemized and thus relatively easy to see (Figs. 6, 7, 8, 9). For both arms of SBI-01, the BAC cocktail probes to, detectable hybridization sites were confined to terminal regions of three *S. brachypodum* chromosomes. Dual-color FISH of the two BAC pools co-localized the respective FISH sites, even though they were derived from different arms of SBI-02 (Fig. 8, 9). The findings suggest that *S. brachypodum* and *S. versicolor* contain one or more tandemly repeated sequences closely related to one or more sequences present in at least one BAC in each of the two SBI-02 BAC pools. It is most likely this sequence is of *S. bicolor* origin, even though specific FISH experiments were not conducted to exclude possible homology of BAC vector sequence.

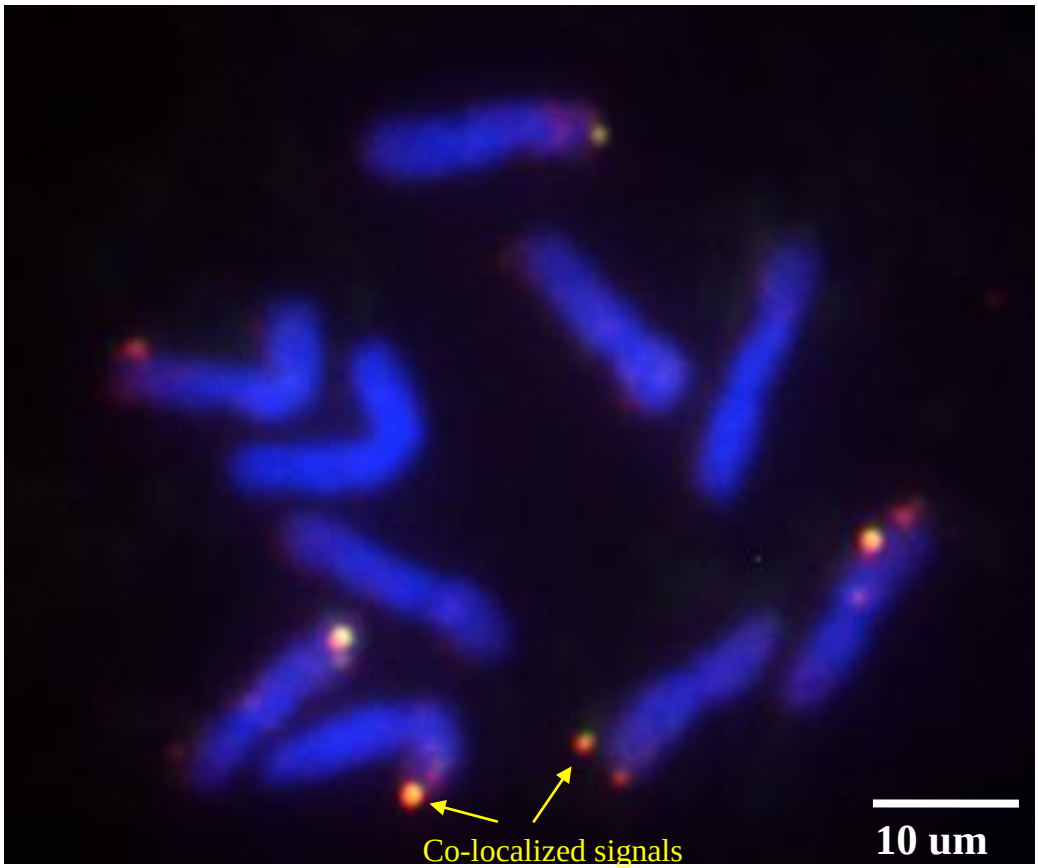


Fig. 8. FISH signals hybridized on somatic metaphase *S. brachypodum* chromosomes using two multi-BAC probe cocktails for opposing arms of SBI-02. The over-lap between red and green signals co-localized hybridization of pooled BACs from p-arm (red) and q-arm (green) of SBI-02.

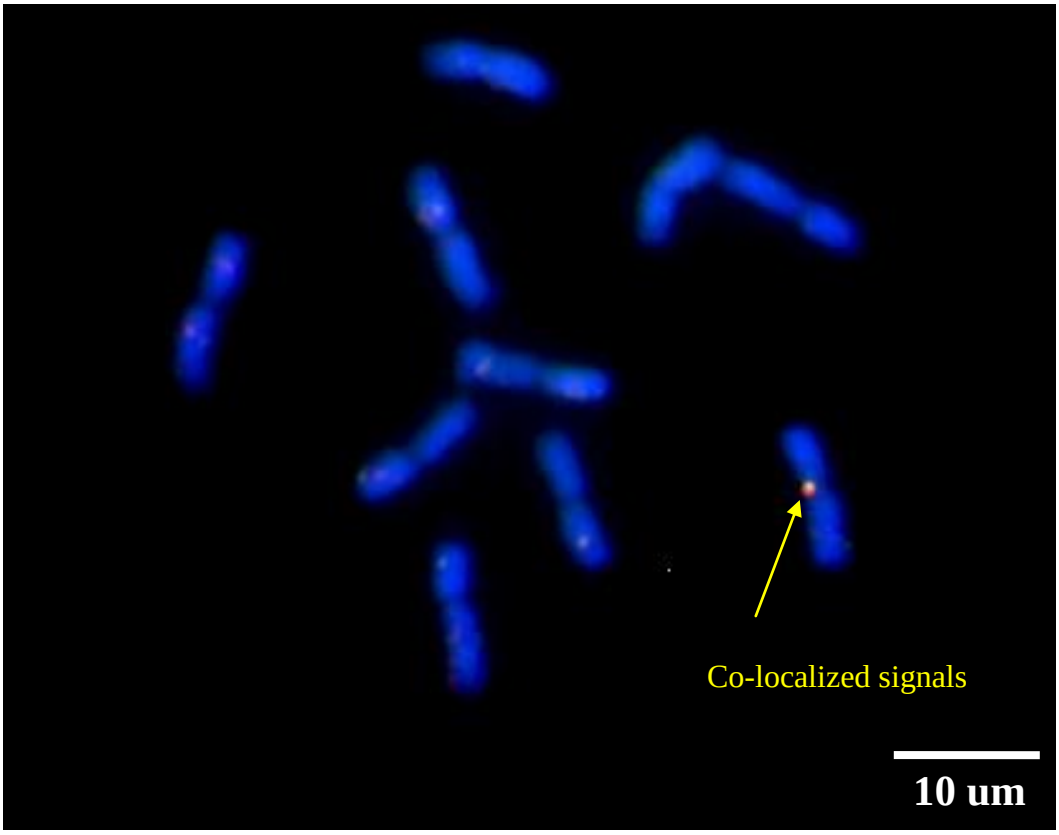


Fig. 9. FISH signals hybridized on somatic metaphase *S. versicolor* chromosomes using a 20-probe BAC cocktail of SBI-02. Pooled BACs from the p-arm (short) were labeled with digoxygenin (DIG) and detected with anti-digoxygenin-Cy3 conjugate system (red signal). Pooled BACs from the q-arm (long) were labeled with biotin and detected with streptavidin-DTAF conjugate signal (green signal). The "painting" FISH pattern is due to repetitive hybridization.

Southern Hybridization

All of the Southern hybridizations of individual SBI-02 BAC clones to *Hind*III-restricted DNA samples of select *Sorghum* species resulted in "smeared" patterns, though they varied from light to dark (Figs. 10, 11). The "smeared" distributions indicated that all of these BACs contain sequence(s) homologous to repetitive elements present in genomes of all of these *Sorghum* species, and that most of those elements are dispersed. The variations in intensity reflect relative abundance and/or degrees of homology.

Careful examination of the Southern hybridization revealed while a few BACs (BAC 112a4, 120e10, 105b9, 39d3, 99d7) revealed similar hybridization patterns in *S. angustum* in relation to other *x*=5 species, BACs 48d12, 109b1, 245a8 did not. Those BACs (48d12, 109b1, 245a8) revealed multiple, conserved fragments *in S. angustum* that were of different sizes or not present in the other *x=5 Sorghum* species.

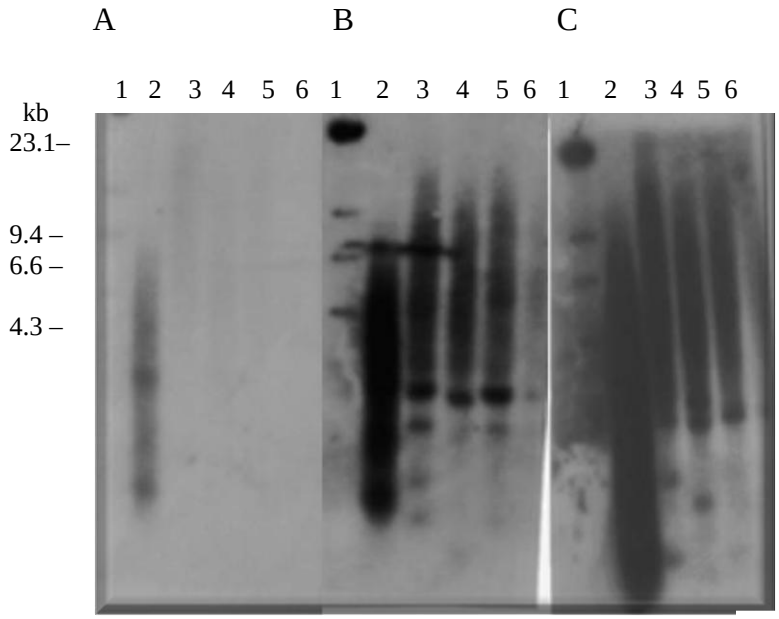


Fig. 10. Comparative Southern hybridization of *S. bicolor* (SBi-02) BACs (A) 112a4, (B) 48d12 and (C) 109b1 to genomic DNA of *S. bicolor* (lane 2), *S. angustum* (lane 3), *S. versicolor* (lane 4), *S. brachypodum* (lane 5) and *S. intrans* (lane 6). λ-*Hind*III marker (lane 1) serves a molecular marker and control.

