THE USE OF CEN38 IN ASSESSING EVOLUTIONARY RELATIONSHIPS IN THE GENUS *SORGHUM*

A Thesis

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

MASTER OF SCIENCE

August 2005

Major Subject: Plant Breeding

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Approved by:

Chair of Committee,	H. James Price
Committee Members,	David M. Stelly
	Hongbin Zhang
	Patricia Klein
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ABSTRACT

The Use of CEN38 in Assessing Evolutionary Relationships in the Genus *Sorghum*.

(August 2005)

Jason Correnth Anderson, B.S., Southern University

Chair of Advisory Committee: Dr. H. James Price

A DNA sequence-based phylogenetic tree (Dillon *et al*., 2004) places the species of the genus *Sorghum* into two sister lineages, one with $x = 5$ and the other with $x = 10$ as a basic chromosome number. It has not been resolved whether or not these lineages are monophyletic or polyphyletic. A repetitive sequence, CEN38, found only in *Sorghum* and sugarcane, was used to assess evolutionary relationships among *Sorghum* species. The objectives of this research were to determine the taxonomic distribution of CEN38, its chromosomal position(s), and its organization in DNA. CEN38 was detected by filter hybridization to be present in the DNA of 16 of 21 *Sorghum* species analyzed, ranging from 15 to ~21,000 copies. It was detected by fluorescence *in situ* hybridization (FISH) only in chromosomes of species of the section *Eu-sorghum*, where it had a pericentromeric distribution. The low copy number and/or chromosomal distribution of CEN38 in other *Sorghum* species apparently does not allow for its detection by FISH. Analysis of restriction enzyme digested DNA with homology to CEN38 and of fragments amplified by PCR using primers selected to amplify *S. bicolor* CEN38 sequences showed that *S. laxiflorum* and *S. macrospermum* have tandemly arranged CEN38 sequences as is found in *S. bicolor*. This supports the close evolutionary affinity

of the species in the $x = 10$ lineage. In the $x = 5$ lineage, DNA of 11 of 16 species analyzed hybridized with CEN38 by filter hybridization. In *S. versicolor*, large DNA fragments (4.36 kb to 23 kb) generated by digestion with restriction enzymes hybridized to CEN38. Since a ladder of smaller fragments was not detected, CEN38 may have been inserted into a transposable element in this species and dispersed throughout the genome. Among species of the $x = 5$ lineage, PCR using primers for *S. bicolor* CEN38 amplified only DNA fragments from *S. timorense* and these formed a ladder based on a ~125 bp repeat. Since hybridization of the CEN38 sequence to DNA of *S. timorense* was not detected by filter hybridizations, these sequences apparently are not similar to CEN38. Cloning and sequencing of DNA from species of the $x = 5$ lineage that hybridizes to CEN38 are needed to determine whether or not they are in the CEN38 family. A monophyletic or polyphyletic origin of the $x = 5$ and $x = 10$ lineages was not resolved.

DEDICATION

This thesis is dedicated to my Lord and Savior Jesus Christ for allowing me to do His will. I also dedicate this to my loving family and friends for their encouragement and support.

ACKNOWLEDGMENTS

I would like to acknowledge Dr. H. James Price for his guidance, assistance and support. A special thanks to Dr. Wayne Smith for welcoming me into the Department of Soil and Crop Sciences. I would also like to thank my committee members, Dr. David Stelly, Dr. Hongbin Zhang and Dr. Patricia Klein for their wisdom and support. Finally, I would like to thank all the members of the laboratories of Drs. Price, Stelly, Zhang and Klein for welcoming me into their laboratories.

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INTRODUCTION

Background

Sorghum [*Sorghum bicolor* (L.) Moench] is the fifth major cereal crop in the world. Cultivated *Sorghum bicolor* constitutes as a major economical crop in the agriculture community of many countries in the world (Garber, 1950). Sorghum is also an intregal 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. In Africa, the straw of traditional tall sorghums is used to make palisades in villages or around a homestead. The plant bases are 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. In the United States, sorghum is a principal feed ingredient for both cattle and poultry.

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, whereas selected varieties are widely grown in temperate climates (Lazarides *et al*., 1991). The

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This thesis follows the style of Crop Science.

wild races of *S. bicolor* serve as an extensive pool of germplasm for breeders around the world (Rooney and Smith, 2000; Rosenow and Dahlberg, 2000).

Taxonomy and cytology

The genus *Sorghum* consists of 25 recognized species, 17 of which are indigenous to Australia (Dillon et al., 2001). The other species of *Sorghum* are found in Africa, Central America and India (Garber, 1950). Species in the *Sorghum* genus have chromosome numbers of $2n$ = 10, 20, 30, and 40 (Garber, 1950; Lazarides et al., 1991). The genus *Sorghum* has been traditionally classified into five subgenera or sections: *Eu-sorghum*, *Chaetosorghum*, *Heterosorghum*, *Para-sorghum* and *Stiposorghum* (Garber, 1950). Although this classification is convenient, it does not represent evolutionary relationships (Dillon et al., 2004). Phylogenetic relationships based on DNA sequence comparison are presented in Figure 1. The section *Eu-sorghum* includes the cultivated species *S. bicolor* (L.) Moench, its subspecies *drummondii* and *arundinaceum*, and wild species *S. x alum* Parodi, *S. halepense* (L.) Pers., and *S. propinquum* (Kunth) Hitchc (deWet, 1978). The section *Eu-sorghum* originated in Africa or Asia (DeWet and Harlan, 1971; Doggett, 1976; DuVall and Doebley, 1990). *Sorghum bicolor*, which has a basic chromosome number of 10, has a relatively small genome of ~818 Mbp (Price *et al*., 2005a). Individual chromosomes are hard to recognize morphologically at metaphase I due to their small size and general lack of morphologically distinctive characters. In fact, Kim *et al*. (2002) used BAC-FISH to establish a means of metaphase karyotyping. The position of the nucleolus-organizing region in *S. bicolor* is intercalary, residing near the centromere of the largest chromosome (Garber, 1950). The

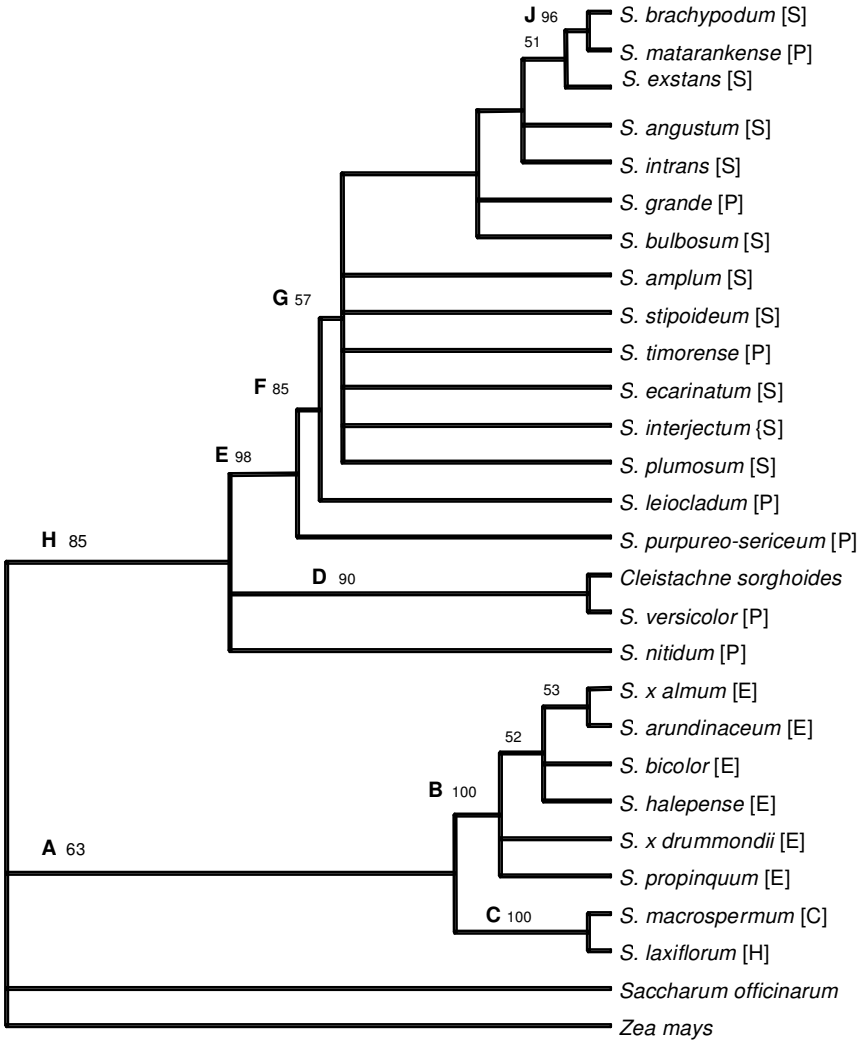


Figure 1. Phylogeny of the genus *Sorghum* derived by Dillon *et al*. (2004). It is a strict consensus tree for the combined ITS1/*ndh*F data using maximum parsimony analyses. Numbers above branches are percentages of bootstrap replicates in which the clade was recovered. Letters A and H designate sister lineages. Trees were rooted using *Zea mays*. Letters in parenthesis indicate taxonomic sections within *Sorghum* where P = *Para-sorghum*, S = *Stiposorghum*, H = *Heterosorghum* and E = *Eu-sorghum.*

centromeres are clearly distinct after differential staining at pachynema. Occasional multivalents (trivalents or quadrivalents) have been reported at metaphase I of *S. bicolor* chromosomes (Kidd, 1952; Celarier, 1958). This notion might imply the tetraploid origin of *S. bicolor*. With the advent of bacterial artificial chromosomes (BACs) and techniques such as fluorescent *in situ* hybridization (FISH), Gomez *et al*. (1998) provided evidence seemingly congruent with the hypothesis that sorghum is at least of tetraploid origin. However, the acceptance of this hypothesis is not widespread. *Sorghum halepense*, also known as Johnsongrass, is a tetraploid derived from a natural cross between *S. arundinaceum* and *S. propinquum* (Doggett, 1976). Garber (1950) reported a somatic chromosome number of $2n = 4x = 40$ for *S. halepense.* Meiotic observations have varied, however the existence of pairings higher than quadrivalents have been seen at a low frequency (Garber, 1944; Endrizzi, 1957). *Sorghum propinquum* is a perennial rhizomateous species related to *S. bicolor* (Doggett, 1976, Chittenden *et al*., 1994; Sun *et al*., 1994). It is a $2n = 20$ species that is chromosomally similar to *S. bicolor* and has been crossed to it. F_2 progeny resulting from crosses of *S. bicolor* and *S. propinquum* have been evaluated to generate RFLP (restriction fragment length polymorphism) loci used in mapping (Chittenden et al., 1994). Hybrids of *S. bicolor* and *S. propinquum* are meiotically regular with ten bivalents observed at meiotic metaphase I (Doggett, 1988).

Sections *Chaetosorghum* and *Heterosorghum* consist of *S. macrospermum* and *S. laxiflorum* respectively. Both species are annuals and polyploids, displaying 20 bivalents at metaphase I of meiosis (Garber, 1950; Lazarides *et al*., 1991; Wu, 1990).

Sorghum laxiflorum and *S. macrospermum* were identified as the Australian species most closely related to cultivated *Sorghum* (Dillon *et al*., 2004).

Section *Stiposorghum* consists of ten species indigenous to northern Australia (Lazarides *et al*., 1991). Section *Para-sorghum* comprises seven African, Asian, Australian and Central American species. The basic chromosome number of species in each section is five. Most of the species in *Parasorghum* and *Stiposorghum* are diploid ($2n = 10$) with a few species being tetraploid or hexaploid. The species of sections *Para-sorghum* and *Stiposorghum* may be separated into two groups based on number of nucleolus chromosomes at pachytene (Garber, 1950). The species of *Parasorghum* have 1 nucleolus organizer per genome whereas the species of *Stiposorghum* have 2 or 4 nucleoli genome. Morphological and positional differences of nucleolus-organizing regions at pachynema were also observed (Garber, 1950). In general, the chromosome sizes of species in sections *Parasorghum* and *Stiposorghum* are large, relative to those of *Eu-sorghum* (Price *et al*., 2005a).

The taxonomic distribution and organization of CEN38 in the genus *Sorghum* have not previously been revealed. Dillon *et al*. (2001, 2004) used ITS1 and *ndh*F gene sequence comparison to analyze the genus *Sorghum*. Two main lineages were revealed, one containing species with a basic chromosome number of 5 and the other lineage containing species with a basic chromosome number of 10 (Figure 1). In the current study, the presence of CEN38 is analyzed in relationship to the *Sorghum* phylogenetic tree to see if it can be used as an evolutionary marker. Specifically, its distribution will

be superimposed on the phylogeny of Dillon et al. (2004) to see if it can be used to support a monophyletic or polyphyletic evolution of species in the sister lineages, A and H, of the genus *Sorghum*.

Genetic maps and molecular cytogenetics

Before the era of genetic mapping using molecular markers, over 200 genes had been discovered in sorghum (Rooney, 2000). Collectively, these genes controlled many different phenotypes including morphology, maturity, fertility, disease resistance and drought tolerance. Only seven linkage groups containing three or more (8 maximum) genes were identified (Doggett, 1988). Construction of sorghum genome maps based on DNA markers began in the early 1990's, with several moderately marker-dense maps being reported (Bhattramakki et al., 2000; Chittenden *et al*., 1994; Pereira *et al*., 1994; Peng et al., 1999). These maps were developed using SSR (simple sequence repeat) and/or RFLP markers from F_2 populations and recombinant inbred lines. Current RFLP/SSR/AFLP-based genetic maps in sorghum are high density and include approximately 3000 loci (Klein *et al*., 2000; Menz *et al*., 2002). More recently, an integrated *Sorghum bicolor* genome map has been constructed utilizing a combination of methodologies, including high-throughput amplified fragment length polymorphism (AFLP) DNA marker technology (Klein *et al*., 2000; Menz *et al*., 2002), six-dimensional pooling of BAC clones (Klein *et al*., 2000), cDNA capture technology (Childs *et al*., 2001), sequenced-based alignment of the genomes of sorghum and rice (Klein *et al*., 2003) and BAC-based fluorescence *in situ* hybridization (FISH) (Islam-Faridi *et al*., 2002; Kim *et al*., 2002, 2005a, b). FISH of genetically mapped BACs has permitted all

linkage groups to be associated to specific sorghum chromosomes and to integrate the genetic recombination frequency with physical distances along each chromosome (Islam-Faridi *et al*., 2002; Kim *et al*., 2002, 2005b). It also provided an excellent means of karyotyping sorghum chromosomes using landed sorghum BACs (Kim *et al*., 2002, 2005a). Recent application is leading to a more thoroughly integrated sorghum genomic map that will provide a valuable resource to researchers.

