Published November, 2010

ORIGINAL RESEARCH

Genome-size Variation in Switchgrass (*Panicum virgatum*): Flow Cytometry and Cytology Reveal Rampant Aneuploidy

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Abstract

Switchgrass (*Panicum virgatum* L.), a native perennial dominant of the prairies of North America, has been targeted as a model herbaceous species for biofeedstock development. A flow-cytometric survey of a core set of 11 primarily upland polyploid switchgrass accessions indicated that there was considerable variation in genome size within each accession, particularly at the octoploid ($2n = 8X = 72$ chromosome) ploidy level. Highly variable chromosome counts in mitotic cell preparations indicated that aneuploidy was more common in octoploids (86.3%) than tetraploids (23.2%). Furthermore, the incidence of hyper- versus hypoaneuploidy is equivalent in tetraploids. This is clearly not the case in octoploids, where close to 90% of the aneuploid counts are lower than the euploid number. Cytogenetic investigation using fluorescent in situ hybridization (FISH) revealed an unexpected degree of variation in chromosome structure underlying the apparent genomic instability of this species. These results indicate that rapid advances in the breeding of polyploid biofuel feedstocks, based on the molecular-genetic dissection of biomass characteristics and yield, will be predicated on the continual improvement of our understanding of the cytogenetics of these species.

Published in The Plant Genome 3:130–141. Published 16 Nov. 2010.
doi: 10.3835/plantgenome2010.04.0010

5585 Guilford Rd., Madison, WI 53711 USA
An open-access publication

"*Species therefore evidently vary in their fundamental chromosome organization or, as we may say, genetic structure. In some this structure is less rigid, more flexible, better buffered, than in others: and it is clear that where it is more flexible, where the chromosomes are more nearly neutral, changes in the basic number by gain, and less often by loss, of whole chromosomes will more readily come about…*" (Darlington, 1956)

"*From a practical standpoint, a varietal improvement program can be outlined more intelligently when the cytogenetical behavior of the species is understood.*" (Myers and Hill, 1940)

THE GENETIC STRUCTURE of an organism has profound effects on all aspects of its basic biology. The manner in which its genes are distributed, whether in few or many chromosomes, the numbers of whole sets of chromosomes (ploidy levels) present, and the presence or absence of individual copies of chromosomes, all contribute to its phenotype. Ploidy differences among individuals in a population will determine mating patterns and gene flow, affecting the distribution of genetic variation both within and among individuals. In natural populations, the resulting phenotypic variation is the foundation for ecotype formation, and, ultimately, over time, can lead to speciation. In a breeding program, it is the raw material for cultivar development and improvement of the crop. Thus, a thorough knowledge of the extent of any form of genome-size variation in a study organism is a critical first step for any type of genetics or genomics research program.

Switchgrass (*Panicum virgatum* L.) has been the subject of intensive and extensive agronomic and breeding research for biofeedstock production since it was

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identified by the United States Department of Energy (US-DOE) as the model herbaceous species for biofeedstock development (McLaughlin and Kszos, 2005). As part of the first phase of an association mapping study of this known polyploid, we reviewed the literature pertaining to chromosome-number variation in switchgrass cultivars and natural populations, and determined that there was sufficient potential for a high diversity in chromosome numbers to warrant an in-depth study of the germplasm in our own association and linkage populations. Of the 22 grass species screened by Oak Ridge National Laboratory as potential biofeedstock candidates (Turhollow et al., 1988), virtually all were polyploid, and switchgrass held the dubious distinction of having not only one of the highest numbers of known ploidy levels, but also the highest observed incidence of aneuploidy among the candidate species (Supplemental Table 1).

The extent of the chromosome-number variation found in switchgrass is illustrated by the long list of vegetative chromosome numbers ($2n$) given in its description in the most recent Flora of North America: 18, 21, 25, 30, 32, 35, 36, 54–60, 67–72, 74, 77, 90, 108 (Barkworth et al., 2003). The majority of these have been confirmed by consultation of the primary literature (Supplemental Table 2). With a base chromosome number of 9, the species-wide range in ploidy levels found in natural populations is diploid ($2X$) to duodecaploid ($12X$) (Nielsen, 1944). Aneuploid counts have been reported sporadically in the older literature (Brown, 1948; Barnett and Carver, 1967; Brunken and Estes, 1975). Populations containing plants of multiple ploidies were regularly discovered (Church, 1940; Nielsen, 1944; McMillan and Weiler, 1959; Porter, 1966). In the most extensive cytological survey of chromosome number variation in switchgrass to date, McMillan and Weiler (1959) counted chromosomes in pollen mother cells from clones originating from 43 populations spanning the central Midwest, from southern Canada to northern Texas. Of the 29 populations where they sampled more than two plants, 31% contained plants from three ploidy levels ($2n = 4X = 36$ chromosomes; $6X = 54$; $8X = 72$), 31% with two, and 38% with one (either $2n = 36$ or 72). The pattern of chromosome number variation that was revealed by all of these studies was eventually incorporated in the descriptions of ecotypes in switchgrass: it was determined that the lowland type is exclusively tetraploid, whereas the upland type can vary in ploidy ($4X$, $6X$, and $8X$), both within and among populations (Porter, 1966).