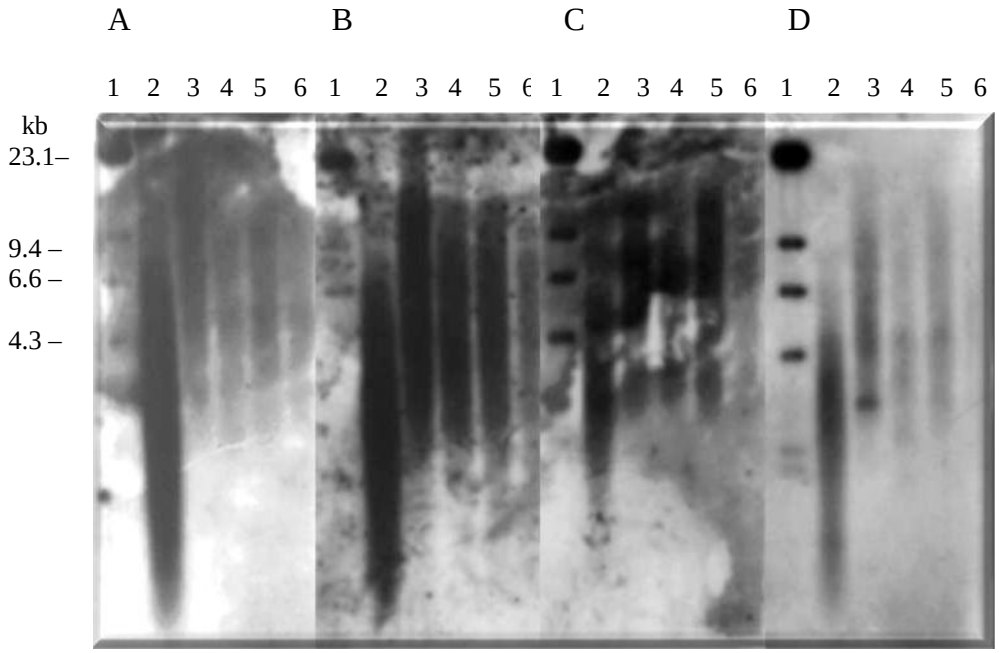


Fig. 11. Comparative Southern hybridization of *S. bicolor* (SBI-02) BACs (A) 105b9, (B) 39d3, (C) 99d7 and (D) 245a8 to *HindIII*-restricted genomic DNA of *S. bicolor* (lane 2), *S. angustum* (lane 3), *S. versicolor* (lane 4), *S. brachypodum* (lane 5) and *S. intrans* (lane 6). λ-*Hind*III marker (lane 1) serves a molecular marker and control.

Discussion

The objective of this study was to determine if the extensive karyotypic rearrangements previously observed in *S. bicolor* versus *S. angustum* (Kim et al. unpublished) are unique to *S. angustum*, or common to other *x*=5 *Sorghum* species. If the *S. angustum* pattern were common to *x*=5 genomes, the findings would underscore the importance of the cytogenomic evolutionary pattern, and also the organizational stability of *Sorghum x*=5 genomes. If the rearrangements were unique to *S. angustum*, and other patterns exist for the other x=5 genomes, the findings would highlight/point to an underlying mechanism for rearrangements, and complexity of *Sorghum* x= 5 genome evolution. FISH of SBI-02 BAC-FISH cocktails to somatic metaphase chromosomes of *S. versicolor* and *S. brachypodum* and single-BAC FISH to *S. intrans* (data not shown) failed to yield localized site-specific BAC-FISH signals. The lack of typical BAC-FISH signals could be due to inadequate homology of low-/single-copy sequences, or their evolutionary redistribution relative to *S. bicolor* and *S. angustum*. In *S. brachypodum*, detectable signals occurred on only three chromosome pairs, but these signals were shown to be due to tandemized repeats, because probes from opposite SBI-02 arms also produced co-localized dual-color FISH signals. A number of signals were observed on *S. versicolor* chromosomes; at least some of these were also attributable to repeated sequences, based on co-localization of FISH signals from BAC probes from opposite SBI-02 arms. Against this "noisy" background, it was not possible to discern the presence or locations of low-copy FISH signals. Thus, for both *S. brachypodum* and *S. versicolor*, the expected types of data were not obtained.

Southern hybridization of all eight BACs from the BAC pools to *Hind*III-restricted DNA from the various species (Figs. 10, 11) gave strong, "smeared" distributions. This pattern indicated that the BACs used to make FISH probes contain sequences sufficiently homologous to interspersed repeats to enable hybridization to all of the targeted genomes. Exceptions would be expected when there is insufficient abundance of one or more specific unlabeled repetitive sequences relative to the abundance in labeled probe and/or target where repeats are in high density, e.g., tandem repeats. Such differences would perhaps be most expected when probes (labeled and unlabeled) and targets are derived from different species.

The Southern hybridization expanded many questions that were revealed by the BAC-FISH study. First, how could *in situ* hybridizations of pooled BACs from SBI-02 yield a recognizable hybridization pattern on *S. angustum* (Kim et al. unpublished), but not *S. brachypodum* and *S. versicolor* (Figs. 7, 8, 9, 10)? Careful examination of the Southern hybridization revealed while a few BACs (BAC 112a4, 120e10, 105b9, 39d3, 99d7) revealed similar hybridization patterns in *S. angustum* in relation to other *x*=5 species, BACs 48d12, 109b1, 245a8 did not. Those BACs (48d12, 109b1, 245a8) revealed multiple, conserved fragments in *S. angustum* that were of different sizes or not present in the other *x*=5 *Sorghum* species.

The direction of evolution is usually not evident from only karyological comparisons (Guerra 2008). The occurrence of both *x*=5 and *x*=10 species in *Sorghum* raises questions regarding the base number of the genus (Price et al. 2005). There is genetic, molecular and cytological evidence that implies that *S. bicolor* (*x*=10) may be a

tetraploid or at least be of disomic tetraploid origin (Brown 1943; Kidd 1952; Endrizzi and Morgan 1955; Gomez et al. 1998; Zwick et al. 2002). However, Peng et al. (1999) concluded that the distribution of duplicated loci in sorghum does not support the hypothesis that sorghum is of tetraploid origins. As a result, the ancestral chromosome number of the genus *Sorghum* (x=5 vs. x=10) remains unresolved. Kim et al. (unpublished) defined homologous regions from *S. bicolor* (x=10) in *S. angustum* (x=5), yet the direction of karyotype evolution remains unresolved.

Dillon et al. (2007) reported 25 *Sorghum* species form a distinct monophyletic group. However, speciation and divergence could have caused many of the x=5 species to lose much of their homology with *S. bicolor*. For example, Dillon et al. (2007) stated that within clade H of the x=5 lineage (F), the cluster of *S. extans*, *S. intrans* and *S. angustum* has little bootstrap support (24%) relative to the rest of the lineage. *S. extans*, *S. intrans* and *S. angustum* have been classified as the cluster most distant from *S. bicolor*; whereas the seven species that form an unresolved polytomy within the same clade were collectively closer (Fig. 1). Furthermore, Lysak et al. (2005) reported that chromosomal rearrangements exhibit a low-level homoplasy because the same chromosomal patterns are unlikely to occur independently in different phylogenetic lineages. Rokas and Holland (2000) stated when chromosomal rearrangements are shared by different species, such rearrangements represent "rare genomic changes" indicative of phylogenetic relatedness and common ancestry.

Swigonova et al. (2004) described a hypothesis in which the ancient progenitor genome of genus *Sorghum* had a basic number of five (x=5) and diverged from the

maize genome approximately 11 mya. They proposed a scenario in which divergence and eventually a polyploidization event gave rise to the *x*=10 *Sorghum* species. Consequently, the two lineages would theoretically share the same genes. Genes in plants can evolve at very different rates, perhaps due to difference in selective pressures or different local rates of mutation (Wolfe et al. 1987; 1989 *a*, *b*; Gaut 1997; Zhang et al. 2001). For example, retrotransposons and gene silencing events could have taken place, essentially eradicating most syntenic genes originally shared in most *x*=5 species.

Polyploidization and subsequent events have greatly influenced plant speciation, thus making polyploidization an important evolutionary force (Soltis and Soltis 1999). Extensive and rapid genome restructuring can occur after polyploidization (Soltis and Soltis 1999) which can be mediated by retrotransposons (Fedoroff 2000; Lonnig and Saedler 2002). Transposable elements (TEs) might facilitate rapid genome restructuring after polyploidization (Soltis and Soltis 1999). The end results could be higher genomic restructuring in polyploids compared with their diploid progenitors.

The cytogenomic findings reported here did not suffice to determine if the gross structural rearrangements found by Kim et al. (unpublished) also apply specifically or in kind to other *Sorghum* species. The cytogenomic data clearly indicated that more extensive phylogenetic analyses need to be carried out to resolve this phylogetically-convoluted genus. Relative to the existing phylogenetic interpretations and methodologies used for this experiment, the BAC-FISH results indicate that *S. angustum* shares a closer genetic relationship to *S. bicolor* than previously envisioned for the large-genome *x*=5 lineage, and a more distant relationship from *S. brachypodum, S. versicolor*

and presumably some of the other *x*=5 *Sorghum* species. Is there an unresolved dichotomy within the *x*=5 lineage? If so, it will be of interest to determine how it arose.

Polyploidization is accepted as a major force in plant genome evolution, as is the accumulation of repetitive elements (retrotransposons and transposons). In contrast, mechanisms for large-scale genome reduction are yet to be clearly defined and substantiated. It will thus be of great interest to determine the roles of repetitive elements in *Sorghum* genome evolution-- genome expansion at *x*=5 levels and/or diploidization and/or genome reduction at *n*=10 levels. Analogous processes implicitly permeate angiosperm evolution, as large differences in genome size exist (Soltis et al. 2002; Specht and Bartlett 2009). Given large variations within a relatively narrow phylogenetic range, *Sorghum* may be an ideal model for investigating these questions. It is conceivable that such investigations will contribute significantly to the understanding of how repetitive elements drive evolution up and down in plant DNA content and/or chromosome number.

CHAPTER III

PHYSICAL MAPPING AND CHROMOSOMAL LOCALIZATION OF TELOMERIC DNA IN THE GENUS *SORGHUM*

Introduction

Major structural chromosomal rearrangements including deletions, duplications, translocations and inversions are often associated with cytogenetically detectable heterochromatic regions composed of repetitive DNA, and frequently appear in heterochromatin-euchromatin borders (Robertson et al. 2007). The termini of eukaryotic chromosomes form a unique chromatin domain that comprises the telomere and adjacent subtelomeric region (Zellinger and Riha 2007). The primary roles of telomeres are to counteract incomplete replication of linear chromosomes and to protect chromosome termini from deleterious DNA repair activities. Telomeres also serve as protective caps that prevent end-to-end fusions, recombination and degradation of chromosome ends (Linger and Cech 1998; McEachern et al. 2000; McKnight and Shippen 2004; Mefford and Trask 2002; Mewborn et al. 2005).