Repetitive DNA and CEN38

There is remarkable variability in genome size among eukaryotes that does not correlate with the evolutionary complexity or the number of coding genes of an organism (Price, 1976; Charlesworth *et al*., 1994). Much of this variation is due to non-coding, repeated DNA. For many years, repetitive sequences were believed by some to be "selfish elements" or "junk DNAs". A major fraction of the genomes of many eukaryotes is comprised of repetitive DNA sequences, in which short sequences are repeated in small to huge tandem arrays and/or are dispersed throughout the genome (Flavell, 1986). Families of repetitive DNA sequences are differentiated by their degree of sequence homology, distribution among species and/or genome and physical organization (Schmidt and Heslop-Harrison, 1998). The concept that variation in DNA content is due to the accumulation of "junk" DNA has recently been strongly challenged (Cavalier-Smith, 2005).

The definition of centromere-associated sequences and their functions is likely to improve the understanding of centromere action and genome evolution (Zwick *et al*., 2000). It is widely known that centromere functions are highly conserved throughout

many species. However, there is a notable lack of sequence homology among the centromeres of distantly related species, highlighting the extremely rapid rate of centromere DNA evolution (Hall *et al*., 2004). In *S. bicolor*, CEN38 is a tandemly repeated DNA sequence that was subcloned (Zwick *et al*., 2000) from a bacterial artificial chromosome, BAC 22B2 (Gomez *et al*., 1998). CEN38 belongs to a family of repeats from *Sorghum* that also includes the sequence, p*Sau*3A10 (Miller *et al*., 1998) and the sugarcane sequence SCEN (Nagaki *et al*., 1998). It is an AT-rich DNA sequence that is amplified as a <280 bp dimer, each dimer consisting of two divergent <140 bp monomers (Zwick *et al*., 2000). Results from Miller *et al*. (1998) and Zwick *et al*. (2000) reported that this repeat family was found only in the section *Eu-sorghum* and *Saccharum* and not in related species such as rice, maize and wheat.

CEN38 yielded differentially strong hybridization signal in pericentromeric regions of 10 out of 20 *Sorghum* mitotic chromosomes (Gomez *et al*., 1998; Zwick *et al*., 2000). CEN38 has been used to provide insight onto the architecture and karyotype of sorghum chromosomes (Islam-Faridi *et al*., 2002; Kim *et al*., 2002). The aim of this research is to evaluate CEN38 as an "evolutionary tool" to assess phylogenetic and evolutionary relationships among members of the genus *Sorghum*. Since CEN38 has been reported in only the *Eu-sorghum* section of *Sorghum* and *Saccharum* and not in related gramineous species, *Sorghum* species possessing CEN38 may be derived from a common ancestor with CEN38 in its genome. Therefore, if CEN38 is found in both the $x = 10$ and $x = 5$ sister lineages detected by Dillon *et al.* (2004), it would be evidence for a monophyletic origin of the genus *Sorghum*. If it is not present in both sister lineages, it

would not help resolve the issue of whether or not the two *Sorghum* lineages are monophyletic or polyphyletic. The objectives of this research are: (1) To determine the taxonomic distribution of the *S. bicolor* centromere-associated CEN38 sequence; (2) To determine the chromosomal distribution of CEN38 in at least two additional *Sorghum* species using fluorescence *in situ* hybridization (FISH); and (3) To determine the organization of CEN38 in the DNA of *Sorghum* species.

MATERIALS AND METHODS

Plant materials

The *Sorghum* species used in this research were propagated from seeds and grown in a glasshouse, except *S. halepense*, which was sampled directly from the field. Accession numbers, herbarium voucher numbers, life forms and origins are listed in Table 1.

DNA extraction

DNA from 21 species of *Sorghum* was extracted carefully following a modification of CTAB (cetytrimethylammonium bromide) method by Zhang (1995). Fresh young leaf tissue, approximately five to 10 g, was collected early in the morning to avoid accumulating unwanted photosynthetic compounds. 200 mL of extraction buffer (1275.4 g Sorbitol, 242.0 g Tris, 33.6 g EDTA; final volume to 20 L with dd H_20, adjust pH to 7.5 before adding sodium bisulfite) combined with 0.76 g of dissolved sodium bisulfite were used to homogenize the leaf tissue in a kitchen blender. The leaf tissue was pureed for 30-40 sec to form a slurry. This slurry was filtered through two layers of cheesecloth and one layer of Miracloth® into a 50 mL Falcon tube on ice. Subsequently the tube was centrifuged on a JA-14 rotor at 1880 rcf (relative centrifugal field) at 4ºC for 20 min. The supernatant was discarded and the pellet was re-suspended with a paintbrush in 10 ml of extraction buffer. Ten mL of nuclei lysis buffer (1.0 M Tris, HCL, 0.5 M EDTA, 5.0 M NaCl, CTAB, dd H_2O) and 4 mL of 5% sarkosyl (50g/L N Lauroyl sarcosine) were added and mixed gently by inverting several times. The mixture was incubated at 60ºC in a water bath for 15-20 min. Following incubation,

Table 1. Accession number, life form and origin of 21 *Sorghum* species used in this study (Modified from Price *et al*., (2005a).

Species	Herbarium voucher	Accession number[†]	Life form	Collection date and site, or source of seeds
Sorghum amplum Lazarides	CANB 480260[‡]	302455[‡]	Annual	17-Mar.-94, 1.4 km E of Lake ArgleT/O on Great Northern Hwy, WA, Australia
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, Ausralia
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. bulbosum Lazarides	DNA D129483[¶]	302645	Annual	25-Apr.-96, 29.1 km S Wyndham T/O on Halls Creek Rd (Great Northern Hwy) NT, Australia
S. ecarinatum Lazarides	DNA D129449	302648	Annual	1.9 km W of Quanbun Stn T/O on Great Northern HWY (Approx 5 km W of 2321), NT
S. exstans Lazarides	BRI AQ586005	302577	Annual	2-Apr.-95, 37 km N Pickataramoor on Melville Island, Australia
S. halepense (L.) Pers.			Perennial	14-June-01, Hwy 60, 0.5 miles W of Brazos River, T.A.E.S. Field Laboratory, Burleson County, TX USA
S. interjectum Lazarides	BRI AQ585985	302563	Perennial	0.5 km E of Angurugu River crossing, map unit 6, Groote Eyland
S. intrans F. Muell. Ex Benth	DNA D133021	302668	Annual	Rod to Howard river floodplain, SE of Darwin
S. laxiflorum Bailey	BRI AQ773635	302510	Annual	15-Apr.-94, 67.9 km N of Wollogorang on Wollogorang Station Rd to coast, NT, Australia
S. leiocladum (Hack.) C E. Hubb	DNA D0155521	300170	Perennial	16-Dec-97, 2-3 km W from Drake on roadside on range in State Forest, NSW, Australia
S. macrospermum Garber	DNA C867	302367	Annual	4-Apr.-95, 7.9 km N Katherine River bridge on Stuart Hwy, NT Australia
S. matarankense Garber & Snyder	DNA D129470	302636	Annual	25.5 km N Newcastle Waters on Stuart Hwy
S. nitidum (Vahl.) Pers.	BRI AQ496360	316930	Perennial	6-Jun.-00, 450m down road from summit of Mt. Stuart on both sides of road, QLD, Australia
S. plumosum (R. Br.) P. Beauv.	BRI AQ773634	302489	Perennial	11-Apr.-94, Einslie River, 26.4 km W of Georgetown on Gulf Development Rd QLD, Australia,
S. propinquum (Kunth) Hitch.			Perennial	Africa, seeds from W. Rooney, Texas A&M University
S. purpureosericeum (A. Rich). Aschers & Schweinf	IS 18945[#]	318068	Annual	Sudan, Northeast tropical Africa
S. stipoideum (Ewart & Jean White) C. Gardner and C. E. Hubb	DNA D129471	302632	Annual	Old Victoria Riv crossing, NT
S. timorense (Kunth) Buse	DNA D129479	302440	Annual	28.5 km W Top SpringsT/O on Victoria Hwy, 0.5 km E Innesfan Stn T/O, NT
S. versicolor Anderss.			Annual	East Africa, seeds obtained from G. Liang, Kansas State University

[†]AusTRC number, Australian Tropical Crops and Forages Collection, Queensland Department of Primary Industries
[‡]CANB = Australian National Herbarium, Canberra, ACT Australia
[§]BRI = Queensland Herbarium, Mt Coot-tha, QLD Australia
[¶]DNA = Northern Territory Herbarium, Darwin, NT Australia
[#]IS = ICRISAT

approximately 17 mL of 24:1 chloroform/isoamyl were added to the mixture in a hood. The mixture was inverted 10-15 times to get an emulsion and then centrifuged at 1880 rcf at room temperature for 20 min. After centrifugation, the aqueous layer (top layer) was pipetted into a new tube and one volume of isopropanol was added to precipitate the DNA. To promote precipitation of the *Sorghum* DNA, tubes were centrifuged at 8820 rcf at 4ºC for 20 min, the isopropanol was decanted, and 4 mL of 70% ethanol was added to the mixture. Again the tubes of DNA were centrifuged at 8820 rcf for 10 min. The ethanol was decanted off and the DNA pellet was allowed to air dry 5 min. Afterwards various amounts of TE buffer (200-800 µl) were added to dissolve the DNA pellet (depending on size of pellet). The DNA was subsequently stored at 4ºC until needed.

Extraction, amplification and purification of pCEN38

The plasmid pCEN38 was extracted from *E. coli* cells by the standard protocol by Sanbrook, Fritsch, and Maniatis (1989). One L of LB medium (950 mL of dd H_2O, 10 g of bacto-tryptone, 5 g bacto-yeast extract and 10 g NaCl) was prepared and autoclaved for 20 min. One mL of ampicillin was added per L of LB medium. Fifty mL of LB medium was poured into a separate flask. This flask of LB medium was inoculated with ~25 µL of the glycerol stock of CEN38. Another batch of LB medium was prepared (without ampicillin) and added to the small flask containing the CEN38 glycerol stock. This doubled the volume of the flask (45 mL to 90 mL) and halved the concentration of ampicillin to the correct concentration (50 µg/µL to 25 µg/µL). The flask with cells was then covered with aluminum foil and incubated at an environmental

shaker at 37ºC for 250 cycles/min overnight. The next day the flask was removed from the shaker. The original LB medium's (containing ampicillin) concentration remained 50 μg/μL. The concentration was halved when ~870 mL of regular LB medium was added. This large volume was poured into 5 smaller flasks (~300 mL – 600 mL) and 10 mL of the late-log phase culture was added to each flask. These cultures were incubated for 3 h at 65ºC with vigorous shaking (250 cycles/min) on a rotary shaker. After incubation, the cultures were combined and the optical density (OD) was checked with a spectrophotometer. Serial dilutions were made (1:10 and 1:100) and the optical density was recorded at ODλ600 and ODλ450. The large culture was aliquotted into 5 smaller flasks and again incubated at 37ºC onto a rotary shaker with vigorous shaking overnight. The next morning, the cultures were poured into 4 large centrifugal bottles and centrifuged at 4ºC at 3000 rpm on a JA-14 rotor for 15 min. The supernatant was subsequently decanted and drained. Fifty mL of ice-cold TE was added to each bottle to resuspend the pellet. After resuspension, the DNA was combined into 1 bottle and centrifuged again at 4ºC for 15 min. After centrifugation, the DNA was released during lysis of the bacteria cells. Twenty mL of ice-cold Solution I (50 mM glucose, 25mM Tric/HCL pH 8.0, 10 mM EDTA pH 8.0) were added to resuspend the pellet. The bottle was kept on ice for 10 minutes. Forty mL of freshly prepared Solution II (0.2N NaOH, 1% SDS) were added and mixed thoroughly, but gently. This is very important because this is the stage when the plasmid is released from the bacteria. The solution was kept on ice for an additional 10 min. Thirty ml of ice-cold Solution III (5M potassium acetate, glacial acetic acid, H_2O) was added to the solution and mixed well. The mixture

was kept on ice for 30 min. Afterwards, the solution was centrifuged at 4ºC at 3000 rpm for 15 min. The supernatant was subsequently filtered through 4 layers of cheesecloth and 2 layers of Miracloth®. Nearly 1 volume (0.6) of isopropanol was added to the supernatant and centrifuged at 4ºC at 5000 rpm for 20 min. The isopropanol was decanted and the pellet was washed with 70% ethanol. The resuspended pellet was centrifuged at 4ºC at 5000 rpm for 3 min. Ten ml of TE was added to the tube to dissolve the pellet and inverted at 4ºC overnight. The following day, the quality of plasmid DNA containing CEN38 was verified by 1% agarose gel electrophoresis. A large amount of RNA existed, so the pCEN38 was treated with RNase to remove the RNA. The quality of DNA was checked again via agarose gel electrophoresis, which showed no presence of RNA. After removing the RNA from the sample containing pCEN38, the plasmid DNA was quantified using DyNA Quant 200 Fluorometer. After quantification, CEN38 was amplified using polymerase chain reaction (PCR) and purification through a Sephadex G-50 column (5% Sephadex saturated with TE).

Detection and quantification of DNA by dot blot hybridization

The presence of CEN38 in genomic DNA from *Sorghum* species was quantified by dot blot hybridization using a modified protocol by Zhang (2005b). Genomic DNA from 21 *Sorghum* species and *Cleistochne sorghoides* listed in Table 1 on page 11 was quantified by a DyNA fluorometer. Various concentrations of genomic DNA were used for dot blot analysis (100 ng/µL, 200 ng/µL, 300 ng/µL and 2 µg/mL). The concentration of the probe DNA (CEN38) used was 32 ng/µL. CEN38 was used as a

positive control and subdivided into 10,000 copies, 50,000 copies and 100,000 copies to estimate the copy numbers present. TE was used a negative control.