Cultivars appear to reflect their ecotype of origin, generally being described as tetraploid, hexaploid, octoploid, or some combination of these. Therefore, particularly in the cultivars derived from upland populations, the chromosome number of individual clones can vary. A survey of the cytogenetic information available for the primarily upland cultivars included in this study supports this: different authors give different chromosome numbers for the same cultivar, usually based on meiotic chromosome counts in pollen mother cells or mitotic counts in shoot or root meristematic cells from a few individual plants (Table 1). In some cases, ploidy is estimated by flow cytometry, which provides a measure of the nuclear DNA content in leaf cells, without any chromosome count data to confirm the assigned ploidy level (Hultquist et al., 1996; Casler et al., 2006). This practice has lead to some confusion in the past, particularly with the identification of hexaploids versus octoploids (Wullschleger et al., 1996), and also has required caveats about the potential for the presence of aneuploids and hexaploids (as opposed to octoploids) in the germplasm being characterized (Casler et al., 2006).

In polyploids, the copy number of functional polymorphisms (i.e., allele dosage) may be an important factor underlying trait variation. In addition, switchgrass is an outcrossing, highly heterozygous species. To deal with potentially 16 different alleles per locus, we are developing genetic markers that quantitatively score polymorphism frequency within an individual. However, to facilitate the application of the newest genotyping by sequencing technologies in highly diverse polyploid species, such as switchgrass and other perennial grasses, it will be highly advantageous to work initially with germplasm in which the copy-number of individual chromosomes in the sequenced genomes is fully known. In this study we conduct a flow-cytometric survey of a core set of switchgrass cultivars and follow this with a cytological study of chromosome-number variation in a subset of this germplasm. We address the following questions:

1. What is the extent of genome-size variation within and among switchgrass accessions?
2. How is this variation related to chromosome-number variation?
3. Is flow cytometry an accurate predictor of chromosome number?

Materials and Methods

Germplasm and Cultivation

All seed and clones were provided by MDC, unless otherwise indicated in Table 2. All plants were grown in a greenhouse at Cornell University, Ithaca, NY, in Cornell potting mix, under ambient light conditions, watered as needed, and fertilized once weekly with 300 ppm 21–5–20 solution. Germination of seed was enhanced by incubation in damp soil in a 40°F cold room for 2 to 6 wk before transfer to the greenhouse.

Flow Cytometry

The protocol for the preparation of leaf samples for flow cytometry used in this study is based on that of Arumuganathan and Earle (1991), with various modifications. One leaf per plant was harvested the evening before or the morning of the experiment and kept refrigerated until preparation of samples. A 1 cm^2 piece of leaf was finely chopped in 600 uL of $MgSO_4$ buffer solution (Solution "A") in a Petri dish kept on ice. Solution A is prepared fresh on the day of the experiment as follows (per sample): 585 µL $MgSO_4$ stock buffer [10 mmol L^{-1} $MgSO_4.7H_2O$,50 mmol L^{-1} KCl,

Table 1. Background information for the switchgrass accessions used in this study, including published chromosome numbers based on cytological data and ploidy determinations based solely on flow cytometry.

Accession	Type†	Ecotype‡	PI No.	2n or Ploidy§	Sample *n*	Data source¶	Authority
Blackwell	C	U	Grif 16409	54	12	PMC	(Riley and Vogel, 1982)
				72	8	AMC,PMC	(Hopkins et al., 1996)
				72	3	RT	(Lu et al., 1998)
				4X,8X	3,18	FC	(Narasimhamoorthy et al., 2008)
Carthage	C	U	421138	6X or 8X?	10	FC	(Hultquist et al., 1996)
Cave-in-Rock	C	U	569228	72	7	AMC,PMC	(Hopkins et al., 1996)
				72	1	RT	(Lu et al., 1998)
				4X,6X,8X	1,3,18	FC	(Narasimhamoorthy et al., 2008)
Dacotah	C	U	537588	36	20	MC	(Barker et al., 1990)
				4X	2	FC	(Narasimhamoorthy et al., 2008)
Forestburg	C	U	478001	36	20	MC	(Barker et al., 1988); Barker, personal communication, 2010
Kanlow	C	L	421521	36	7	PMC	(Riley and Vogel, 1982)
				36	1	AMC;PMC	(Hopkins et al., 1996)
				36	5	RT;PMC	(Lu et al., 1998)
				4X	2	FC	(Narasimhamoorthy et al., 2008)
KY 1625	C	U	431575	72	4	AMC;PMC	(Hopkins et al., 1996)
				36,54,72	9	n.i.#	(Henry and Taylor, 1989)
				4X	2	FC	(Narasimhamoorthy et al., 2008)
Pathfinder	C	U	642192	54	12	PMC	(Riley and Vogel, 1982)
				72	15	AMC; PMC	(Hopkins et al., 1996)
Sunburst	C	U	598136	6X or 8X?	10	FC	(Hultquist et al., 1996)
				4X	2	FC	(Narasimhamoorthy et al., 2008)
WS4U	BP	U	639191	4X	162	FC	(Casler et al., 2006)
WS8U	BP	U	639192	8X	98	FC	(Casler et al., 2006)
WS98-IP	NP	U		n.i.			(Casler et al., 2007)

†C = cultivar; BP = breeding population; NP = natural population.