Plant telomeric DNA sequence was first described in *Arabidopsis thaliana* by Richards and Ausubel (1998). They sequenced clones enriched in telomeric DNA and revealed that the *Arabidopsis* telomeres were composed primarily of tandemly repeated blocks of 5´-TTTAGGG-3´, which is similar to the modal vertebrate telomeric standard, TTAGGG. The *Arabidopsis*-type telomeric repeat sequence (A-type TRS) is considered

to be the archetype of plant telomeric sequences (Zellinger and Rhia 2007). It is widely conserved among the majority of plant species ranging from the unicellular green alga *Chlorella vulgaris* (Higashiyama et al. 1995) to monocot crops such as maize and barley (Burr et al. 1992; Kilian et al. 1995). However, notable exceptions where telomere sequence variation were discovered as the lower alga *Chlyamdomonas reinhardtii*, which contains TTTTAGGG repeats (Petracek et al. 1990) and a group of plants within the monocot order *Asparageles*, whose chromosome termini contain vertebrate-type TTAGGG repeats (Puizina et al. 2003; Sykorova et al. 2003; Weiss and Scherthan 2002; Weiss-Schneeweiss et al. 2004). Sykorova et al. (2003) also reported that the onion (*Allium cepa*) is devoid of any known telomeric sequence and identity of its telomeric DNA sequence remains unknown.

Various telomere-like repeats have also been found at interstitial chromosomal regions in diverse species, including higher plants and animals (Richards and Ausubel 1988; Meyne et al. 1990; Fuchs et al. 1995; Gortner et al. 1998; Hizume et al. 1998; Uchida et al. 2002*a*, *b*). These interstitial-telomeric repeats, ITRs, are thought to have originated from ancestral chromosomal rearrangements, such as telomere-telomere chromosomal fusions (Meyne et al. 1990; Ijdo et al. 1991). Cytological studies suggest that ITRs are fragile sites that are involved in chromosomal aberrations (Bouffler 1998). The presence of ITRs has been correlated with general genomic instability, including the creation of recombination hotspots, chromosomal breakage and subsequent telomere-mediated healing (Hastie et al. 1989; Biessmann et al. 1994).

Reports to date indicate that the frequency of ITRs tends to be lower in plants than in mammals. Studies from chromosomes of tissue-cultured cells of *Sigmidon mascotensis* (Meyne et al. 1990), chromosomes of canine tumor cells (Reimann et al. 1994), human (Ijdo et al. 1991*a*), Indian muntjac (Lee et al. 1993), and *Gonatodes taniae* chromosomes (Schubert 1992) verified that ITRs were remnants of mammalian chromosome fusions. Interstitial telomeric repeats (ITRs) were observed in nine plants species by Fuchs (1995) and in *A. thaliana* by Uchida et al. (2002*a*, *b*), who suggested that these ITRs probably originated from telomere-mediated chromosomal rearrangements.

Little is known about the localization of A-type TRS in the genus *Sorghum*. Paterson et al. (2009) reported the sorghum telomere signature sequence as $(AAACCCT)_n$. They noted that chromosomes 1, 4, 5, 7 and 10 share evidence of having localization of telomeric DNA on both chromosomal arms, whereas chromosomes 2, 3, 6, 8 and 9 include localization of telomere DNA on only one chromosomal arm.

The objective of this study was to examine and compare the locations, organization and distribution of *Arabidopsis*-type telomeric repeats (A-type TRS) in the x=5 and x=10 lineages of *Sorghum*.

Materials and Methods

Plant Materials

The *Sorghum* species used in this research were propagated from seeds and grown in a glasshouse. Accession numbers, herbarium voucher numbers, life forms and origins are listed in Table 1.

Somatic Chromosome Preparation

Somatic metaphase chromosome spreads were prepared following techniques a derived from the methods of Kim et al. (2002) and Andras et al. (1999).

Probe Labeling and *In situ* Hybridization

Purified A-type TRS $(TTTAGGG)_n$ (kindly prepared for by T. McKnight and Anjali Misra) was labeled with biotin-dUTP and digoxygenin-11-dUTP by the BioNick Labeling system (Roche Molecular Biochemicals, Indianapolis, Indiana, USA). *In situ* hybridization techniques were performed following modifications of the protocol described in Jewell and Islam-Faridi (1994).

Microscopy

Digital images were recorded from an AxioImager Z-1 Epi-fluorescence microscope with suitable filter sets (Chroma Technology, USA), using a COHU High Performance CCD Camera and the Metafer v4 MetaSystems Finder digital image system (MetaSystem Inc., USA) (Thanks to Dr. Nurul Faridi for kindly allowing us to use his imaging system while our equipment was being repaired). Images were processed with Ikaros and ISIS v5.1 and then further processed with Adobe Photoshop CS v8 (Adobe Systems, USA).

Results

In situ Hybridization of A-type TRS

The A-type telomeric repeat sequence (TRS) was detected in all *Sorghum* species tested in this study, for which results are summarized in Table 5. Below, the results of each species are detailed.

S. bicolor

In *S. bicolor*, hybridization of A-type TRS was localized to the terminal regions of all somatic metaphase chromosomes, and no interstitial hybridization of A-type TRS was detected (Fig. 12). Though signal strengths varied and were not visible in all cells, all chromosomes displayed A-type TRS signals at both termini.

S. angustum

Hybridization of A-type TRS was localized to the termini or sub-termini regions of all *S. angustum* chromosomes (Fig. 13). Four pairs of chromosomes displayed signals on both termini, whereas one pair displayed signals only on one terminal end of the chromosome. No interstitial hybridization of A-type TRS was detected.

Table 5. Summary of A-type TRS (*Arabidopsis*-type telomeric repeat sequence) hybridization in *Sorghum* species.

Species	Chromosome number (haploid)	Terminal Signals	Non-terminal (interstitial) signals
S. bicolor	*n*=10	+[a]	-
S. angustum	*n*=5	+[a]	-
S. intrans	*n*=5	+[a]	-
S. versicolor	*n*=5	+[a]	-
S. brachypodum	*n*=5	-[b]	+[c]

[a] Terminal signals were observed on both arms of most chromosomes

[b] Faint signals were observed in a few cells on one chromosome pair at maximum exposure (~4 sec)

[c] Interstitial signals were observed on one chromosome pair

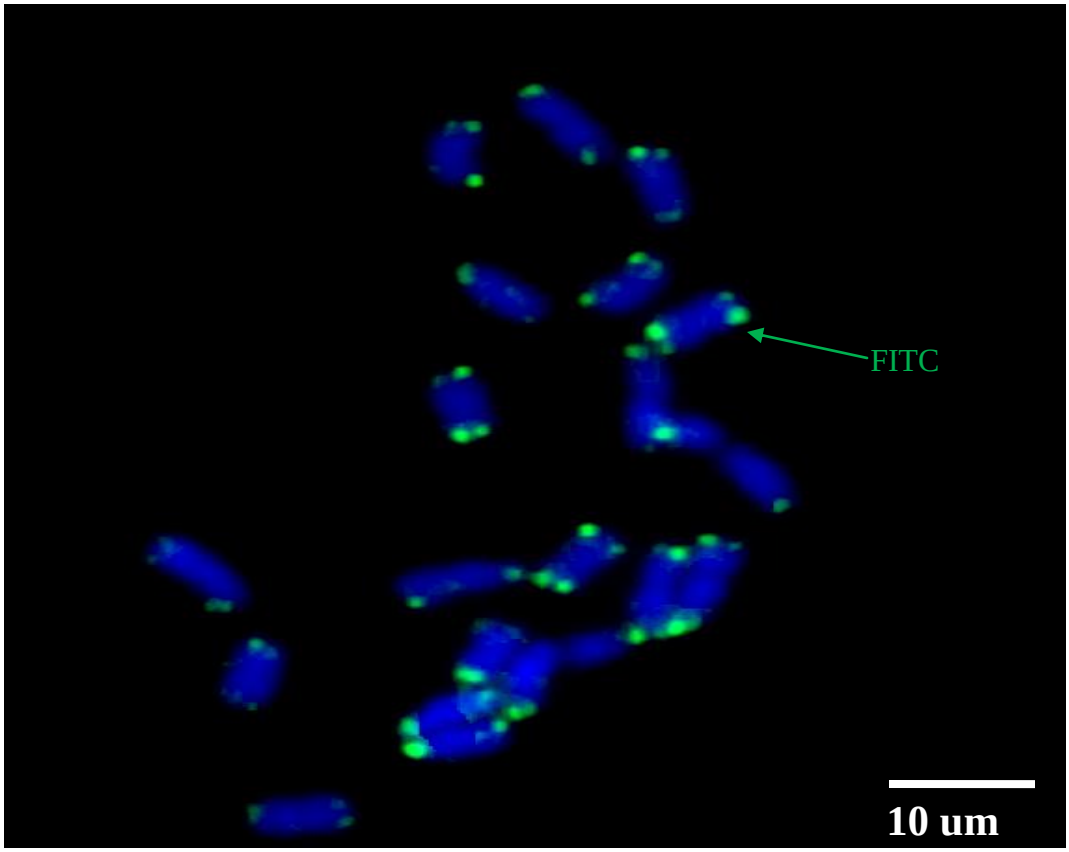


Fig. 12. *Arabidopsis*-type telomeric repeat sequence (A-type TRS) FISH signals hybridized onto both telomeres of all somatic metaphase *S. bicolor* chromosomes. A-type TRS was labeled with digoxygenin and detected with anti-digoxygenin-FITC conjugate (green).