The nylon membrane was soaked in 325 mL of 2x SSC. While the filter was soaking, 50 μL of denaturation buffer were added to each tube of genomic DNA and allowed to remain on ice for 15 min. All of the samples were incubated at 80ºC for 10 min, and 50 μL of neutralization buffer were added to each tube. The tubes remained on ice for 15 min. The filter was placed in the Bio-Dot apparatus and 100 μL of 20x SSC were added to the template and vacuum-filtrated gently. The samples (110 μL each) were loaded with a multi-tip pipette and vacuum-filtered gently onto the membrane. Afterwards, 100 μL of 0.4 N NaOH (each) were filtered onto the membrane. The membrane was then removed from the apparatus and rinsed in 2x SSC for 5 min. The filter was wrapped in SaranWrap® and incubated at 80ºC for exactly 2 h. After incubation, the filter was stored at 4ºC. On day 2, the membrane was incubated in hybridization solution (250 mL of 20x SSC, 25mLof 0.5% SDS, 25 mM 0.5 M KPB, pH 6.5, 100x Denhardt's and 625 mL of dd H_2O) at 65ºC for >2 h. Afterwards, the probe DNA (CEN38) was radioactively-labeled by incubating at 37ºC for 30 min in LS (labeling solution), 0.5U/uL Klenow, P32-dCTP, and dd H_20. The labeled probe DNA was denatured by adding one volume of 0.4 N NaOH and incubating the reaction at 95ºC for 10 min. The labeled probe was carefully transferred into the hybridization solution (without touching the membrane) and incubated overnight at 65ºC with gentle shaking. On the third day, the membrane was washed several times at 65ºC with a washing buffer (20x SSC [0.2x], 20% SDS [0.1%] and ddH_20). After the third washing, the membrane

was blotted with paper towels to remove excess fluid and wrapped with SaranWrap®. The membrane was covered with x-ray film and placed in an autoradiograph cassette at -80ºC for 2 h exposure. The x-ray film was developed and the exposed areas were quantified on the autoradiograph using a phosphoimager. The copy numbers were calculated using the densiometric values of *Sorghum* genomic DNA relative to the value of the control DNA (CEN38). This value is multiplied by the copies of CEN38 present in the control. This value is divided by the number of cells present in each species' genome. This quantified value was the copy number of CEN38 present in each cell per diploid complement (Table 2).

Seed germination and somatic chromosome preparation

To obtain young, actively dividing root tips for preparing slides with chromosome spreads, seeds of *Sorghum* species (*S. versicolor*, *S. macrospermum*, *S. laxiflorum* and *S. bicolor*) were germinated on a 0.7% agar medium (200 mL of dd H_20, 4 g of sucrose, 1.4 g of agarose) using aseptic techniques. The quality of root tips used for somatic chromosome spreads from plants grown in a greenhouse showed much seasonal and environmental variability, therefore it is often difficult to obtain good metaphase chromosome spreads using root tips from plants grown in a greenhouse. However, it was observed that seeds germinated on a 0.7% agar medium (containing dd H_20, 0.4% sucrose) produced roots that were optimal for preparing chromosome spreads year round. This method can be used to propagate an ample number of actively dividing roots that are free of soil debris. The use of aseptic techniques lowers the rate of

Table 2. Chromosome number and estimated copy number of CEN38 for 21 species of *Sorghum* and *Cleistochne sorghoides*.

Number	Species†	Chromosome number (2*n*)	Estimated CEN38 copy numbers‡
1	*S. brachypodum* (S)	10	0
2	*S. matarankense* (P)	10	15
3	*S. extans* (S)	10	34
4	*S. angustum* (S)	10	37
5	*S. intrans* (S)	10	0
6	*S. bulbosum* (S)	10	129
7	*S. amplum* (S)	30	164
8	*S. plumosum* (S)	30	0
9	*S. stipoideum* (S)	10	51
10	*S. timorense* (P)	10	0
11	*S. encarinatum* (S)	10	0
12	*S. interjectum* (S)	30	97
13	*S. leiocladum* (P)	10	92
14	*S. purpureo-sericeum* (P)	10	90
15	*S. versicolor* (P)	10	278
16	*S. nitidum* (P)	20	205
17	*S. bicolor* (E)	20	20,708
18	*S. halepense* (E)	40	20,828
19	*S. propinquum* (E)	20	12,008
20	*S. macrospermum* (C)	40	71
21	*S. laxiflorum* (H)	40	436
22	*Cleistochne sorghoides*	36	0

†*Sorghum* subgenera – C=*Chaetosorghum*, E=*Eu-sorghum*, H=*Heterosorghum*, P=*Para-sorghum*, and S=*Stiposorghum*

‡Estimated copy numbers based on haploid (1C) genome size (Price et al., 2005a)

contamination. This new procedure is overall a cleaner, quicker and more optimal technique to produce root tips for somatic chromosome spreads.

The agar medium was heat-sterilized and allowed to cool (35-45 min) before it was poured into Petri plates. While the agar medium was cooling, seeds were surfaced-sterilized in 30% sodium hypochlorite for 20 min. The agar medium was carefully poured into the Petri plates and allowed to solidify. While solidifying, the seeds were removed from the bleach and rinsed with sterilized water. The seeds were gently placed onto the agar using aseptic techniques. The plates were covered, labeled, sealed with Parafilm® and placed in the dark for at least 2 d. The time the seeds remained in the dark depended on germination and the length of the primary root. The root was excised at ~ 2.5 cm – 7.6 cm. Somatic chromosome spreads were prepared by the protocol of Jewell and Islam-Faridi (1994). Excised roots were treated with aqueous α-monobromonaphthalene at room temperature in the dark for 1 h and 45 min. The roots were fixed with 4:1 ethanol: acetic acid. The following day, the root tips were rinsed with dd H_20 for an h (every 15 min) and treated with 0.2 N HCL for 10 min. The roots were rinsed again with ddH_20 for 10 min before the root tips were excised and submerged into enzyme solution (5% cellulose, 2.5% pectolyase in 0.2 M citrate buffer). The root tips were incubated at 37ºC for at least 20-25 min (the time depended on the size of root tip, i.e. very large tip required 35 min). After digestion, the enzyme was removed and dd H_20 was added to the root tips. The tips were spread on the slide with the aid of 3:1 ethanol: acetic acid and tweezers. The slides were allowed to dry and

analyzed under a light microscope. After visualization of the cells under the microscope, the slides were kept in a slide box at -80ºC.

Fluorescence *in situ* hybridization (FISH)

Visualization of the chromosomal distribution of CEN38 and 18S-28S rDNA in specific *Sorghum* species followed a modified protocol of Jewell and Islam-Faridi (1994), as described by Hanson *et al*. (1995) and Kim *et al*. (2002). First, the purifed pCEN38 was indirectly labeled with digoxigenin-11 dUTP (DIG) by nick-translation. Labeled probe was hybridized to chromosomal DNA. On day 1, the hybridization mixture was prepared (25 µL/slide). The mixture consisted of deionized formamide (50%), 50% dextran sulfate (10%) and 20x SSC (2x concentration). The probe DNAs (1 µL of CEN38), 18S-28S rDNA (1 µL) and TE (3 µL) were added. Afterwards, 100 µL of formamide was added to each slide, covered with a glass coverslips, and incubated at 70ºC for 1.5 min to denature the chromosomal DNA. While the chromosomal DNA was denaturing, the graded EtOH series was setup. The 70% EtOH coplin jar was placed in -20ºC freezer to be chilled. The 80%, 95% and absolute EtOH series were kept at room temperature. After, denaturation, the coverslips were removed and the slides were dehydrated in the graded ethanol series. While the slide were air-drying (10 min), the probe DNA (CEN38 and 18S-28S rDNA) was added to the hybridization mixture and denatured at 90ºC for 10 min. The denatured probes were immediately quenched on ice for 5 min. Twenty-five µL of hybridization mixture were added per slide. Glass coverslips were placed on the slides and sealed with rubber cement. The slides were incubated in a humidity chamber at 37ºC overnight. On day 2, the rubber cement and

glass coverslips were removed from the slides. It was important to not let the slides dry out. The slides were washed with 2x SSC at 40ºC for 5 min in a shaking incubator. The 2x SSC was removed and fresh 2x SSC was added to the slides at room temperature with the lid off the coplin jar for 5 min. The 2x SSC was removed and the slides were washed with 4x SSC (with 0.2% Tween-20) at room temperature for 5 min. After washing, 200 µL of 0.1 g of 5% BSA solution was added to 2 mL of 4x SSC (0.2% Tween-20) was added to each slide to block non-homologous DNA from hybridizing to the conjugated dyes. The next step was done in the dark to avoid exposing the fluorescent dyes to light. The hybridization of probe DNA (DIG-labeled) to its homologous sequences in chromosomal DNA was detected with fluorescein isothiocyanate (FITC) conjugated with antidigoxygenin ([1.3 µg/mL]/100 µL of 5% BSA/4x SSC/0.2% Tween-20). The 18S-28S rDNA was detected with (Cy-3) conjugated with streptoavidin ([5 µg/mL]/ 100 µL of 5% BSA/4x SSC/0.2% Tween-20). Plastic coverslips were placed on the slides and incubated for 20-30 min at 37ºC. The coverslips were removed and the slides were washed three times, 1-2 min with 4x SSC/0.2% Tween-20 at 37ºC. The chromosomes are detected by adding 100-200 µL of 3 µg/mL DAPI with Vectashield®. The slides were then covered with clean glass coverslips and observed under an Olympus AX-70 fluorescent microscope.

Southern hybridization

The presence and organization of CEN38 in the genus *Sorghum* was detected by Southern blotting. Procedures for Southern blotting and hybridization followed the protocol by Zhang (2005b). Genomic DNA from twenty-one species of *Sorghum* was

digested with specific restriction enzymes, *HindIII* and *PstI*, with each having a specific restriction site in CEN38 (Table 3). The DNA was digested 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 was added to each sample and the samples were inserted into the gel. Two lanes of marker DNA (λ DNA digested with *Hind*III) were loaded per gel. The gel was run at 24 v 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. 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 edge of the gel without samples was cut with a razor blade (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. The areas of the gel and the blotting paper wick that was not covered by the membrane was covered with Parafilm® to block transfer through these areas. Two sheets of blotting paper with sizes slightly larger than the gel were cut, soaked in the reservoir

Table 3. Lane assignments, species, and restriction enzymes used to digest DNA prior to Southern blotting.

Lane #†	Species/Marker	Restriction Enzyme
1	*λDNA marker*	*Hind*III
2	*S. brachypodum*	*Hind*III
3	*S. brachypodum*	*Pst*I
4	*S. matarankense*	*Hind*III
5	*S. matarankense*	*Pst*I
6	*S. extans*	*Hind*III
7	*S. extans*	*Pst*I
8	*S. angustum*	*Hind*III
9	*S. angustum*	*Pst*I
10	*S. intrans*	*Hind*III
11	*S. intrans*	*Pst*I
12	*S. bulbosum*	*Hind*III
13	*S. bulbosum*	*Pst*I
14	*S. amplum*	*Hind*III
15	*S. amplum*	*Pst*I
16	*S. plumosum*	*Hind*III
17	*S. plumosum*	*Pst*I
18	*S. stipoideum*	*Hind*III
19	*S. stipoideum*	*Pst*I
20	*S. timorense*	*Pst*I
21	*S. timorense*	*Hind*III
22	*S. encarinatum*	*Hind*III
23	*S. encarinatum*	*Pst*I
24	*S. interjectum*	*Hind*III
25	*S. interjectum*	*Pst*I
26	*S. leiocladum*	*Hind*III
27	*S. leiocladum*	*Pst*I
28	*S. purpureo-sericeum*	*Hind*III
29	*S. purpureo-sericeum*	*Pst*I
30	*λDNA marker*	*Hind*III
1a	*λDNA marker*	*Hind*III
2a	*S. versicolor*	*Hind*III
3a	*S. versicolor*	*Pst*I
4a	*S. nitidum*	*Hind*III
5a	*S. nitidum*	*Pst*I

Table 3. Continued

Lane #†	Species/Marker	Restriction Enzyme
6a	*S. bicolor*	*Hind*III
7a	*S. bicolor*	*Pst*I
8a	*S. halepense*	*Hind*III
9a	*S. halepense*	*Pst*I
10a	*S. propinquum*	*Hind*III
11a	*S. propinquum*	*Pst*I
12a	*S. macrospermum*	*Hind*III
13a	*S. macrospermum*	*Pst*I
14a	*S. laxiflorum*	*Hind*III
15a	*S. laxiflorum*	*Pst*I
16a	*Cleistochne sorghoides*	*Hind*III
17a	*Cleistochne sorghoides*	*Pst*I
18a	*λDNA marker*	*Hind*III

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

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 probe DNA (CEN38) was 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 probe DNA was denatured by adding one volume of 0.4 N NaOH and incubating the reaction at 95ºC for 10 min. The labeled probe was 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 the exposed ladder patterns on the autoradiograph were analyzed using a phosphoimager.

PCR amplification

The presence and organization of CEN38 in the genus *Sorghum* were detected by PCR amplification. Procedures for PCR amplification followed the protocol by Zhang (2005a). Ten *Sorghum* species were chosen for PCR amplification, depending on the presence and absence of CEN38 displayed by Southern hybridization (Table 4). Gene-specific forward and reverse primers (4 sets) were synthesized based on the nucleotide sequence of CEN38 found in GenBank. The sequence for each primer set is as follows:

Primer set 1

Forward Primer 5' TTTGCAGGCAACGTACCATA 3',

Reverse primer 5' GAACCGAGCTTCCACTTGAG 3'

Primer set 2

Forward primer 5' TGGAATCTTGCTTCGGTTTC 3',

Reverse primer 5' GCCTGCAAATTGTGCAACTA 3'

Primer set 3

Forward primer 5' TCACATGGAATCTTGCTTCG 3',

Reverse primer 5' GCCTGCAAATTGTGCAACTA 3'

Primer set 4

Forward primer 5' CCGTTGGAACTCCTTGAGAA 3',

Reverse primer 5' CGAGCTTCCACTTGAGCTTC 3'.