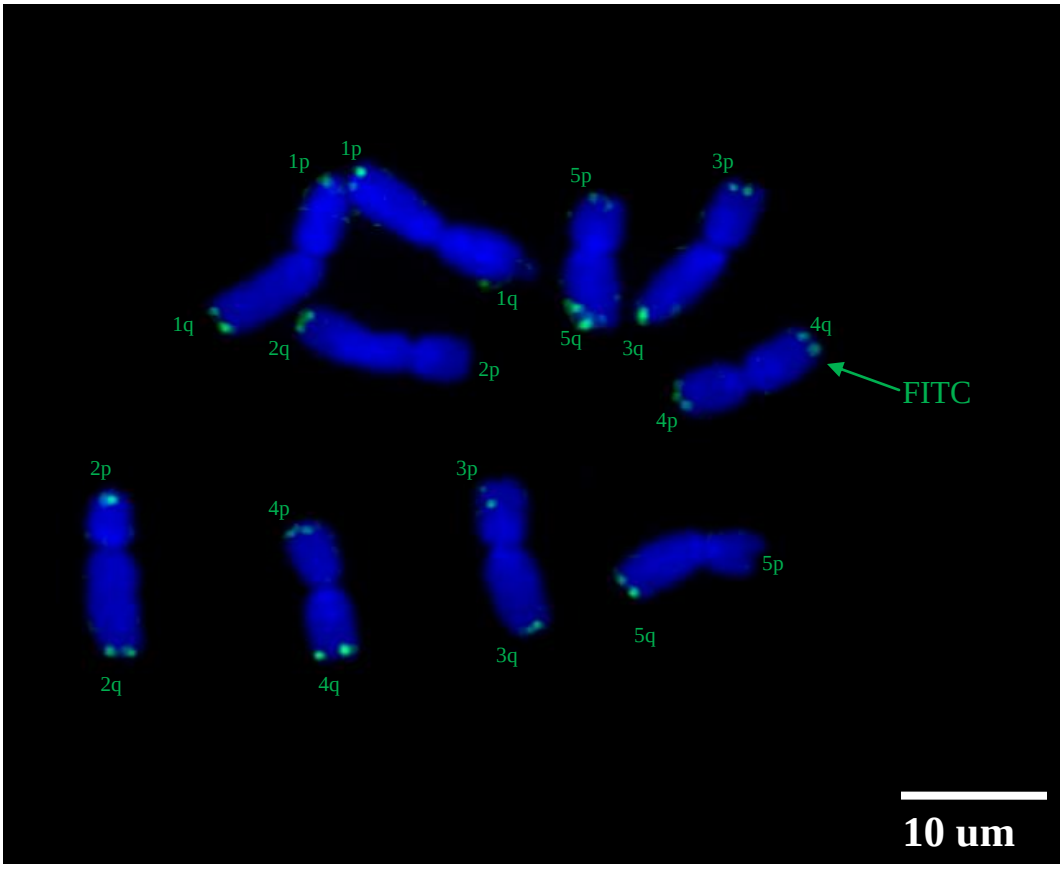


Fig. 13. *Arabidopsis*-type telomeric repeat sequence (A-type TRS) FISH signals hybridized onto the somatic metaphase *S. angustum* chromosomes. Signal is readily observed at or near 17 or the 20 telomeres, and for each of the remaining 3, the homologous telomere has observable signal. Homologous chromosomes are numbered according to size and karyotypic features. Chromosomes A-type TRS was labeled with digoxygenin and detected with anti-digoxygenin-FITC conjugate (green).

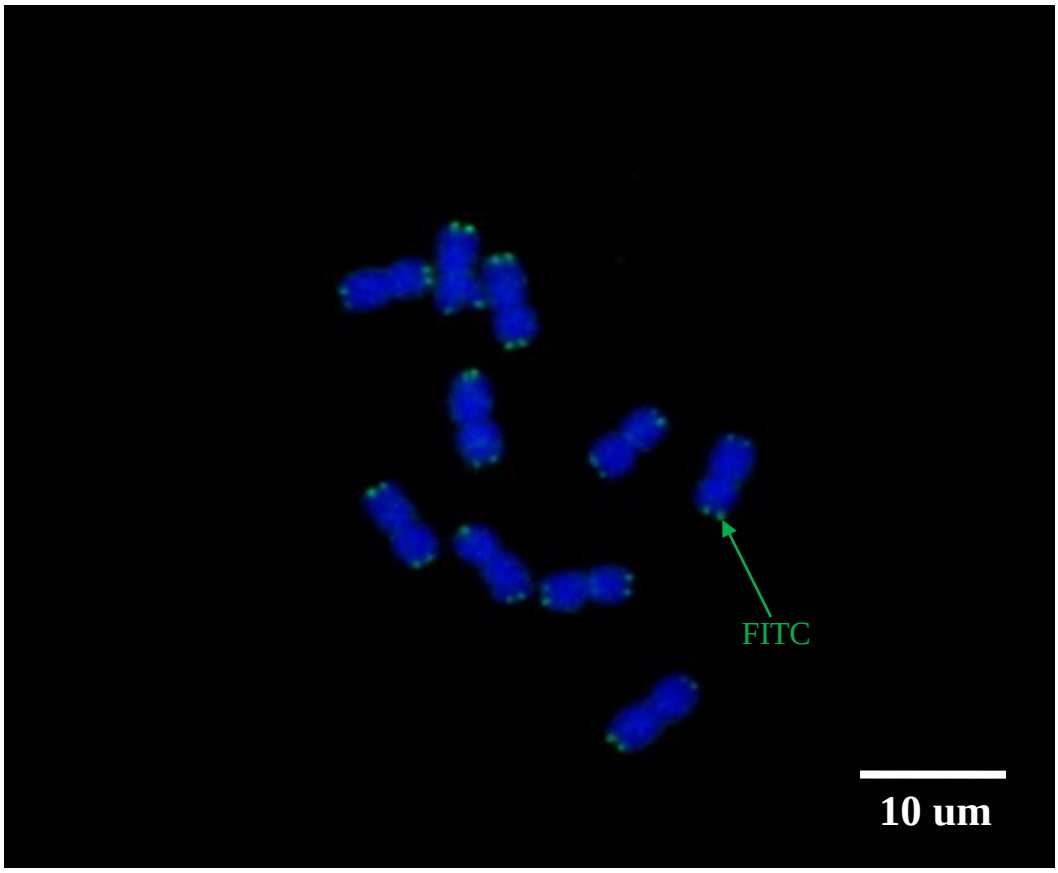


Fig. 14. *Arabidopsis*-type telomeric repeat sequence (A-type TRS) FISH signals hybridized on somatic metaphase *S. versicolor* chromosomes. A-type TRS was labeled with digoxygenin and detected with anti-digoxygenin-FITC conjugate (green).

S. versicolor

Hybridization of A-type TRS was localized to the termini or sub-termini regions of all chromosomes of *S.versicolor* (Fig. 14). All five pairs of chromosomes displayed signals on both termini regions. No interstitial hybridization of A-type TRS was detected.

S. intrans

Hybridization of A-type TRS was localized to the termini or sub-termini regions of all chromosomes of *S. intrans* (Fig. 15). Four pairs of chromosomes exhibited signals on both termini regions (one homologous chromosome of one pair lacked both termini signal detection) whereas, one pair of chromosomes exhibited signals only on one terminal end. No interstitial hybridization of A-type TRS was detected.

S. brachypodum

The hybridization pattern of A-type TRS was quite disparate in respect to other *Sorghum* species tested in this study (Fig. 16). No consistent hybridization of A-type TRS was detected, even when exposure for the reporting fluorophore was maximized (~ 4 sec) (image not shown).

S. brachypodum was the only *Sorghum* species in which interstitial hybridization of A-type TRS was detected (Fig. 16). The A-type TRS hybridized intercalary to only one chromosome pair. This unique hybridization pattern indicates a possible loss of canonical A-type TRS in most chromosomes and a translocation of A-type TRS to the intercalary region of one chromosome pair.

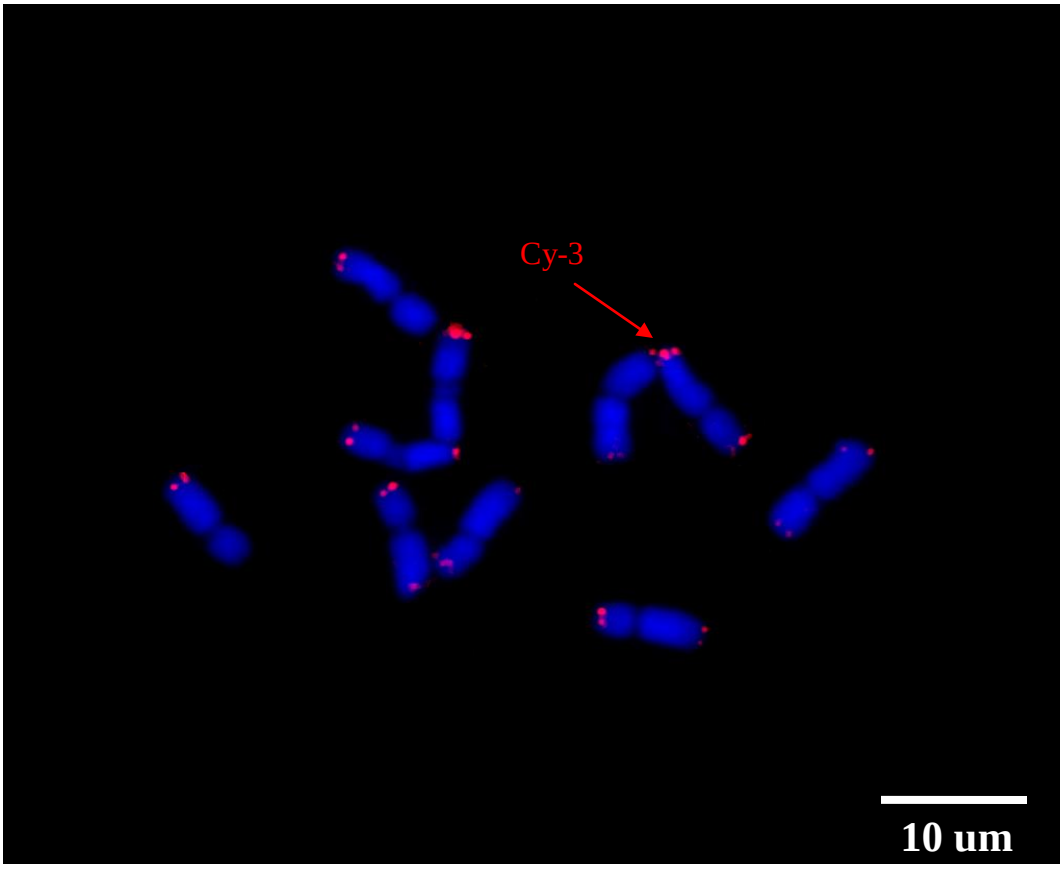


Fig. 15. *Arabidopsis*-type telomeric repeat sequence (A-type TRS) FISH signals hybridized on somatic metaphase *S. intrans* chromosomes. A-type TRS was labeled with biotin and detected with streptavidin-Cy3 conjugate (red).