DNA from each *Sorghum* species was amplified separately with each of the primer sets. A cocktail containing 30 µl of 10x PCR buffer, 9 µL of 50 mM $MgCl_2$, 30 µL of dNTPs (2 mM each), 15 µL of forward primer (8 µM), 15 µL of reverse primer (8 µM) and 3 µL

of AmpliTaq (5 units/μL) was made for each set of reactions (4). From these 4 cocktails, 8.5 μL was aliquotted into 40 Ultra PCR tubes. Then 15.50 μL of dd H_20 and 1 μL of DNA were added to each tube, increasing the total volume of the reaction to 25 μL. The reactions were briefly centrifuged before undergoing amplification in a Perkin-Elmer 9600 thermal cycler. The program consisting of heating and cooling steps were linked as follows: (94°C, 4 min- hot start - 94°C, 40 sec - 42°C, 2 min - 72°C, 5 min x 25 cycles; extension step - 72°C, 7 min – 4°C, infinite. After amplification, the PCR product of each reaction was electrophoresed on a 1% agarose gel in 1% NEB, stained with ethidium bromide and counterstained with dd H_20 for analysis.

Table 4. Lane assignments and description for *Sorghum* species used in PCR amplification

Lane #†	Species/Marker	Primer Set‡
1	1 kb plus DNA ladder	N/A
2	*S. bicolor*	Set #1
3	*S. halepense*	Set #1
4	*S. propinquum*	Set #1
5	*S. laxiflorum*	Set #1
6	*S. macrospermum*	Set #1
7	*S. versicolor*	Set #1
8	*S. timorense*	Set #1
9	*S. nitidum*	Set #1
10	*S. brachypodum*	Set #1
11	*Cleistochne sorghoides*	Set #1
12	*S. bicolor*	Set #2
13	*S. halepense*	Set #2
14	*S. propinquum*	Set #2
15	1 kb plus DNA ladder	N/A
16	*S. laxiflorum*	Set #2
17	*S. macrospermum*	Set #2
18	*S. versicolor*	Set #2
19	*S. timorense*	Set #2
20	*S. nitidum*	Set #2
21	*S. brachypodum*	Set #2
22	*Cleistochne sorghoides*	Set #2
23	*S. bicolor*	Set #3
24	*S. halepense*	Set #3
25	*S. propinquum*	Set #3
26	*S. laxiflorum*	Set #3
27	*S. macrospermum*	Set #3
28	*S. versicolor*	Set #3
29	*S. timorense*	Set #3
30	1 kb plus DNA ladder	N/A

Table 4. Continued

Lane #†	Species/Marker	Primer set‡
1a	1 kb plus DNA ladder	N/A
2a	*S. nitidum*	Set #3
3a	*S. brachypodum*	Set #3
4a	*Cleistochne sorghoides*	Set #3
5a	*S. bicolor*	Set #4
6a	*S. halepense*	Set #4
7a	*S. propinquum*	Set #4
8a	*S. laxiflorum*	Set #4
9a	*S. macrospermum*	Set #4
10a	*S. versicolor*	Set #4
11a	*S. timorense*	Set #4
12a	*S. nitidum*	Set #4
13a	*S. brachypodum*	Set #4
14a	*Cleistochne sorghoides*	Set #4
15a	λDNA marker	N/A

† letter "a" designates the lanes on the bottom-half of the gel
‡ Four different primer sets, each set consisting of forward and reverse primers

RESULTS

Taxonomic distribution of CEN38 in the genus *Sorghum*

Hybridization of CEN38 was detectable for only 3 of 21 Sorghum species in the initial dot blots, where loading values were 0.1, 0.2, and 0.3 μg of genomic DNA (Figure 2). Due to the lack of hybridization displayed in the other 18 species of *Sorghum*, the amount of all genomic DNA was increased to 2 μg in a second dot blot. The second dot blot displayed hybridization of CEN38 for 15 of the 21 *Sorghum* species (Figure 3). Hybridization intensities of each species were individually quantified and scored by a phosphoimager. Densiometric values for each species relative to the value of CEN38 control DNA plus the genome size of the specific species were used to calculate approximate copy numbers of CEN38 present in each cell (Table 4).

Comparisons of the two dot blot hybridizations indicated that the small amount of DNA used in the initial hybridization (0.1 μg, 0.2 μg and 0.3 μg) was probably not adequate to detect hybridization. Once the amount of DNA was increased for the second dot blot, it revealed a broader range of hybridization intensities. Strong hybridization signals were detected for species of the section *Eu-sorhgum* (*S. bicolor*, *S. halepense* and S. *propinquum*), with reduced hybridization to species outside the *Eu-sorghum* section, e.g. *S. versicolor* and *S. laxiflorum* (Figure 3).

FISH of CEN38 and 18S rDNA

Chromosomes of four species (*S. bicolor*, *S. versicolor*, *S. laxiflorum*, *S. macrospermum*) were analyzed by FISH of CEN38 to determine its relative abundance and locations. CEN38 was labeled with digoxygenin (DIG) and detected with

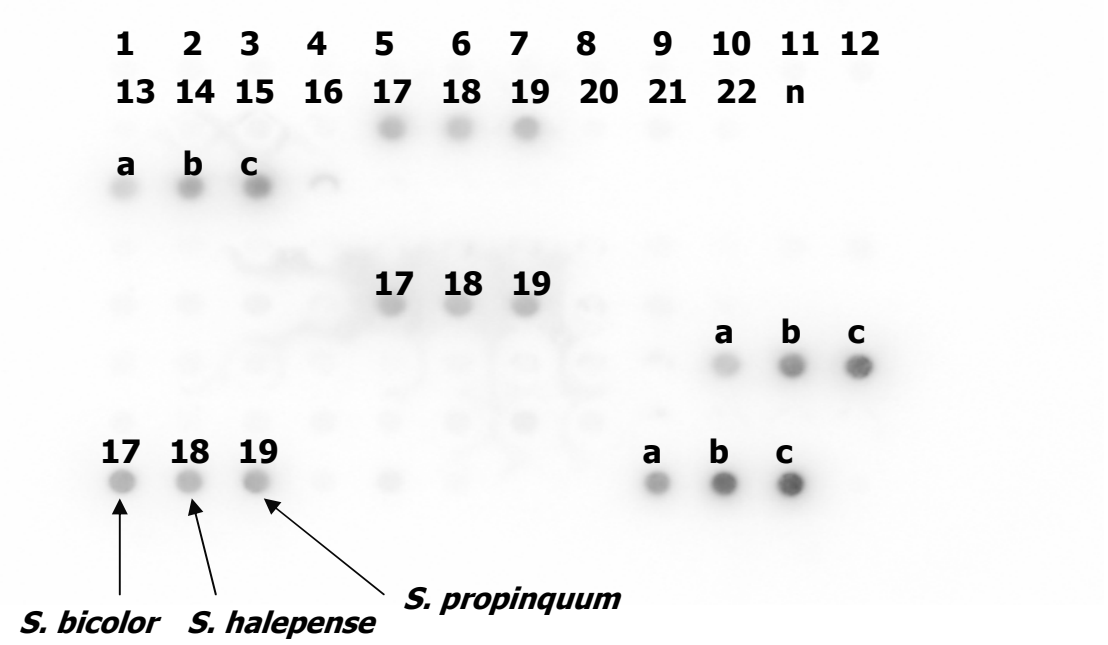


Figure 2. Autoradiogram of dot blot hybridizations of radioactively-labeled-pCEN38 to genomic DNA from 21 species of *Sorghum*. The amount of genomic DNA and positive controls were 0.1 µg (rows 1, 2, and 3), 0.2 µg (rows 4, 5, and 6), and 0.3µg (rows 7, 8, and 9). The positive controls were subdivided into 10,000 copies, 50,000 copies and 100,000 copies. Autoradiogram lanes: a) positive control (10,000 copies of CEN38); b) positive control (50,000 copies of CEN38); c) positive control (100,000 copies of CEN38), n) negative control (TE); 17) *S. bicolor;* 18) *S. halepense*; 19) *S. propinquum*; 1) *S. brachypodum*; 2) *S. matarankense*; 3) *S. extans*; 4) *S. angustum*; 5) *S. intrans*; 6) *S. bulbosum*; 7) *S. amplum*; 8) *S. plumosum*; 9) *S. stipoideum;* 10) *S. timorense*; 11) *S. encarinatum*; 12) *S. interjectum*; 13) *S. leiocladum*; 14) *S. purpureo-sericeum*; 15) *S. versicolor;* 16) *S. nitidum*; 20) *S. macrospermum*; 21) *S. laxiflorum*; 22) *Cleistochne sorghoides* (see Table 4)

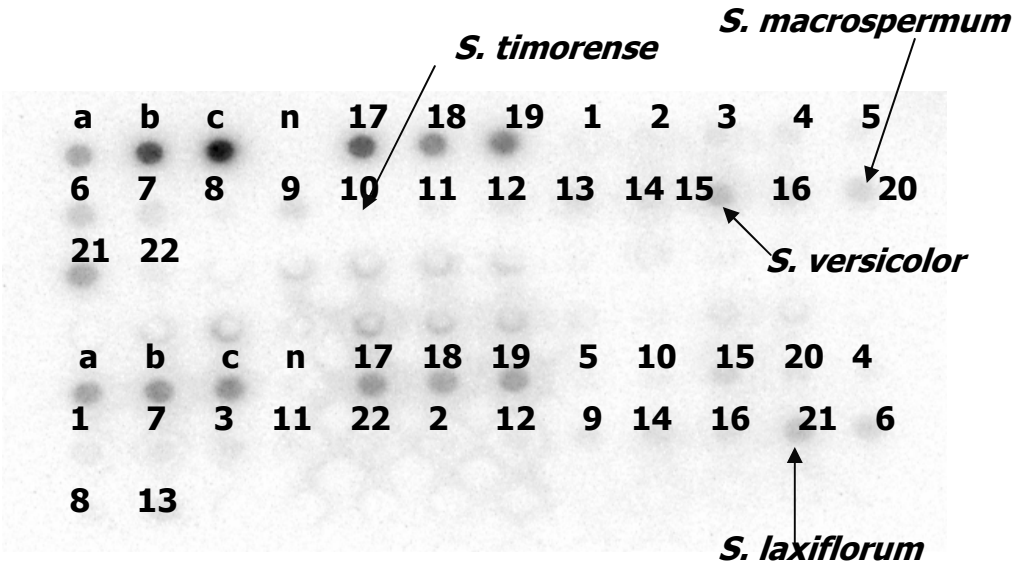


Figure 3. Autoradiogram of 2nd dot blot displaying hybridizations of radioactively-labeled pCEN38 to genomic DNA from 21 species of *Sorghum*. The amount of genomic DNA and positive controls were 2 µg . The positive controls remained subdivided into 10,000 copies, 50,000 copies and 100,000 copies. Autoradiogram lanes: a) positive control (10,000 copies of CEN38), b) positive control (50,000 copies of CEN38), c) positive control (100,000 copies of CEN38), n) negative control (TE), 17) *S. bicolor;* 18) *S. halepense*; 19) *S. propinquum*; 1) *S. brachypodum*; 2) *S. matarankense*; 3) *S. extans*; 4) *S. angustum*; 5) *S. intrans*; 6) *S. bulbosum*; 7) *S. amplum*; 8) *S. plumosum*; 9) *S. stipoideum;* 10) *S. timorense*; 11) *S. encarinatum*; 12) *S. interjectum*; 13) *S. leiocladum*; 14) *S. purpureo-sericeum*; 15) *S. versicolor;* 16) *S. nitidum*; 20) *S. macrospermum*; 21) *S. laxiflorum*; 22) *Cleistochne sorghoides* (see Table 4)

fluorescein isothiocyanate (FITC) conjugated to anti-digoxygenin antibody. The 18S-28S rDNA probe was labeled with biotin and detected with Cy-3-conjugated streptavidin antibody. Surprisingly, CEN38 was detected by FISH to chromosomes of *S. bicolor* (Figure 4), but not to *S. versicolor* (Figure 5, 6), *S. macrospermum* and *S. laxiflorum* (results not shown). CEN38 hybridized strongly to just 10 of 20 *S. bicolor* chromosomes, which is consistent with the reports of Gomez *et al*. (1998) and Zwick *et al*. (2000). The other species displayed no hybridization of CEN38. *Sorghum versicolor* ($2n = 10$), which has the highest copy numbers of CEN38 of the H lineage species, did not display CEN38 FISH signals (Figure 5). *Sorghum macrospermum* ($2n = 40$), which is a relative of *S. bicolor* (Dillon *et al*., 2004), displayed no CEN38 hybridization (Figures 6). *Sorghum laxiflorum* ($2n = 40$) (results not shown), which has the highest copy numbers of CEN38 for species outside the section *Eu-sorghum*, also displayed no hybridization of CEN38. After observing these results, the hybridization-detecting fluorochromes were switched (green-fluorescent FITC to red-fluorescent Cy-3). Cy-3 is noted to have a stronger affinity and higher sensitivity than FITC. Also, CEN38 was allowed to hybridize for 4 d to chromosomes. However, this revealed no detectable hybridization of CEN38 by FISH (Figures 7, 8, 9). Somatic chromosome spreads of *S. bicolor* were used as a positive control side by side on the same slides as *S. versicolor*, *S. laxiflorum* and *S. macrospermum* (data not shown). This also revealed no detectable hybridization of CEN38 by FISH.

FISH of 18S-28S rDNA was detected in all of the species. In *S. bicolor*, 18S-28S rDNA was detected at the submetacentric region of the chromosome 1 (Figure 4).