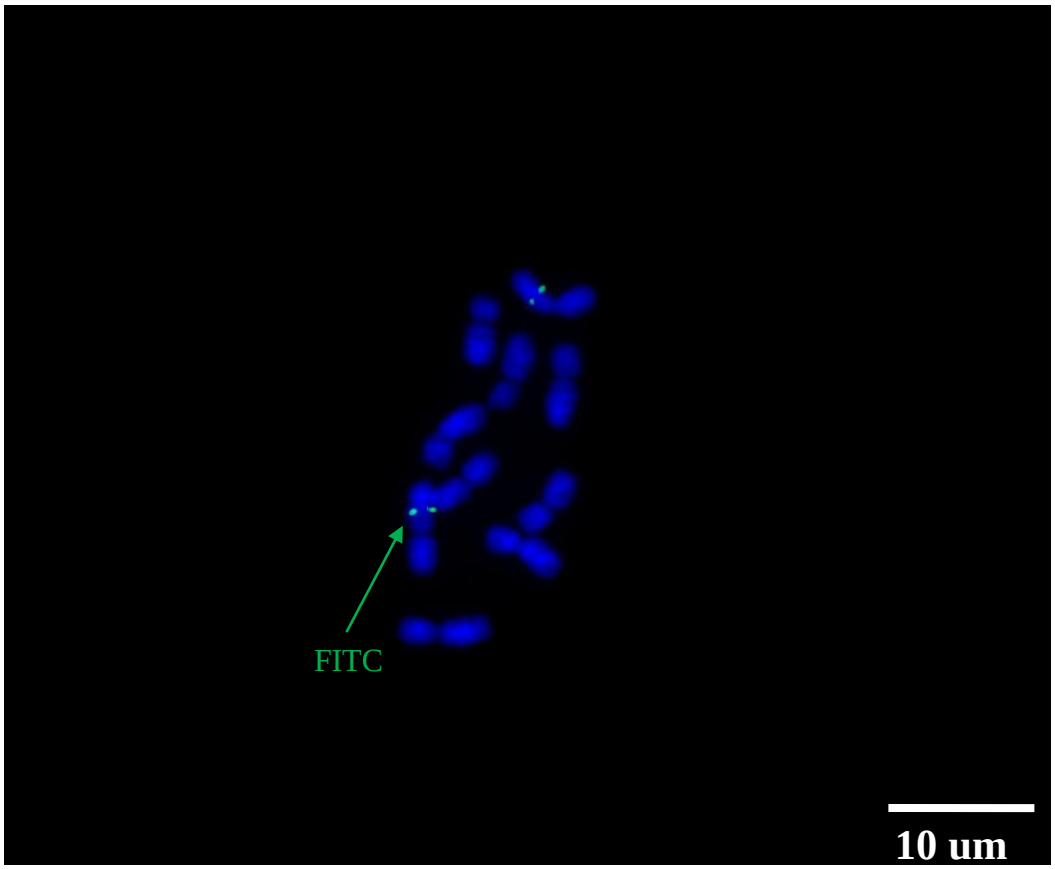


Fig. 16. *Arabidopsis*-type telomeric repeat sequence (A-type TRS) FISH signals hybridized on somatic metaphase *S. brachypodum* chromosomes. A-type TRS was labeled with biotin and detected with anti-digoxygenin-FITC conjugate (green).

Discussion

Fluorescent *in situ* hybridization (FISH) of the A-type telomeric repeat sequence (TRS) resulted in signals at the ends of all chromosomes in the genomes of *Sorghum bicolor*, *S. angustum*, and *S. intrans*, as are found in most plants. The FISH signals were visible on one or both chromatids, usually on both homologs of each chromosome pair, and located at or very near the ends the chromosomes. Minor variations in FISH signal position are common, and so isolated instances where the signals were slightly subterminal were simply attributed to that expected variation. In contrast, an unorthodox distribution of FISH signals for TRS was observed on the *x*=5 genome of *S. brachypodum*. In that genome, no telomeric sites were detected, and a solitary interstitial site was observed. The findings suggest that *Sorghum* genome evolution is more complex than anticipated, even among *x*=5 genomes.

The hybridization of A-type TRS to the ends of all *S. bicolor* (*x*=10) chromosomes is highly discordant with the recent report that the signature telomere sequence of sorghum is $(AAACCCT)_n$ (Paterson et al. 2009). Paterson et al. (2009) further reported that chromosomes 1, 4, 5, 7, and 10 have both telomeres attached and chromosomes 2, 3, 6, 8 and 9 having only one telomere in the assembly. Based on integrated sequencing data, they deduced that $(AAACCCT)_n$ occurs near the termini of multiple chromosomes, but seemingly not all. However, results using *in situ* hybidization revealed the A-type TRS, $(TTTAGGG)_n$, occurs at ends of all sorghum chromosomes. Subsequent to the FISH, *in silico* investigations of the A-type TRS using

integrated sequence data corroborated the Paterson et al. (2009) finding. The two telomeric sequences are located on complementary DNA strands, but with staggered repeating units. Given its widespread role as the archetype plant telomeric repeat and subsequent *in situ* localization, it seems most likely that A-type TRS is the principal canonical telomeric sequence of sorghum.

One major impetus for conducting this study was the possibility of obtaining evidence that could strongly differentiate between the two diametrically opposed hypothesis about the origins of x=5 vs. x=10 *Sorghum* genomes. One of these hypotheses supposes that an ancestral x=10 genome was reduced in number to x=5, e.g., by telomere-telomere fusions between non-homologous chromosomes, *sensu* Meyne et al. (1990). Under such a hypothesis, remnant A-type TRS might have persisted, in which case one would expect it possible to see interstitial hybridization of A-type TRS in *S. angustum*. It is thought that interstitial telomeric repeats (ITRs) generally originate from ancestral chromosome rearrangements (Uchida et al. 2002*a*, *b*). Chromosomal rearrangements such as fusion, fissions or inversions plus the conservation of remnant A-type TRS at the site of these rearrangements are thought to account for such a observations. Marrero (1992) suggested that variation present in *Sideritis* could be due to changes in chromosome structure caused by centric fission. Raskina et al. (2008) also reported that the presence of high numbers of acrocentric chromosomes in *S. dendrochahorra* is consistent with Robertsonian fissions. Fuchs et al. (1995) reported interstitial loci of A-type TRS in nine species. Biessmann (1994) reported that ITRs could have developed by numerous scenarios, such as extension of random short

sequence arrays by slippage during replication, attachment via telomerase, or by chromosomal rearrangements such as fusions and inversions.

The lack of intercalary A-type TRS in *S. angustum* does not necessarily prove that there are no ITRs present. Eight ITRs have been reported in the *Arabidopsis* genome and have been grouped into three classes based on the degeneracy of their telomeric repeats (Uchida et al. 2002*a*). Consequently, Uchida et al. (2002*a*, *b*) proposed ITRs probably originated from telomere-mediated chromosomal rearrangements in the *A. thaliana* genome.

Even though the direction of genomic base-number evolution is not known in the genus *Sorghum*, it is obvious that extensive karyotypic rearrangements have occurred in *S. angustum* (Kim et al. unpublished). The presence of such structural rearrangements is an incentive to search for ITRs, but not a guarantee of their presence. The presence of other ITRs besides A-type TRS in the *A. thaliana* genome has demonstrated that other sequences could be present at intercalary sites of chromosomes that have undergone some form of karyotypic rearrangements. Therefore, the results do not preclude that no ITRs are present in *Sorghum* genomes; the results merely have stated that conserved A-type TRS tandem repeats are not present in large numbers at intercalary positions of *S. angustum* (x=5), *S. intrans* (x=5), *S. versicolor* (x=5) and *S. bicolor* (x=10) chromosomes.

In situ hybridization of A-type TRS to *S. brachypodum* revealed a unique hybridization pattern (Fig. 16); there was a lack of termini A-type TRS signal (Table 5), but the presence of an interstitial A-type TRS signal. Telomere lengths vary among

plant species and cell types (Zellinger and Riha 2007). Also, FISH requires high homology and significant target size to be successful. The amount of A-type TRS could be below our FISH resolution threshold; if the telomere clusters are less than 3Mbp, consistent detection would be difficult by routine FISH procedures. Thus, without complementary data, one cannot refute the possibility that small numbers of terminal A-type TRS repeats exist at *S. brachypodum* telomeres.

Fuchs et al. (1995), Ganal et al. (1991), Schwarzacher & Heslop-Harrison (1991), Wang et al. (1991) and Cox et al. (1993) all reported that variation of terminal signals exists not only between species, but even between individual chromosome termini of one metaphase plate. One cause for this phenomenon could be attributed to karyotypic rearrangements within a species. These rearrangements could be responsible for the variation of copy numbers of telomeric sequences within a species' genomic constitution. Karyotypic rearrangements, such as fusions or fissions, could also be deleterious to telomeric sequences. Once conserved A-type TRS could have been lost and replaced by another telomeric sequence or terminal heterochromatin consisting of satellite repeats and transposable elements, which has been proposed for the chromosomal termini of *Allium cepa* (onion) (Pich et al. 1998). This hypothesis is quite intriguing and may be true for *S. brachypodum.*

Results of CCP of *S. brachypodum* utilizing *S. bicolor* BACs from SBI-02 revealed terminal hybridization of one or more conserved repetitive elements on multiple chromosomes (Figs. 6, 7, 8). Further BAC screening to identify which BACs contain this repetitive element plus subsequent sequencing may reveal another telomeric

sequence and substantiate why I observed hardly any terminal hybridization of A-type TRS. If so, the *Sorghum* genus might be a superb model for studying telomeric evolution.

CHAPTER IV

PHYSICAL MAPPING AND CHROMOSOMAL LOCALIZATION OF RIBOSOMAL DNA IN THE GENUS *SORGHUM*

Introduction

The physical organization of a plant chromosome affects its structure and function. Three regions have traditionally been recognized: euchromatin, which is referred to as "active" chromatin and marked by higher rates of transcription, recombination and gene density, heterochromatin, which is characterized by lower gene densities, transcription and recombination rates (Gill et al. 2008), and nucleolus organizing regions, which contains ribosomal RNA gene clusters ready to be transcribed. In plants, centromeres, pericentromeres, telomeres, and accessory chromosomes such as maize B chromosomes are characterized as heterochromatic regions, whereas nucleolus organizing regions (NORs) are generally present near heterochromatic regions (Alfenito and Birchler 1993; Copenhaver et al. 1999; Franz et al 2000; McCombie et al. 2000). An inherent feature of heterochromatin is a complex composition of repetitive DNA of various types, such as tandem and dispersed repeats, transposable elements (TEs) of all types, satellite DNA and even unique sequences (Raskina et al. 2008; Copenhaver et al 1999; Franz et al. 2000; McCombie et al. 2000). The distribution of heterochromatic content is not uniform, -- even within species, the amount of heterochromatin can vary within and between chromosomes (Gill et al. 2008).

In this study, I concentrated on the localization of ribosomal genes (rDNA) and their impact on *Sorghum* genome evolution. According to genomic organization, repeats are tandemly arranged or dispersed sequences (Schmidt and Heslop-Harrison 1998). Tandem repeats include satellite DNA, micro- and mini-satellites, telomeric repeats and ribosomal genes. In higher eukaryotes, ribosomal genes (rDNAs) are organized into two or more separate locations, one or more locations for 45S rDNA (18S-5.8S-26S) clusters, and one or more locations for 5S rDNA clusters. The major rDNA (45S) repeats are usually found at loci involved in nucleolus formation (NOR), and are thereby subject to unique replication, recombination and other phenomena, one consequence of which is a high rate of uniformity among multiple copies within an organism (Schubert 2007). While parts of the rDNA genes can be highly conserved, they are embedded in clustered repeats that include areas that can exhibit high rates of evolution. Their multiplicity renders them highly amenable to isolation, characterization and usage in phylogenetic research (El Twab and Kondo 2006).