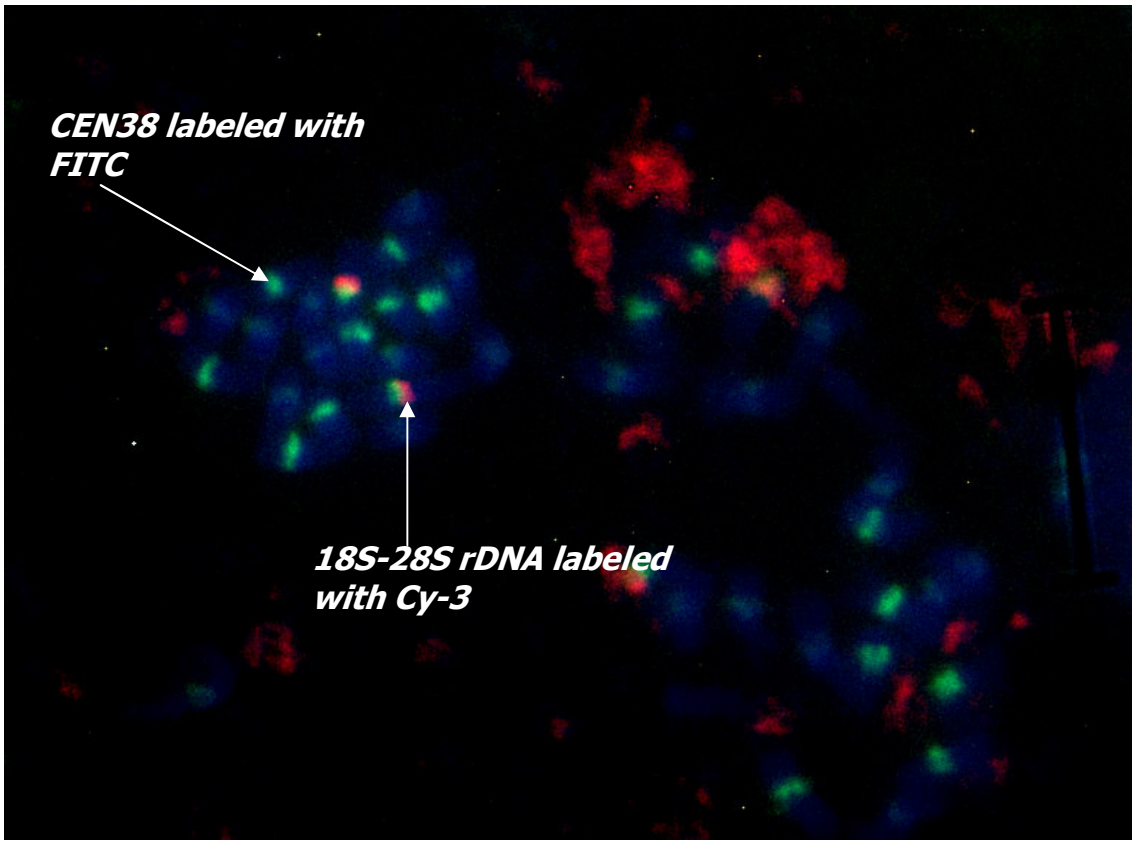


Figure 4. FISH of CEN38 and 18S-28S rDNA to *S. bicolor* chromosomes. FITC-labeled CEN38 (green) hybridizes to 10 of 20 *S. bicolor* chromosomes. Cy-3 labeled 18S-28S rDNA (red) hybridized to the submetacentric regions of the 2 chromosomes (indicated by arrows).

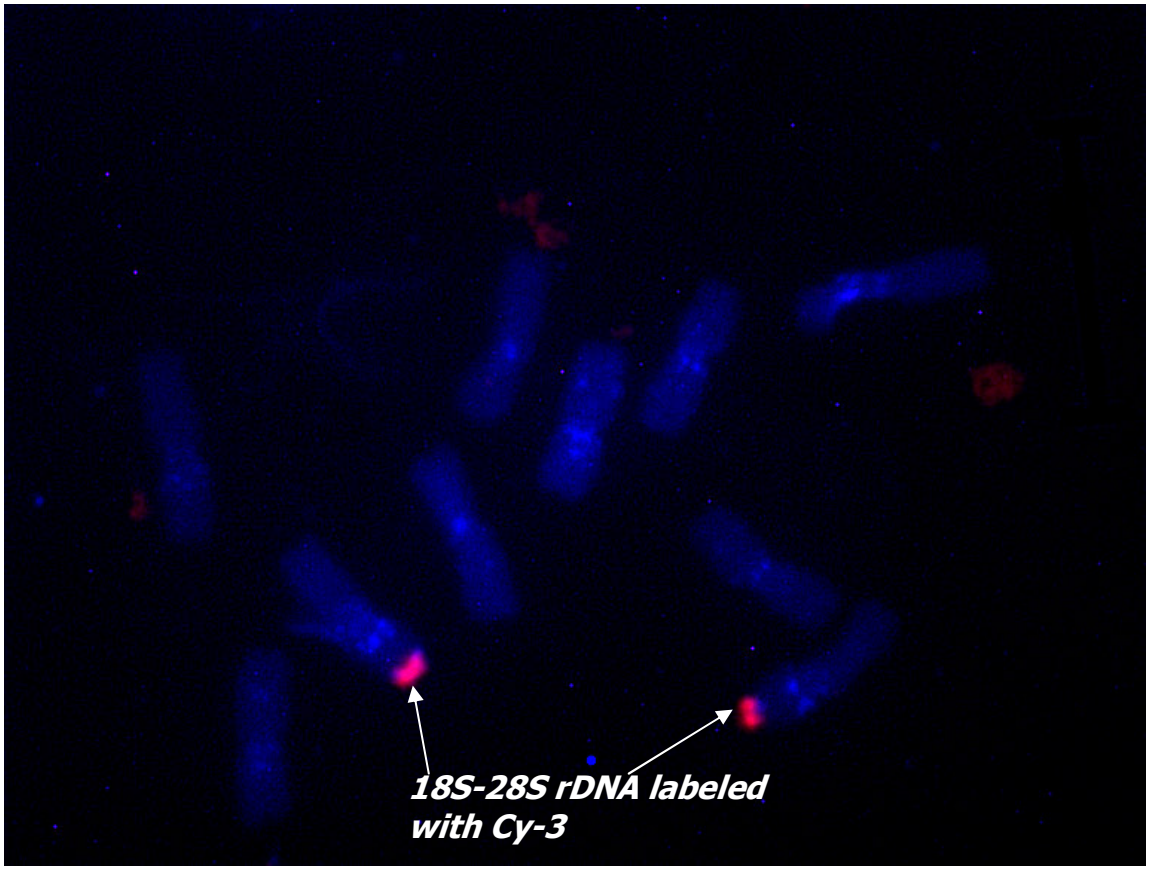


Figure 5. FISH of CEN38 and 18S-28S rDNA to *S. versicolor* (2*n* = 10) chromosomes. FITC-labeled CEN38 (green) displayed no detectable hybridization to *S. versicolor* chromosomes. However, Cy-3 labeled 18S-28S rDNA (red) hybridized to the terminal regions of the chromosomes (indicated by arrows).

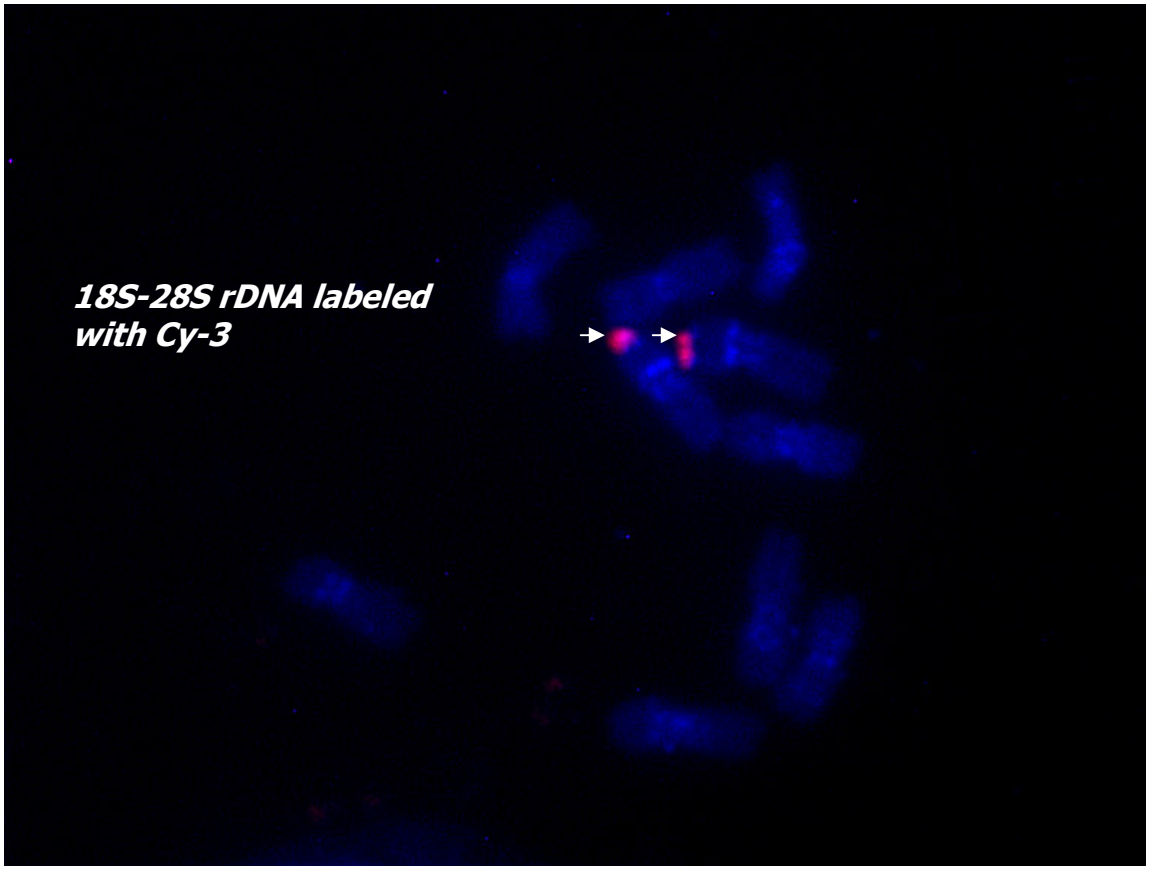


Figure 6. FISH of Cy-3 labeled CEN38 and 18S-28S rDNA to *S. versicolor* ($2n = 10$) chromosomes. Cy-3 labeled CEN38 (red) displayed no detectable hybridization to *S. versicolor* chromosomes. However, Cy-3 labeled 18S-28S rDNA (red) hybridized to the terminal regions of one pair of chromosomes (indicated by arrows).

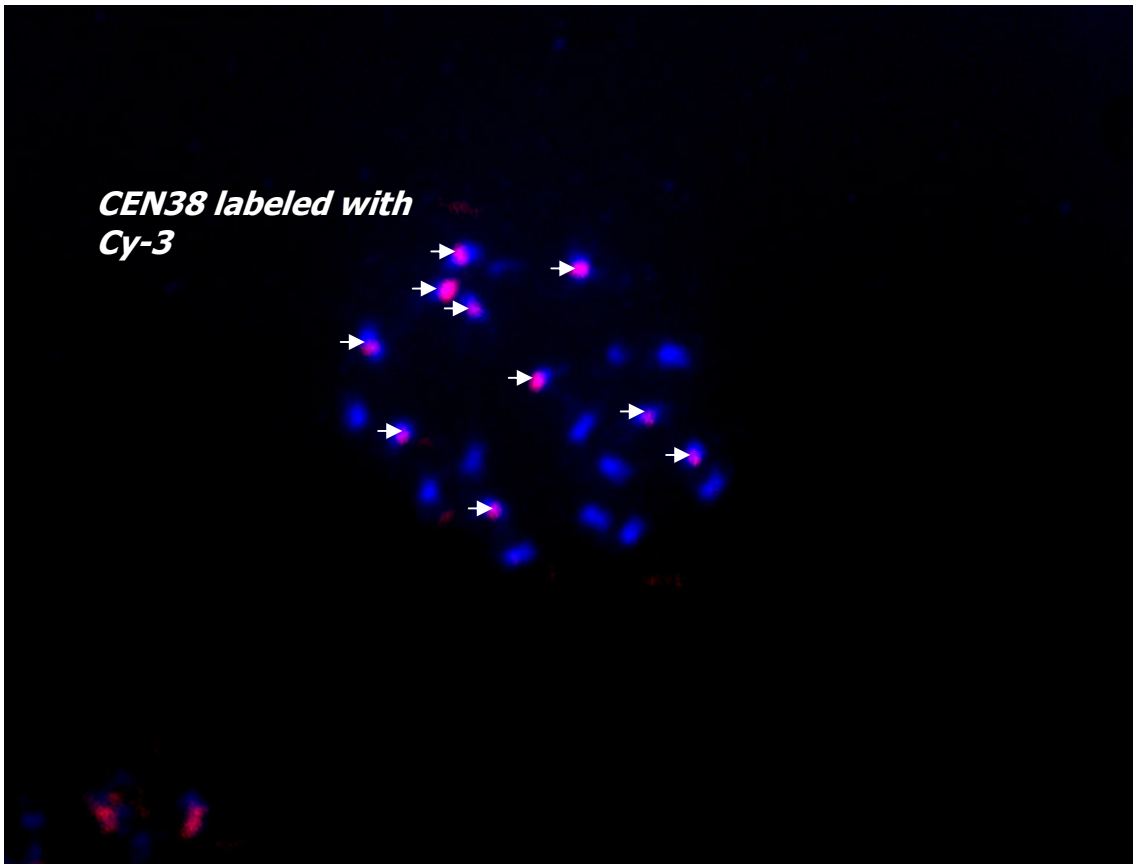


Figure 7. FISH of Cy-3 labeled CEN38 and 18S-28S rDNA to *S. bicolor* chromosomes. Cy-3 labeled CEN38 (red) hybridized to the pericentrimeric regions of 10 of 20 *S. bicolor* chromosomes (indicated by arrows).