In some taxonomic groups, there is wide diversity in the numbers, positions and sizes of 45S clusters (El-Twab and Kondo 2006; Mishima et al. 2002). It is thought their multiplicity and behavior encourages chromosome rearrangements, e.g., humans (Stults et. al 2008). The 5S rDNA repeats involve a conserved transcribed region (~120 bp) and a non-transcribed spacer region (NTS) varying in size and sequence among species (Zhu et al. 2008). The NTS is more rapidly evolving and contains more informative sites to allow for analyses than the conserved transcribed regions (Baker et al. 2000; Baum et al. 2004; Kitamura et al. 2005; Baum and Johnson 2007). The 5S rDNA undergoes

concerted evolution and consequently most species contain a single class of 5S rDNA repeat unit (Nei and Rooney 2005; Besendorfer et al. 2005; Gornung et al. 2007).

Comparative studies of plant repetitive sequences are useful to investigate the evolutionary relationships between plant species (Kamm et al. 1995). *In situ* hybridization is a valuable method for studying the chromosomal distribution of repetitive DNA sequences and to follow evolutionary changes in their physical organization in the genome (Harrison and Heslop-Harrison 1995). Cytological studies have provided evidence that 45S rDNA is located in NOR-bearing chromosomes (Bergey et al. 1989; Sang and Liang 2000; Kondo et al. 1996; Orgaard and Heslop-Harrison 1994; Kondo and Abd El-Twab 2002; Abd El-Twab and Kondo 2003).

The repetitive DNA complex plays an important role in genome evolution (Raskina et al. 2008). Homology among repetitive DNA loci may cause chromosomal rearrangements, which ironically, may cause repetitive DNA changes through mechanisms of concerted evolution (Elder and Turner 1995). I herein report the results of the distribution and characterization of rDNA in select species of the *Sorghum* genus.

Material and Methods

Plant Materials

The *Sorghum* species used in this research were propagated from seeds and grown in a glasshouse. Accession numbers, herbarium voucher numbers, life forms and origins are listed in Table 1.

Somatic Chromosome Preparation

Somatic metaphase chromosome spreads were prepared following a modified technique described in Kim et al. (2002) and Andras et al. (1999).

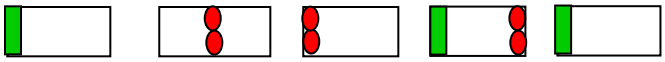
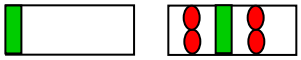
Probe Labeling and *In situ* Hybridization

Purified 45S and 5S rDNA was labeled with biotin-dUTP and digoxygenin-11-dUTP by the BioNick Labeling system (Roche Molecular Biochemicals, Indianapolis, Indiana, USA). *In situ* hybridization techniques were performed following modifications of the protocol described in Jewell and Islam-Faridi (1994).

Microscopy

Digital images were recorded from an AxioImager Z-1 Epi-fluorescence microscope with suitable filter sets (Chroma Technology, USA), using a COHU High Performance CCD Camera and the Metafer v4 MetaSystems Finder digital image system (MetaSystem Inc., USA) (Thanks to Dr. Nurul Faridi for kindly allowing us to use his imaging system while our equipment was being repaired). Images were processed with Ikaros and ISIS v5.1 and then further processed with Adobe Photoshop CS v8 (Adobe Systems, USA).

Table 6. Diagrammatic summary of FISH of rDNA probes to *Sorghum* species.

5S[c]	45S[c]	Species/Ideogram
2	2	***S. bicolor*** 
2	2	***S. angustum*** 
2	2	***S. versicolor*** 
4[d]	4[e]	***S. brachypodum*** 
4	4	***S. intrans*** 

 [a] **Denotes 5S rDNA signal**

 [b] **Denotes 45S rDNA signal**

[c] **Number of sites per diploid complement (2*n*)**

[d] **One interstitial signal was located on one homologous pair; two separate terminal signals were located on two different chromosomes**

[e] **One terminal signal was located on one homologous pair; two separate terminal signals were located on two different chromosomes**

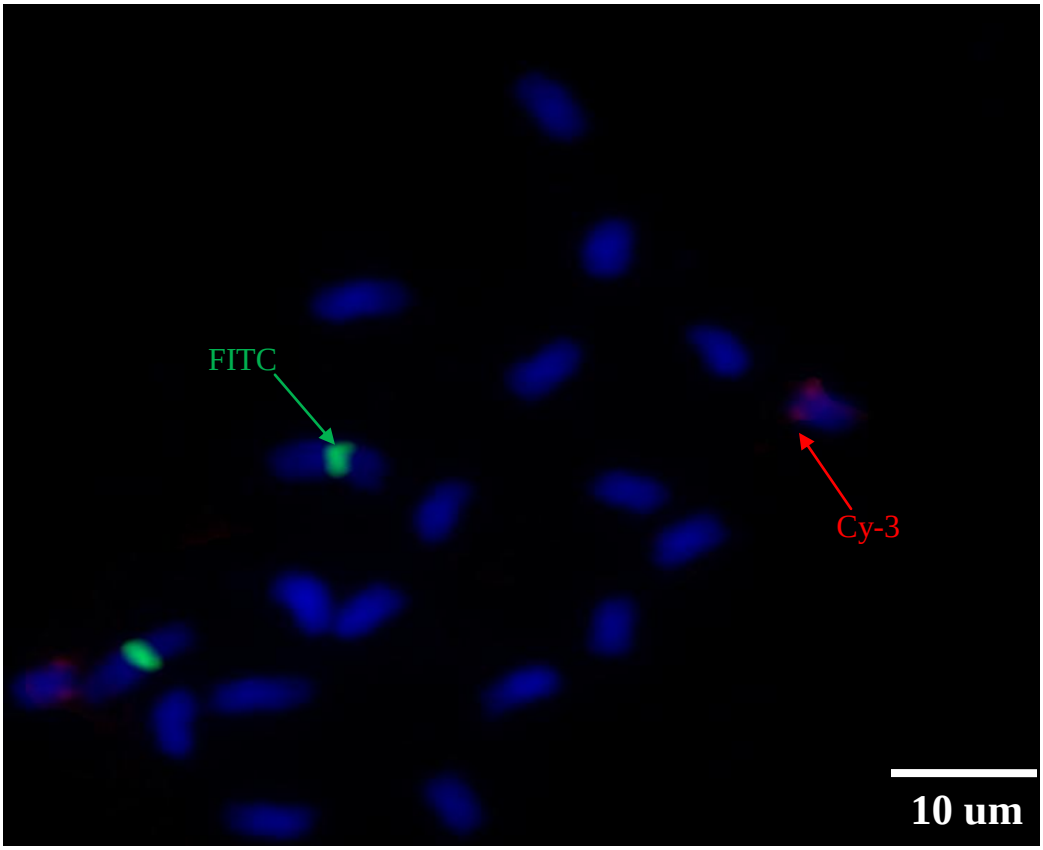


Fig. 17. FISH signals from 45S rDNA (FITC) and 5S rDNA (Cy3) hybridized to somatic metaphase *S. bicolor* chromosomes.

Results

In situ Hybridization of rDNA

FISH results of 45S and 5S rDNA to select species of the *x*=5 and *x*=10 lineages of *Sorghum* revealed different and unique hybridization patterns amongst them (Table 6).

S. bicolor

The hybridization of 45S was previously reported by Sang and Liang (2000). The results of this study concurred with the results reported by Sang and Liang (2000). The 45S rDNA hybridized at an intercalary position of pericentromeric heterochromatin of the longest pair of SBI-01, whereas the hybridization of 5S rDNA yielded one major site at the terminal end of SBI-08 (M. Nurul Islam-Faridi, personal communication) (Fig. 17).

S. angustum

The hybridization pattern of rDNA in *S. angustum* was similar to the pattern of *S. bicolor*, except the 45S rDNA was localized to the terminal region of a chromosome pair and 5S rDNA was localized to the sub-terminal region of another chromosome pair (Fig. 18).

S. versicolor

The hybridization pattern of rDNA yielded results similar to those for *S. angustum*. The 45S rDNA hybridized to one terminal site on one chromosome pair and 5S rDNA hybridized to the sub-terminal region of another chromosome pair (Fig. 19).

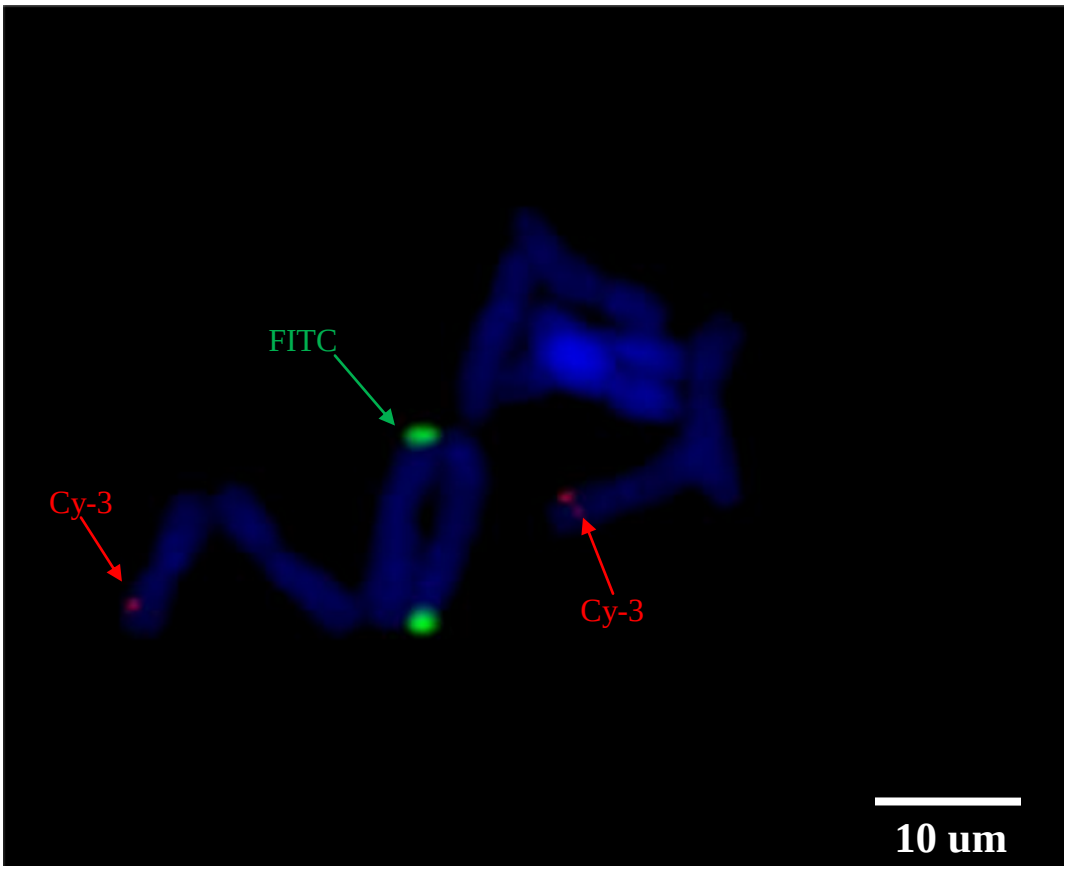


Fig. 18. FISH signals from 45S rDNA (FITC) and 5S rDNA (Cy3) hybridized to somatic metaphase *S. angustum* chromosomes.

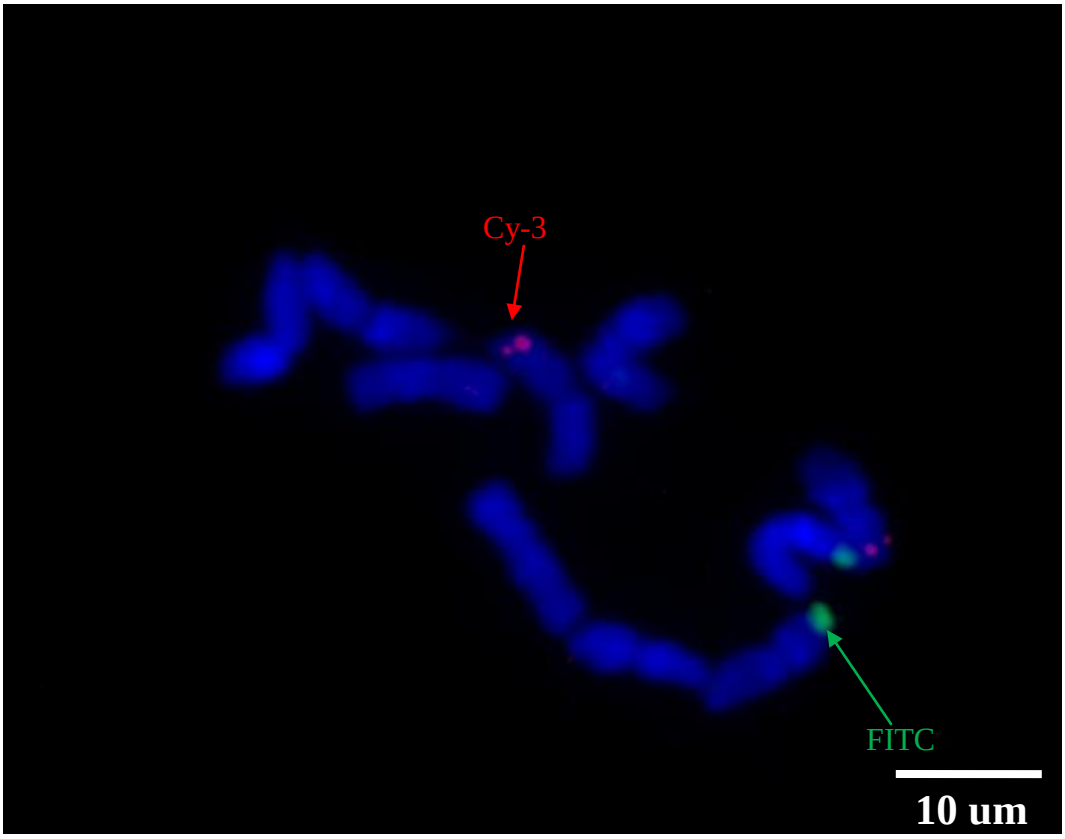


Fig. 19. FISH signals from 45S rDNA (FITC) and 5S rDNA (Cy3) FISH signals hybridized to somatic metaphase *S. versicolor* chromosomes.

S. brachypodum

Hybridization of rDNA yielded intriguing results, displaying the polymorphic nature of rDNA. The 45S rDNA was localized to two terminal sites on two chromosome pairs, whereas 5S rDNA was localized to two different sites on two different chromosome pairs, one terminal site and one intercalary site (Fig. 20).

S. intrans

Hybridization of rDNAs yielded strikingly different hybridization patterns relative to any other *x*=5 species. 45S rDNA was localized to two different sites, one terminal site on one chromosome pair and one intercalary site on another chromosome pair (Fig. 21). 5S rDNA was localized at two sites on the chromosome pair consisting of the intercalary 45S rDNA signal (Fig. 21). Each site was localized at different arms of the chromosomes, and both were proximal to the respective 45S rDNA sites.

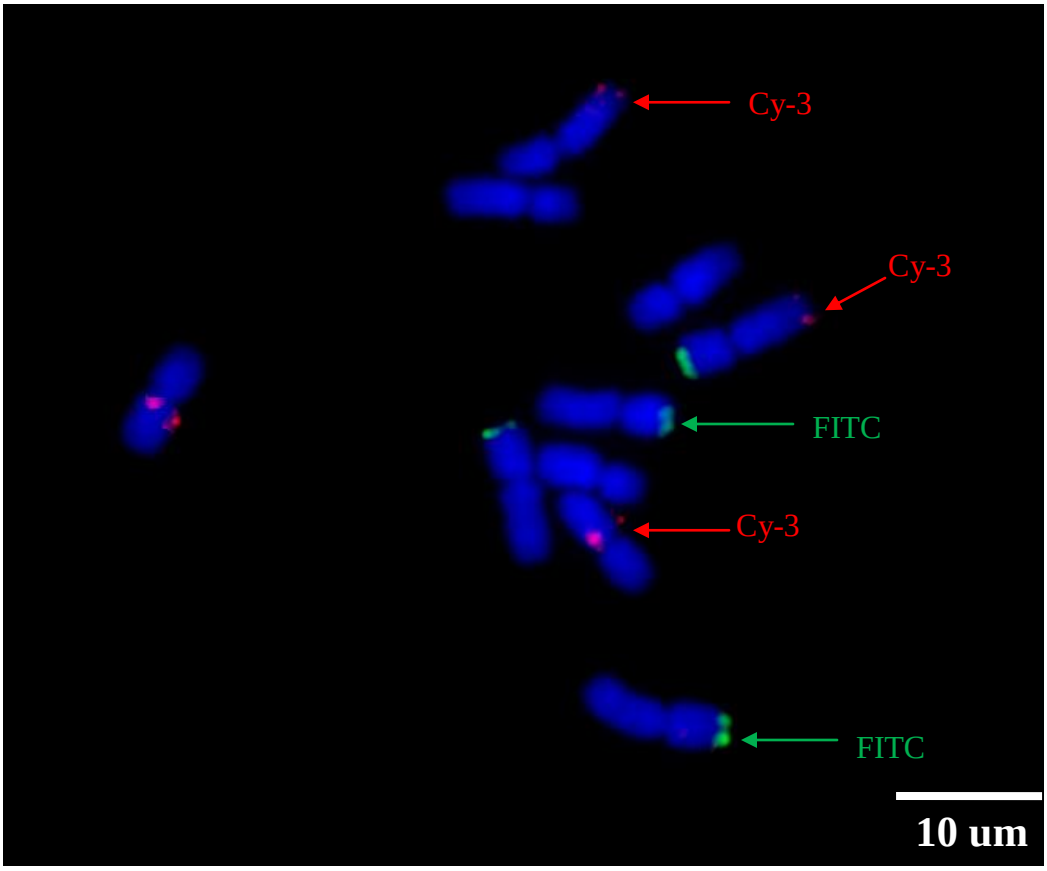


Fig. 20. FISH signals from 45S rDNA (FITC) and 5S rDNA (Cy3) hybridized to somatic metaphase *S. brachypodum* chromosomes.

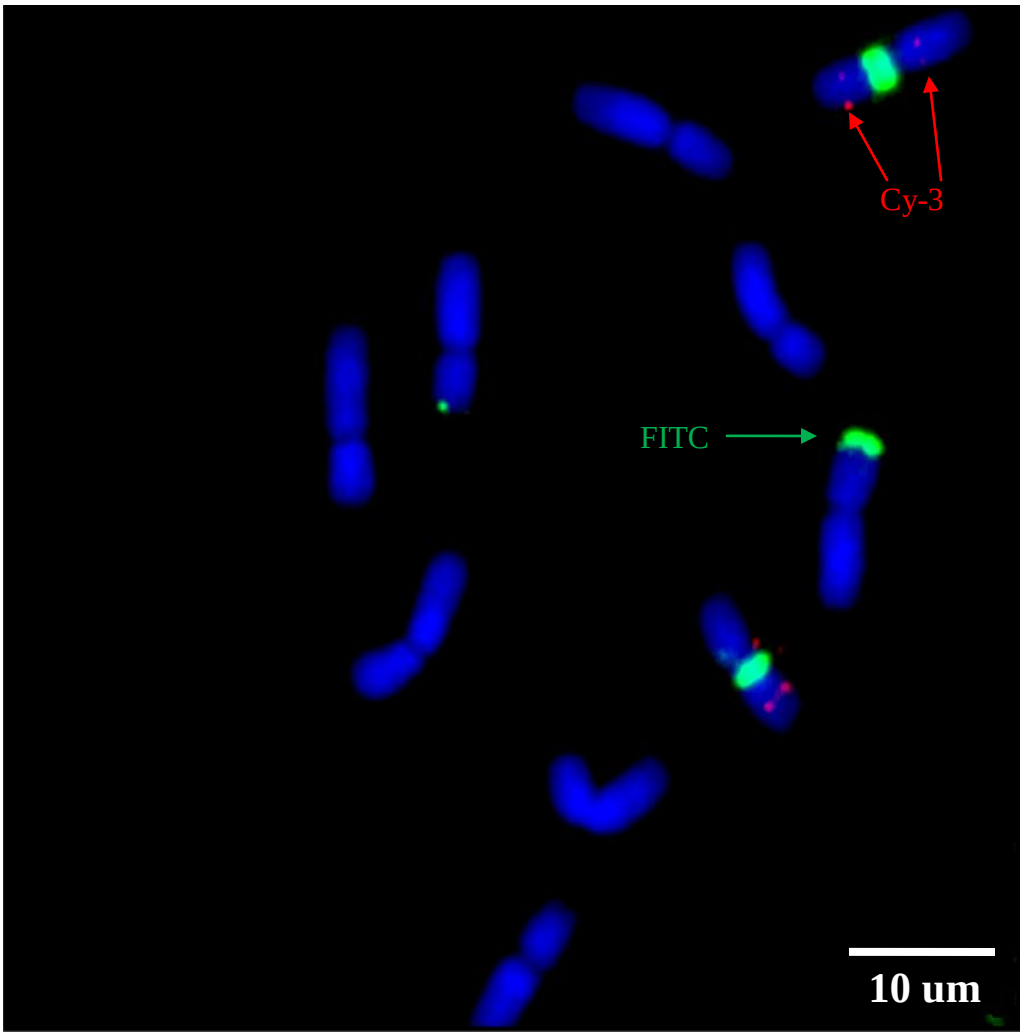


Fig. 21. FISH signals from 45S rDNA (FITC) and 5S rDNA (Cy3) FISH signals hybridized to somatic metaphase *S. intrans* chromosomes.