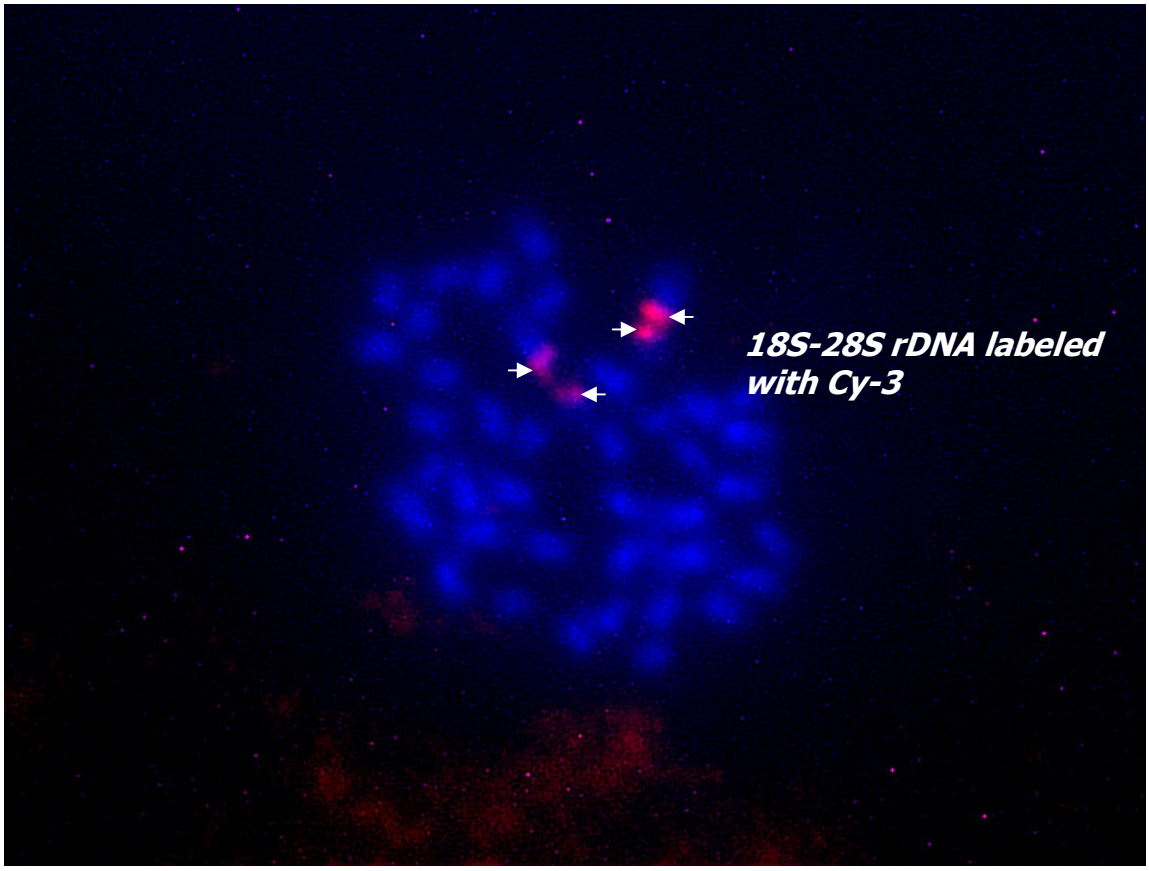


Figure 8. FISH of CEN38 and 18S-28S rDNA to *S. macrospermum* ($2n = 40$) chromosomes. FITC-labeled CEN38 (green) displayed no detectable hybridization to *S. macrospermum* chromosomes. However, Cy-3 labeled 18S-28S rDNA (red) hybridized to the terminal regions of the 4 chromosomes (indicated by arrows).

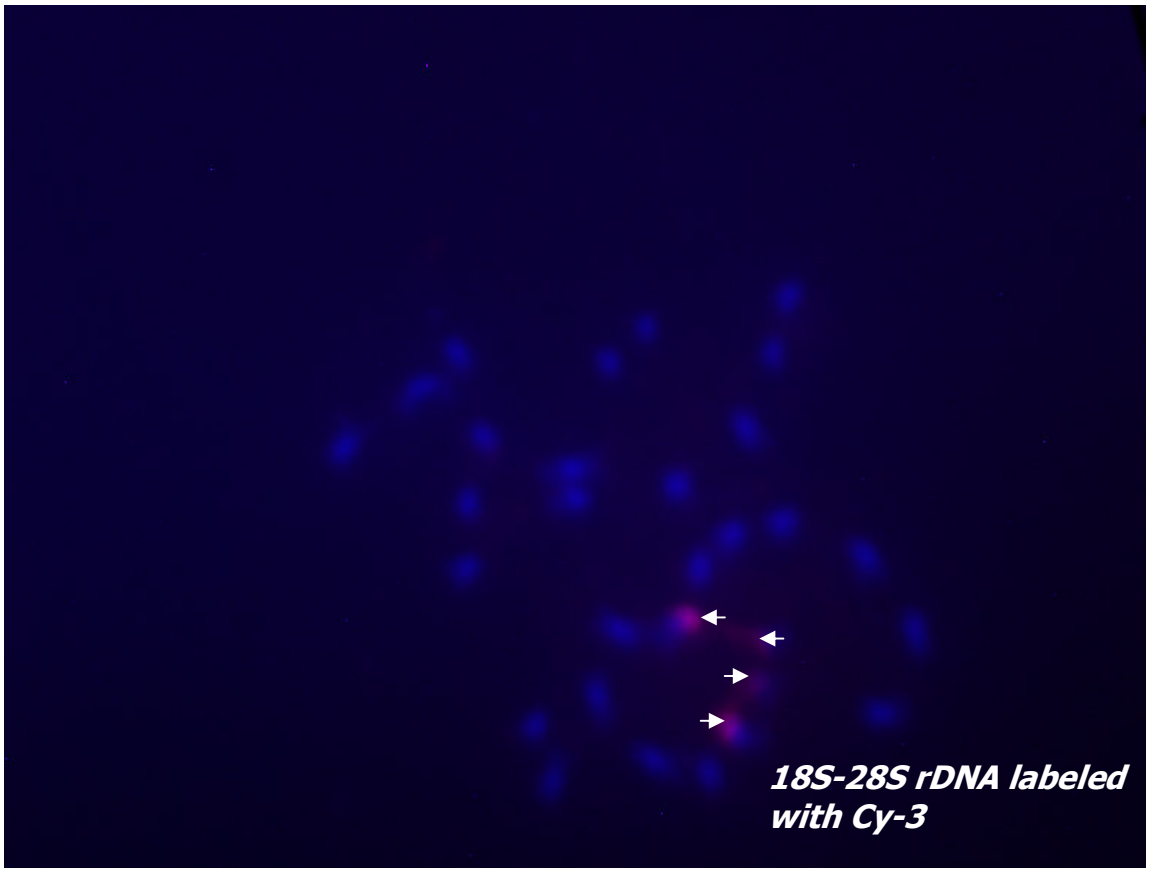


Figure 9. FISH of Cy-3 labeled CEN38 and 18S-28S rDNA to *S. macrospermum* (2*n* = 40 chromosomes. Cy-3 labeled CEN38 (red) displayed no detectable hybridization to *S. macrospermum* chromosomes. However, Cy-3 labeled 18S-28S rDNA (red) hybridized to the terminal region of 4 chromosomes (indicated by arrows) Only a partial chromosome complement is apparent.

Sorghum versicolor (2*n* = 10) yielded 18S-28S rDNA sites on the terminal ends of the largest chromosome (Figures 5, 6). *Sorghum macrospermum* yielded the most intriguing results, having two major and minor sites located on the terminal ends of two chromosome pairs (Figures 8, 9).

Sorghum versicolor chromosomes stained with 4', 6-diamidino-2-phenylindole (DAPI) fluorescent dye yielded dark-staining, heterochromatic regions flanking the centromere (Figure 5, 6), whereas DAPI staining on *S. bicolor* chromosomes yielded relatively more pericentric heterochromatin.

Southern blots and hybridization of restriction-enzyme digested genomic DNA with CEN38

Genomic DNA was digested separately with two restriction enzymes, *Hind*III and *Pst*I. CEN38 used as probes were hybridized to genomic DNA of 21 *Sorghum* species. Three autoradiograms were produced from the Southern blots, each with different post-hybridization washing stringencies. The autoradiogram of the 0.5x SSC washed membrane showed taxonomic-wide hybridization of CEN38 (Figure 10). CEN38 digested with *Pst*I displayed no relevant results in many species compared to species digested with *Hind*III-digested CEN38. *Sorghum plumosum*, *S. leiocladum* and S. *purpureo-sericeum,* displayed a >23 kb fragment was obtained. The presence of CEN38-positive DNA is inconsistent with the dot blot results that did not detect CEN38-positive sequences in *S. plumosum*. However, the CEN38-positive >23 kb fragment detected in *S. plumosum*, *S. purpureo-sericeum* and *S. leiocladum* was not detected (or only barely detected) when post-hybridization washing stringency increased (0.5x – 0.1x

SSC). This indicates that the fragments are not very similar in nucleotide sequence to CEN38.

The most intriguing result of the Southern blot was the 4 bands displayed by *S. versicolor*. The approximate size of these bands is 24 kb, 6.0 kb, 5.0 kb and 840 bp. These bands do not display a pattern expected of tandem repeats. The autoradiograms of 0.2x and 0.1x SSC washed membranes (increased stringency) showed similar results for this species (Figures 11, 12). In the other species, a distinct smear was shown. This suggests that CEN38 may be interspersed in these species. The array of tandem repeats shown in species of the section *Eu-sorghum* is consistent with results from the previous study of Miller *et al*. (1998) where a ~280 bp ladder was apparent.

PCR amplification of CEN38

Four primer sets designed from CEN38 sequence data were used to detect potential CEN38 elements in 10 *Sorghum* species. The PCR products were detected and sized after agarose gel electrophorese (Figures 13). PCR products were not obtained from primer set 1. Primer sets 2, 3 and 4 (results for 4 not shown) generally produced the same results. Primer set 2 produced a ladder-based array of tandem repeats (<280 bp) in species of the section *Eusorghum* (*S. bicolor, S. halepense, S. propinquum*). Species *S. macrospermum* and *S. laxiflorum*, which are related to *S. bicolor* (Dillon *et al*., 2004), yielded one amplified fragment, which was extrapolated as <280 bp. Nevertheless, primer set 3 produced tandem arrays in *S. macrospermum* and *S. laxiflorum*, which were nearly the identical size of the amplified <280 bp repeat

Figure 10. Autoradiogram of Southern blot hybridizations of radioactively-labeled pCEN38 to genomic DNA from 21species of *Sorghum* (0.5x stringency). The amount of genomic DNA was 2 μg per lane. Upper autoradiogram lanes (A): 1) λDNA/*Hind*III; 2) *S. brachypodum*/*Hind*III; 3) *S. brachypodum*/*Pst*I; 4) *S. matarankense*/*Hind*III; 5) S. matarankense/*Pst*I; 6) *S. extans/Hind*III; 7) *S. extans/Pst*I; 8) *S. angustum/Hind*III; 9) *S. angustum/Pst*I; 10) *S. intrans/Hind*III; 11) *S. intrans/Pst*I; 12) *S. bulbosum/Hind*III; 13) *S. bulbosum/Pst*I; 14) *S. amplum/Hind*III; 15) *S. amplum/Pst*I; 16) *S. plumosum/Hind*III; 17) *S. plumosum/Pst*I; 18) *S. stipoideum/Hind*III; 19) *S. stipoideum/Pst*I; 20) *S. timorense/Pst*I; 21) *S. timorense/Hind*III; 22) *S. encarinatum/Hind*III; 23) *S. encarinatum/Pst*I; 24) *S. interjectum/Hind*III; 25) *S. interjectum/Pst*I; 26) *S. leiocladum/Hind*III; 27) *S. leiocladum/Pst*I; 28) *S. purpureo-sericeum/Hind*III; 29) *S. purpureo-sericeum/Pst*I; 30) λDNA/*Hind*III
Lower autoradiogram lanes (B): 1) λDNA/*Hind*III; 2) *S. versicolor/Hind*III; 3) *S. versicolor/Pst*I; 4) *S. nitidum/Hind*III; 5) *S. nitidum/Pst*I; 6) *S. bicolor/Hind*III; 7) *S. bicolor/Pst*I; 8) *S. halepense/Hind*III; 9) *S. halepense/Pst*I; 10) *S. propinquum/Hind*III; 11) *S. propinquum/Pst*I; 12) *S. macrospermum/Hind*III; 13) *S. macrospermum/Pst*I; 14) *S. laxiflorum/Hind*III; 15) *S. laxiflorum/Pst*I; 16) *Cleistochne sorghoides*/*Hind*III; 17) *Cleistochne sorghoides*/*Pst*I; 18) λDNA/*Hind*III (see Table 3)

A) 1 2 3 4 5 6 7 8 9 10 11 12 13141516171819 20 21 222324 25 26 27 282930

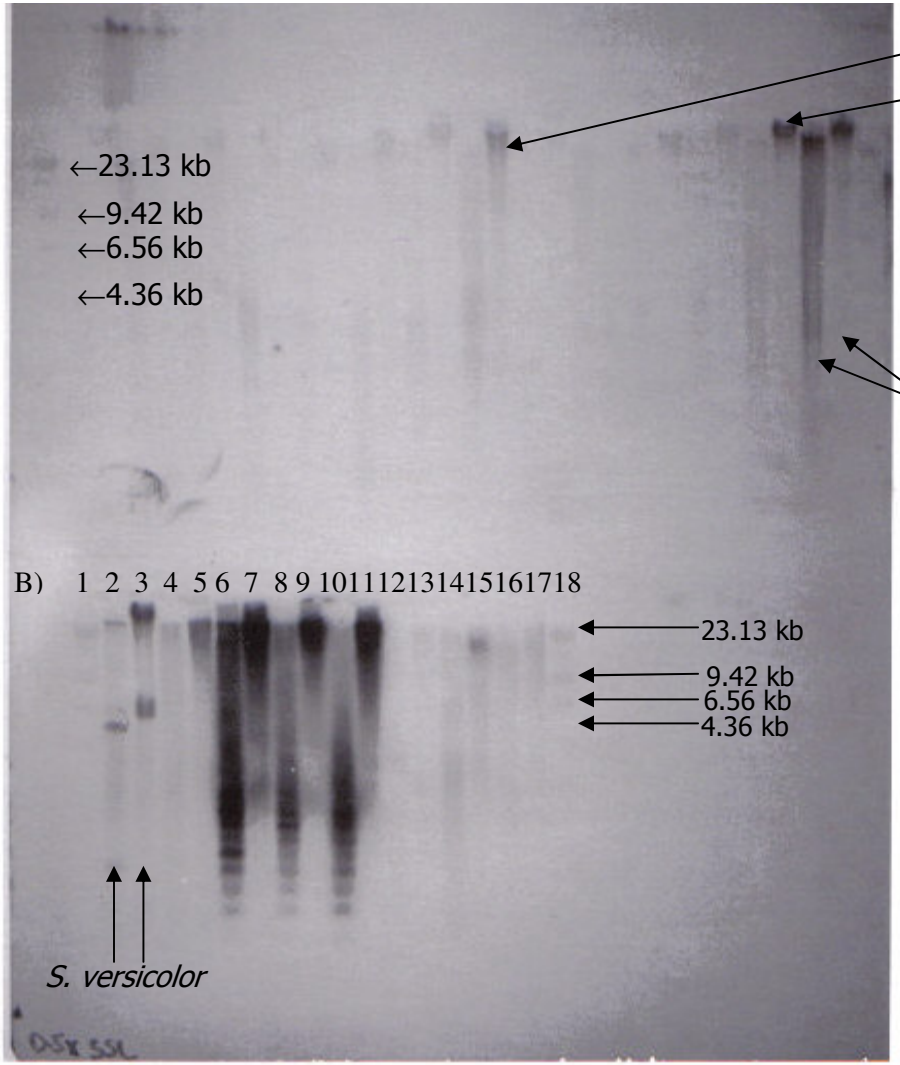


Figure 11. Autoradiogram of Southern blot hybridizations of radioactively-labeled pCEN38 to genomic DNA from 21species of *Sorghum* (0.2x stringency). The amount of genomic DNA was 2 µg per lane. Upper autoradiogram lanes (A): 1) λDNA/*Hind*III; 2) *S. brachypodum*/*Hind*III; 3) *S. brachypodum*/*Pst*I; 4) *S. matarankense*/*Hind*III; 5) S. matarankense/*Pst*I; 6) *S. extans/Hind*III; 7) *S. extans/Pst*I; 8) *S. angustum/Hind*III; 9) *S. angustum/Pst*I; 10) *S. intrans/Hind*III; 11) *S. intrans/Pst*I; 12) *S. bulbosum/Hind*III; 13) *S. bulbosum/Pst*I; 14) *S. amplum/Hind*III; 15) *S. amplum/Pst*I; 16) *S. plumosum/Hind*III; 17) *S. plumosum/Pst*I; 18) *S. stipoideum/Hind*III; 19) *S. stipoideum/Pst*I; 20) *S. timorense/Pst*I; 21) *S. timorense/Hind*III; 22) *S. encarinatum/Hind*III; 23) *S. encarinatum/Pst*I; 24) *S. interjectum/Hind*III; 25) *S. interjectum/Pst*I; 26) *S. leiocladum/Hind*III; 27) *S. leiocladum/Pst*I; 28) *S. purpureo-sericeum/Hind*III; 29) *S. purpureo-sericeum/Pst*I; 30) λDNA/*Hind*III.
Lower autoradiogram lanes (B): 1) λDNA/*Hind*III; 2) *S. versicolor/Hind*III; 3) *S. versicolor/Pst*I; 4) *S. nitidum/Hind*III; 5) *S. nitidum/Pst*I; 6) *S. bicolor/Hind*III; 7) *S. bicolor/Pst*I; 8) *S. halepense/Hind*III; 9) *S. halepense/Pst*I; 10) *S. propinquum/Hind*III; 11) *S. propinquum/Pst*I; 12) *S. macrospermum/Hind*III; 13) *S. macrospermum/Pst*I; 14) *S. laxiflorum/Hind*III; 15) *S. laxiflorum/Pst*I; 16) *Cleistochne sorghoides*/*Hind*III; 17) *Cleistochne sorghoides*/*Pst*I; 18) λDNA/*Hind*III (see Table 3).

A) 1 2 3 4 5 6 7 8 9 10 11 12 13141516 171819 2021 22 23 24 2526 27 28 29 30

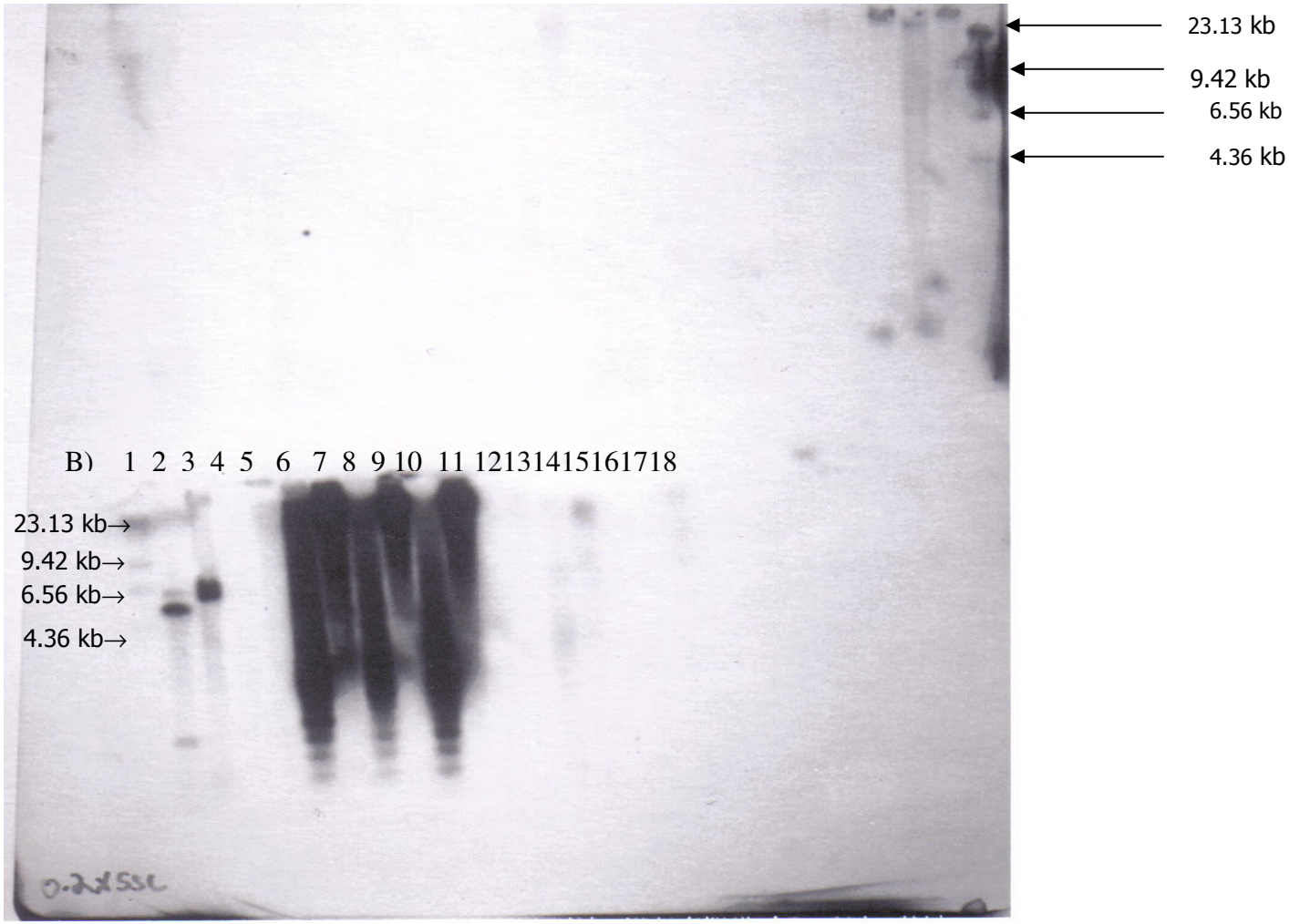
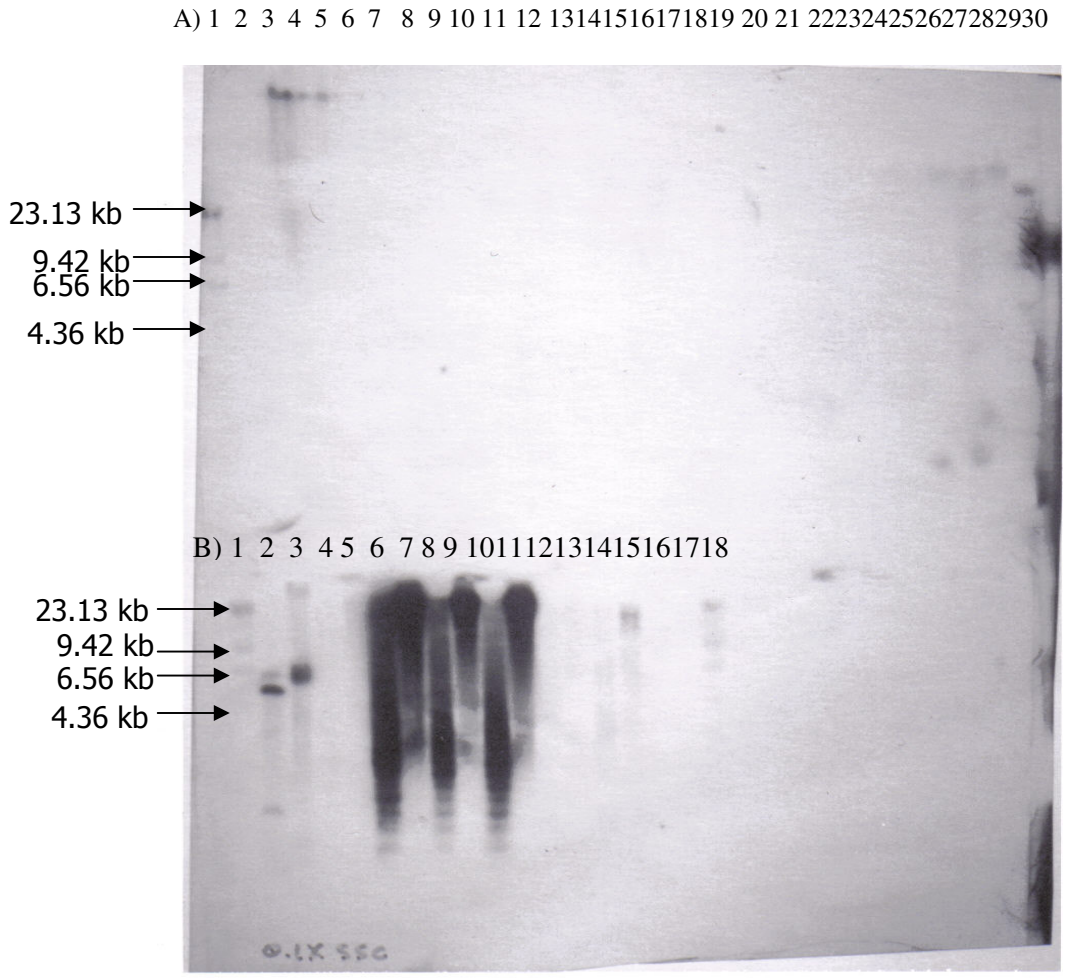
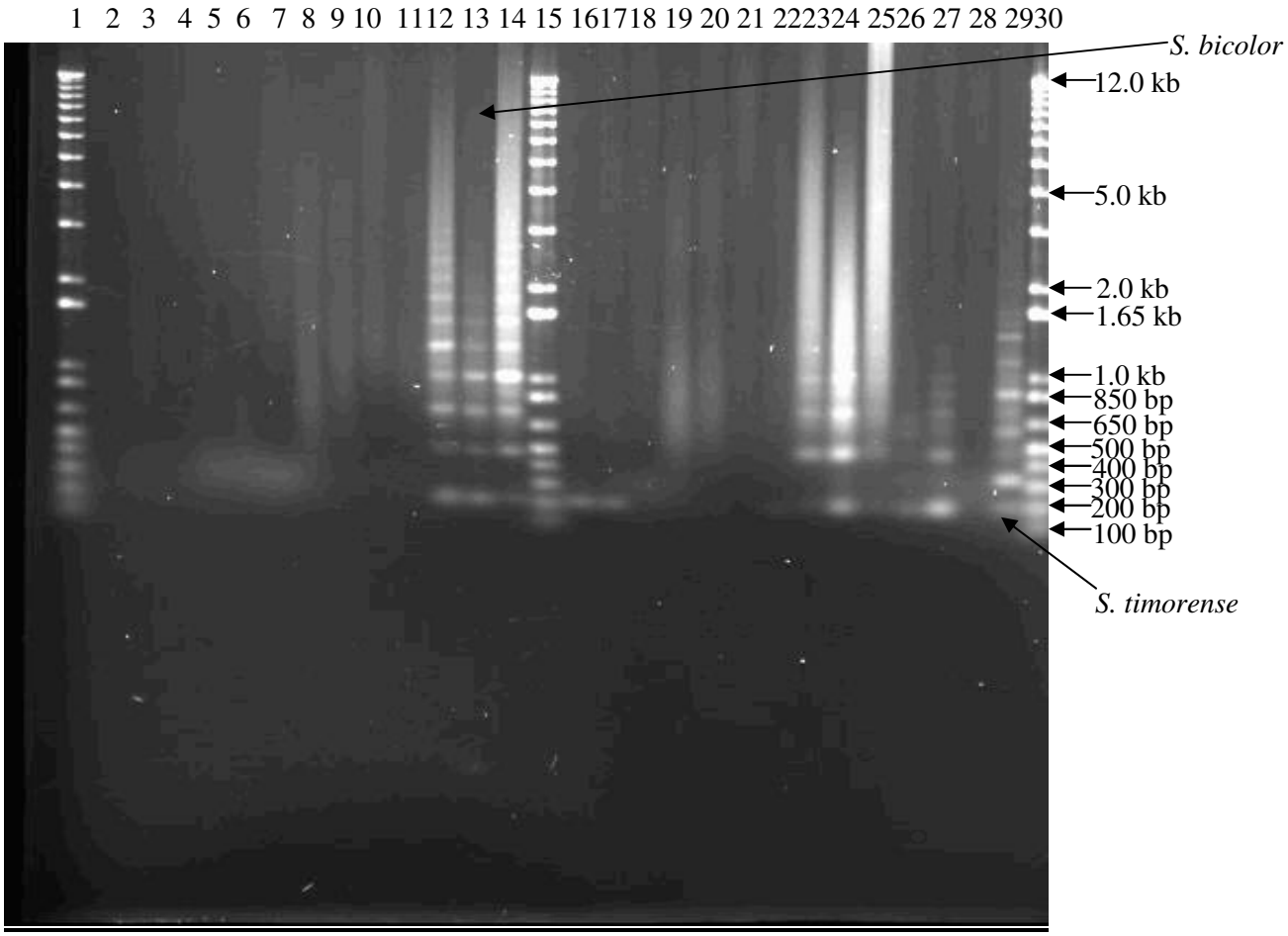