Discussion

The evolutionary conservation of rDNA sequences enables rDNA FISH to extreme diverse taxa. The resulting chromosomal patterns of FISH signals vary extensively in location and size. The variation in rDNA FISH patterns is so extensive that it can often provide evidence of chromosomal polymorphisms between closely related species. Thus, rDNA FISH constitutes a useful tool for phylogentic resolution. Conversely there is a paradox that compromises the effectiveness of rDNA as a cytogenomic tool to study genomic rearrangements. Eukaryotic rDNA is the most conservative fraction in the eukaryotic genome, undergoing minimal changes over hundreds of millions of years (Raskina et al. 2008). Studies in different plant taxa have described the mobility of rDNA clusters (Abd El-Twab and Kondo 2003; Hanson et al. 1996; Ji et al. 1999). Studies within the genus *Chrysanthemum* (Kondo et al. 1996; Khaung et al. 1997; Honda et al. 1997; Abd El-Twab and Kondo 2003) and *Gossypium* (Hanson et al. 1996) have revealed that the numbers and positions of major rDNA sites in chromosomes vary among species. Hanson et al. (1996) reported that variability of size and number of 18S-26S and 5S, and concluded that the behavior of rDNA loci is dynamic. Expanding on Hanson et al (1996), Ji et al. (1999) reported multiplicity and wide differences in size among the 18S-26S loci of *G. hirsutum*. Abd El-Twab and Kondo (2003) further suggested that variation should have phylogenetic implications, since the most similar rDNA FISH patterns are likely to be in the most closely related taxa.

I evaluated the results of comparative 45S and 5S rDNA FISH in *Sorghum* and identical polymorphisms among species, in terms of localizations and numbers of sites of hybridization (Table 6). *S. bicolor* and *S. intrans* possess intercalary 45S rDNA clusters (Figs. 17, 21), whereas *S. angustum*, *S. versicolor*, *S. brachypodum* and *S. intrans* have distal loci (Figs. 18, 19, 20, 21 respectively). The 5S rDNA loci of *S. bicolor*, *S. versicolor* and *S. angustum* (Figs. 17, 19, 18 respectively) are terminal, one per genome, whereas *S. brachypodum* (Fig. 20) has two loci, one terminal and one interstitial site on two different chromosomes per genome. Even more intriguing is the fact that *S. intrans* has two 5S rDNA clusters within one chromosome pair, flanking an interstitial 45S rDNA site (Fig. 21).

Analysis of NOR distribution and organization in rDNA-carrying chromosomes can be helpful in understanding the phylogeny of plant species. As in *Sideritis* speciation (Raskina et al. 2008), the variability among *Sorghum* species for the number and sizes of terminal rDNA sites seems to support the putative role of chromosomal rearrangements in *Sorghum* x=5 genome evolution. Extensive karyotypic rearrangements were observed in *S. angustum* versus *S. bicolor* (Kim et al. unpublished). The data here document that three of four of the x=5 *Sorghum* species vary in number and sizes of rDNA clusters (Table 6). If these differences arose by rearrangements rather than by locus-specific expansion/contraction (Hanson et al. 1996), they would indicate that the specific pattern of rearrangements observed by Kim et al. (unpublished data) is not applicable to other x=5 genomes. Further, they indicate that *ad hoc* comparisons must be made between *S. bicolor* and each x=5 genome. The collective

results of several such comparisons will be needed to firmly establish if the generalities of *S. bicolor*-*S. angustum* rearrangements parallel those for other *x*=5 genomes, which if so, would indicate a similar underlying evolutionary mechanism or force.

Raskina et al. (2004*a*, *b*; 2008) demonstrated in *Zingeria biebersteiniana* that the occurrences of 45S rDNA cluster in the intercalary regions and traces of telomeric sequences inside the 45S rDNA cluster are strong indications of Robertsonian rearrangements. Results indicated that *S. bicolor* (*x*=10) and *S. intrans* (*x*=5), the only two species with intercalary 45S rDNA sites, have no traces of A-type TRS within them. The evidence of structural karyotypic rearrangements in *S. angustum* trumps these contradictory statements and devalues the use of rDNA as an evolutionary tool on a chromosomal level.

By examining the results of comparative rDNA-FISH based solely on their localization pattern, we observed two patterns of synteny. The first type is localization of 45S and 5S rDNA sites on different chromosomes and the second type is localization of 45S and 5S rDNA sites on the same chromosome. Non-synteny was detected in *S. bicolor* (*x*=10) (Fig. 16) and *x*=5 species such as *S. angustum* and *S. versicolor* (Figs. 17, 18), all three of which have only one large 45S cluster and one large 5S cluster. Both S. *intrans* and *S. brachypodum* have more complex rDNA karyotypes and seem to be heterozygous for different translocations or rDNA locus polymorphisms

The pattern of differentiation among species based on the distribution of 5S rDNA in the genus *Sorghum* was similar to that base on 45S rDNA (Table 6). The FISH data indicate that there is one major 5S rDNA cluster per genome in *S. bicolor*, *S.*

versicolor and *S. angustum*, all terminal or sub-terminal (Figs. 16, 18, 17 respectively). The other two *x*=5 species, *S. brachypodum* and S. *intrans* were quite distinct. *S. brachypodum* had four major 5S rDNA sites, one intercalary, and two terminal sites of 5S hybridization (Fig. 20). The latter two 5S rDNA sites were at similar terminal positions of two chromosomes, one containing a syntenic 45S rDNA site at the opposing end and one that lacks 45S rDNA. These results clearly demonstrate polymorphism for 5S rDNA clusters in the genus *Sorghum*. The occurrence of multiple numbers of 5S sites and 45S sites in *S. brachypodum* and *S. intrans* distinguishes them from other *Sorghum* species tested.

The evolutionary dynamics of the rDNA site number remain convoluted. The differences we reported in the genus *Sorghum* indicate that the evolutionary dynamics of *Sorghum* rDNA sites are complex. The polymorphisms in rDNA distribution in the genus *Sorghum* may have future phylogenetic implications since the closeness of taxa may be correlated to the similarity of their rDNA FISH patterns (Hizume et al. 2002; Liu et al. 2003). Variations in rDNA FISH patterns among angiosperms of the same ploidy level have been attributed to chromosomal rearrangements, transpositions events and gene silencing (Moscone et al. 1999; de Melo and Guerra 2003; Marcon et al. 2005). Many hypotheses and mechanisms have been considered to discern the effectiveness of rDNA as a tool for evolutionary studies. My data, which describes polymorphisms of 45S and 5S rDNA in the genus *Sorghum*, may not be as informative on the chromosomal level as it could be on the sequence level. Future analyses, due to the highly polymorphic nature of rDNA, must be performed to prove its suitability as a key resource for genomic

and phylogenetic analyses in the *Sorghum* genus. Dillon et al. (2001; 2004; 2007) utilized only the ITS1 fraction of rDNA to infer phylogenetic relationships. The utilization of more than one rDNA gene in phylogenetic analyses may further resolve the convoluted *Sorghum* genus.

CHAPTER V

CONCLUSIONS

Genome research in the *Sorghum* genus has not progressed as rapidly as it has in model plants such as *Oryza* (Goff et al. 2002; Bowers et al. 2005; Chen et al. 2005) and *Arabidopsis* (Copenhaver et al. 1998; *Arabidopsis* Genome Initiative 2000; Lysak et al. 2003), but the pace is quickening (Paterson et al. 2009). In this study, comparisons of five *Sorghum* species in two different lineages using different cytological markers have given us a "snapshot" view of genome evolution in the *Sorghum* genus. The results of each specific study, albeit limited, allowed for the integration of different evolutionary tools to preliminarily discern evolutionary relationships shared among the species of the *Sorghum* genus.

While FISH made it clearly evident that extensive structural karyotypic rearrangements exist between *S. angustum* and *S. bicolor* BACs (Kim et al. unpublished), the results of my FISH studies indicate that the specific homology relationships between *S. bicolor* and *S. angustum* do not extend to *S. intrans* or *S. brachypodum*. The FISH efforts on *S. versicolor* did not differentiate it from *S. angustum*, except that BAC-FISH did not yield signal on *S. versicolor*. They underscore the diversity of the *x*=5 *Sorghum* taxa, and indicate that relatedness of most species that form the *x*=5 lineage have not been completely described and resolved. If the BAC-FISH results accurately reflect relative homology, they indicate that *S. angustum* shares a closer relationship to *S. bicolor* than previously described. Nonetheless, I conclude

that *Sorghum* phylogenetic relationships cannot be accurately resolved with existing data.

The distribution of repetitive DNA (telomeric DNA and rDNA) in the genus *Sorghum* was highly informative and enabled us to compare A-type TRS, telomeric and rDNA among select *Sorghum* species. *In situ* hybridization of A-type TRS did not elucidate any mechanisms of *Sorghum* genome evolution, but it revealed an intercalary A-type TRS locus, and loss or reduction of canonical A-type TRS at *S. brachypodum* telomeres. If confirmed, the findings suggest *S. brachypodum* and the genus *Sorghum* telomeric behavior might be developed as a telomere evolution model.

In situ hybridization of rDNA provided us with valuable information at the chromosomal level. The overall organization and physical distribution of specific 45S and 5S rDNA clusters in each species was revealed. The variations in number and locations of rDNA sites among x=5 genomes suggests that the specific rearrangements existing today between *S. bicolor* and *S. angustum* probably do not extend to *S. intrans* or *S. brachypodum.* This suggestion is tempered by evidence that variability in rDNA can be extensive, such that changes in rDNA position and number need not involve structural rearrangements within and between chromosomes.

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