Figure 12. Autoradiogram of Southern blot hybridizations of radioactively-labeled pCEN38 to genomic DNA from 21species of *Sorghum* (0.1x stringency). The amount of genomic DNA was 2 μg per lane. Upper autoradiogram lanes (A): 1) λDNA/*Hind*III; 2) *S. brachypodum*/*Hind*III; 3) *S. brachypodum*/*Pst*I; 4) *S. matarankense*/*Hind*III; 5) S. matarankense/*Pst*I; 6) *S. extans/Hind*III; 7) *S. extans/Pst*I; 8) *S. angustum/Hind*III; 9) *S. angustum/Pst*I; 10) *S. intrans/Hind*III; 11) *S. intrans/Pst*I; 12) *S. bulbosum/Hind*III; 13) *S. bulbosum/Pst*I; 14) *S. amplum/Hind*III; 15) *S. amplum/Pst*I; 16) *S. plumosum/Hind*III; 17) *S. plumosum/Pst*I; 18) *S. stipoideum/Hind*III; 19) *S. stipoideum/Pst*I; 20) *S. timorense/Pst*I; 21) *S. timorense/Hind*III; 22) *S. encarinatum/Hind*III; 23) *S. encarinatum/Pst*I; 24) *S. interjectum/Hind*III; 25) *S. interjectum/Pst*I; 26) *S. leiocladum/Hind*III; 27) *S. leiocladum/Pst*I; 28) *S. purpureo-sericeum/Hind*III; 29) *S. purpureo-sericeum/Pst*I; 30) λDNA/*Hind*III.
Lower autoradiogram lanes (B): 1) λDNA/*Hind*III; 2) *S. versicolor/Hind*III; 3) *S. versicolor/Pst*I; 4) *S. nitidum/Hind*III; 5) *S. nitidum/Pst*I; 6) *S. bicolor/Hind*III; 7) *S. bicolor/Pst*I; 8) *S. halepense/Hind*III; 9) *S. halepense/Pst*I; 10) *S. propinquum/Hind*III; 11) *S. propinquum/Pst*I; 12) *S. macrospermum/Hind*III; 13) *S. macrospermum/Pst*I; 14) *S. laxiflorum/Hind*III; 15) *S. laxiflorum/Pst*I; 16) *Cleistochne sorghoides*/*Hind*III; 17) *Cleistochne sorghoides*/*Pst*I; 18) λDNA/*Hind*III (see Table 3).


A) 1 2 3 4 5 6 7 8 9 10 11 12 13141516171819 20 21 222324252627282930
23.13 kb
9.42 kb
6.56 kb
4.36 kb
B) 1 2 3 4 5 6 7 8 9 101112131415161718
23.13 kb
9.42 kb
6.56 kb
4.36 kb
0.1X SSC

produced by primer set 2 in the species of the section *Eu-sorghum*. However, *S. timorense*, a species that displayed no hybridization to CEN38 on the dot or Southern blots, produced a ladder of fragments (~ 225 bp, 350 bp, 475 bp, 600 bp, 725 bp, 850 bp, 975, 1.1 kb, etc…) when amplified with primer set 3. The ladder depicted a tandem array based on repeats of ~ 125 bp.

Figure 13. Photograph of PCR products of 10 *Sorghum* species using 4 different primer sets. Gel lanes: 1) 1 kb plus ladder; 2) *S. bicolor/* Set #1; 3) *S. halepense/* Set #1*; 4) S. propinquum/* Set #1; 5) *S. laxiflorum*/Set #1; 6) *S. macrospermum/* Set #1; 7) *S. versicolor/* Set #1; 8) *S. timorense/* Set #1; 9) *S. nitidum/* Set #1; 10) *S. brachypodum*/ Set #1*;* 11) *Cleistochne sorghoides*/ Set #1; 12) *S. bicolor*/Set #2; 13) *S. halepense/* Set #2; 14) *S. propinquum/* Set #2; 15) 1 kb plus ladder; 16) *S. laxiflorum/* Set #2; 17) *S. macrospermum/* Set #2; 18) *S. versicolor/* Set #2; 19) *S. timorense/* Set #2; 20) *S. nitidum/*Set #2; 21) *S. brachypodum*/ Set #2; 22) *Cleistochne sorghoides*/ Set #2; 23) *S. bicolor/* Set #3; 24) *S. halepense/* Set #3; 25) *S. propinquum/* Set #3; 26) *S. laxiflorum/* Set #3; 27) *S. macrospermum/* Set #3; 28) *S. versicolor/* Set #3; 29) *S. timorense/* Set #3; 30) 1 kb plus ladder, (see Table 4).


1 2 3 4 5 6 7 8 9 10 1112 13 14 15 161718 19 20 21 222324 2526 27 28 2930
S. bicolor
12.0 kb
5.0 kb
2.0 kb
1.65 kb
1.0 kb
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp
S. timorense

DISCUSSION AND SUMMARY

The current study detected the presence of sequences with homology to CEN38 in 16 of the 21 *Sorghum* species analyzed using dot blotting. When detected, the copy number was estimated to range from 15 to ~ 21,000 copies. The taxonomic distribution of CEN38 provides interesting, but limited, data concerning the phylogenetic relationships among species of the genus *Sorghum*. The data obtained have limitations and caution needs to be exercised in its interpretation. The hybridization detected to DNA of dot blots and to Southern transferred restriction fragments does not prove that CEN38 was detected. Hybridizations may have resulted from sequences that share some regions of homology but are not in the CEN38 family. Dillon *et al*. (2004) detected two sister lineages (A and H in Fig. 1) in a *Sorghum* phylogenetic tree constructed from an ITS1 and *ndh*F DNA sequence comparison. Lineage A contains the species of the *Eu-sorghum* section and two other species, *S. laxiflorum* (*Heterosorghum*) and *S. macrospermum* (*Chaetosorghum*). This lineage has a base chromosome number of x = 10 and the chromosomes are relatively small. The sister lineage (H) in the phylogenetic tree of Dillon et al. (2004) contains species with relatively large chromosomes with a basic chromosome number of x = 5. The evolutionary relationship of lineages A and H remains unresolved. CEN38 has a narrow taxonomic distribution and, with exception of sugarcane, has not been detected in gramineous species other than *Sorghum* (Miller *et al*., 1998; Zwick *et al*., 2000). Therefore, the presence of this sequence in species of both lineages A and H would support the hypothesis that they share a common ancestor and are therefore monophyletic in origin. The current study suggests that CEN38 may

reside in the genome of some H lineage species, but the data are not conclusive. However, CEN38 sequences are abundant in DNA of species of the *Eu-sorghum* section. They also occur, albeit at much lower frequencies, in *S. macrospermum* and *S. laxiflorum* as detected by dot blot hybridization of CEN38 to undigested DNA, and by hybridization of CEN38 to Southern blotted restriction fragments. The occurrence of CEN38 in *S. laxiflorum* and *S. macrospermum* supports the hypothesis that they are closely related to *S. bicolor*.

Spangler (2003) proposed that the genus *Sorghum* be split into three genera, *Sorghum*, *Sarga*, and *Vacoparis*. Only species of the *Eu-sorghum* section and *S. nitidum* were retained in the genus *Sorghum*. All the *Stiposorghum* and *Para-sorghum* species, except *S. nitidum*, were reclassified into the genus Sarga. *Sorghum laxiflorum* and *S. macrospermum* were placed into a new genus, *Vacoparis*. Such a reclassification is not totally supported by cytology and DNA sequence comparisons (Price *et al*. 2005a; Dillon *et al*., 2004). This, coupled with the unresolved issue of the monophyletic vs. polyphyletic origin of the $x = 5$ and $x = 10$ lineages, indicates that reclassification of the genus was premature. The similarities in base chromosome number and genome sizes of *S. laxiflorum*, *S. macrospermum*, and species of the section *Eu-sorghum* (Price et al., 2005a), the apparent presence of CEN38 sequences arranged in a ~ 140 bp ladder in *S. laxiforum*, *S. macrospermum*, and species of the *Eu-sorghum* section, and the ability of hybrids to be recovered from crosses of *S. macrospermum* and *S. bicolor* (Price et al., 2005b) provide strong support for retaining these species in the genus *Sorghum*. *Sorghum nitidum*, based on chromosome size, genome size and DNA sequence

comparisons (Price et al., 2005a; Dillon et al., 2004), aligns with the lineage containing the *Para-sorghum* and *Stiposorghum* sections. Furthermore, Garber (1950) showed through meiotic metaphase analysis of hybrids that *S. nitidum* was an allotetraploid containing one genome of 5 chromosomes from *S. leiocladum* and a second unidentified set of 5 chromosomes. Based on this evidence alone, *S. nitidum* aligns with the other x = 5 chromosome *Sorghum* species and not with the *Eusorghum* species as proposed by Spangler (2003).

Gomez *et al*. (1998) and Zwick *et al*. (2000) reported that CEN38 preferentially hybridized to 10 of 20 *S. bicolor* somatic chromosomes. In this study, *S. bicolor* (~ 21,000 copies of CEN38) was used as a positive control to confirm that the FISH procedure was working. No hybridization of CEN38 was detected by FISH for *S. laxiflorum* (copy number = 436)*, S. macrospermum* (copy number = 71) and *S. versicolor* (copy number = 278). Several factors may have contributed to this lack of detection of CEN38. First, there may not be ample amounts of CEN38 in the chromosomes to detect hybridization by the resolution of FISH. Two, the CEN38 sequences may be dispersed and not organized in tandem arrays in the wild species of *Sorghum*. If so, it would be very difficult to detect CEN38 by FISH, given the low copy numbers in species of sections other than *Eu-sorghum*. Three, short tandem arrays may exist but be interspersed in small clusters throughout the genome that would not be detectable by FISH.

The 18S-28S ribosomal DNA sequences encode ribosomal RNA (rRNA). In eukaryotes, rDNA is arranged in tandemly-repeated units. The transcribed sequences are

highly conserved among plants. FISH of 18S-28S rDNA revealed a secondary constriction site in each chromosome where it is detected, which reveals the nucleolus-organizing region (NOR) (Islam-Faridi *et al*., 2002). In this study, 18S-28S rDNA was used as a positive marker and a determinant of the nucleolus-organizing region in four *Sorghum* species. FISH of 18S-28S rDNA to *S. bicolor* chromosomes revealed the NOR at a submetacentric location on the largest chromosome, i.e., chromosome 1 (Islam-Faridi *et al*., 2002; Kim *et al*., 2005a) (Figure 4). FISH of 18S-28S rDNA to *S. macrospermum* yielded 4 discrete signals, which is indicative of its apparent tetraploid nature. Chromosomes of *S. macrospermum* are very small, therefore it is difficult to determine if the NOR is located in an intercalary or terminal position in highly contracted metaphase chromosomes. FISH of 18S-28S rDNA to *S. versicolor* showed that the NOR is located on the terminal end of the largest chromosome. This is consistent with the previous studies by Garber (1950) and Sang and Liang (2000).

Primers based on sequences present in CEN38 amplified fragments in a ~ 280 bp ladder in *S. laxiflorum* and *S. macrospermum*, as was also observed for *Eu-sorghum* DNAs. This indicates that CEN38 is present in a tandam array. As mentioned earlier, the occurrence of CEN38 supports the hypothesis that *S. laxiflorum*, *S. macrospermum*, and the *Eu-sorghum* species are phylogenetically closely related and share ancestors that also possessed CEN38.

The highest estimated copy number in the genome of H lineage species is 278 for *S. versicolor*. Restriction fragments of *S. versicolor* that hybridized to CEN38 did not form a ladder, as did restriction fragments of *Eu-sorghum* DNA, nor were CEN38

sequences amplified by any of the primer sets used for PCR. Detection of a ladder in restriction fragments containing tandemly repetitive CEN38 sequences is contingent on the conservation of restriction sites in CEN38 DNA (*Hind*III or *Pst*I). PCR amplification of CEN38 (if present) in genomic DNA of divergent *Sorghum* species requires the conservation of sequences complementary to the PCR primers used. If these have undergone nucleotide substitutions, especially at the 3' end of the sequence, their ability to anneal to primers may be lost. CEN38 hybridized to large restriction fragments of *S. versicolor* estimated to be 840 bp, 5 kb, 6 kb, and 24 kb. Such a pattern does not support a tandem arrangement of CEN38 repeats in *S. versicolor*. Rather, it suggests that the CEN38 sequences are a part of larger unrelated fragments.

Transposable elements are abundant and ubiquitous in genomes of higher plant species including *Sorghum* (Voytas *et al*., 1992; SanMiguel and Bennetzen, 1998). The CEN38-positive large restriction fragments observed in *S. versicolor* may represent pieces of transposons that have one or more CEN38-like sequences embedded within them.

This study provides the foundation for several lines of future research. Positive CEN38 restriction fragments of *S. versicolor* could be cloned and sequenced to identify if they belong in the CEN38 repeat family. Comparing the sequences surrounding the CEN38 fragments to sequences in GenBank would allow one to determine if they are embedded in transposable elements. The nature of the ~125 bp fragment of *S. timorense* DNA and DNA fragments of *S. laxiflorum* and *S. macrospermum* that were amplified using primer sets for CEN38 of S. *bicolor* are worthy of further investigation. The DNA

fragments of these species electrophoresed in agarose gels could be Southern transferred to nylon filters and hybridized with labeled CEN38. Since CEN38 was not detected in *S. timorense* DNA by other techniques, it would not be expected to hybridize to PCR amplified fragments of *S. timorense* DNA. If no hybridization to CEN38 is observed, the fragments would be members of a different tandemly arranged repeat family. These sequences could be further characterized by cloning into vectors followed by DNA sequencing. On the other hand, it is likely that the ~280 bp amplified repeats of *S. laxiflorum* and *S. macrospermum* would be detected as members of the CEN38 family.

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