‡U = upland; L = lowland.

§Ploidy level shown if only flow cytometry data are given, without direct cytological evidence.

¶PMC = pollen mother cell; AMC = apical meristem cell; MC = meristematic cell (type not specified); RT = root tip; FC = flow cytometry (estimate of ploidy).

#n.i. = no information.

5 mmol L^{-1} Hepes, pH adjusted to 8.0] + 0.6 mg DTT + 15 µL Triton X-100 solution (Sigma-Aldrich no. 93443). The resulting slurry was poured through a 30-µm filter [Partec CellTrics no.04-0042-2316]. Final volume of filtrate for each sample was adjusted to 425 µL. Two solutions were then added to each sample: 7.5 µL propidium iodide-RNase solution [5 µL PI (5 mg mL^{-1}) + 0.25 µL RNase stock (100 mg mL^{-1}) + 2.25 µL Solution A] and 5 µL Size Standard Solution. The size standard used for this experiment was Triploid Trout nuclei from BioSure, Inc. (Product no. 1014). Triploid trout was chosen as the size standard because it did not require additional chopping time during sample preparation, as a plant standard would, and its size was only slightly larger, but not overlapping, octoploid switchgrass, making it an appropriate standard for both tetraploids and octoploids. A stock solution was made fresh the day of the experiment as follows: 1 drop of standard cells + 150 µL Solution A (enough for 30 samples). Samples were kept in a light-protected cooler and transported to the flow cytometer. The samples were placed on the instrument within 2 h after completion of sample preparation.

The instrument used for the cultivar survey (Experiment 1) was a FACS Calibur (BD Biosciences, San Jose, CA), while subsequent work was performed on an LSRII (BD Biosciences). The stained nuclei samples were excited by a 488 nm laser. Propidium iodide fluorescence was measured with a 610/20 nm band pass filter and doublets were excluded using a linear PI fluorescence area and width plot. The order that samples were run on the instrument was randomized.

Genome sizes were calculated for all samples for which the numbers of both trout standard nuclei and plant nuclei exceeded 500 (in the majority of samples, these numbers exceeded 1000). The genome size of the triploid trout standard is 7.8 pg DNA (Biosure, 2010), so the formula used to calculate genome size in switchgrass was (mean fluorescence $_{\text{plant nuclei}}$/mean fluorescence$_{\text{std nuclei}}$) × 7.8. Genome size data were analyzed using the SAS statistical software (SAS Institute, 2002) general linear models procedure (Proc GLM) to examine the effects of ploidy, experimental block, and cultivar.

Table 2. Switchgrass accession survey (Experiment 1): Overall experimental means, standard errors (SE), and ranges of genome sizes (picograms of DNA), as measured by flow cytometry.

Ploidy	Accession name†	N	Mean	SE	Range (min-max)
4X	WS98-IP	15	2.23	0.060	1.81–2.29
	WS4U	14	2.27	0.052	1.95–2.66
	Dacotah	15	2.37	0.051	2.01–2.77
	Kanlow	15	2.51	0.024	2.36–2.68
8X	Cave-in-Rock‡	14	4.75	0.123	3.89–5.43
	Forestburg-E	15	4.77	0.090	4.23–5.24
	Blackwell	15	4.80	0.082	4.15–5.14
	Sunburst	15	4.83	0.090	4.19–5.39
	Carthage-C	14	4.89	0.092	4.07–5.46
	Forestburg-C	15	4.91	0.091	4.24–5.58
	WS8U	15	4.92	0.095	4.36–5.84
	Pathfinder	15	5.00	0.076	4.53–5.53
	Carthage-E	5	5.27	0.197	4.68–5.85

†For two cultivars (Carthage and Forestburg), two seed sources were used and measured separately. These are indicated by a "C," in which case the source is the Casler germplasm collection; an "E" indicates that the source is Ernst Conservation seeds. For all others, the Casler germplasm collection is the source.

‡Single tetraploid Cave-in-Rock plant not included in the calculation of cultivar descriptive statistics.

Cytological Preparation and Microscopy

Root tips for cytogenetics were harvested multiple times over the course of 6 mo from greenhouse-grown plants that had their roots cut back and were transplanted into wet vermiculite. They were left for about 2 wk on the bench in the greenhouse to allow for root re-growth. The tips were then removed from the new, actively growing roots and given a 24-hour cold water pretreatment before fixing in 3:1 ethanol/glacial acetic acid. Root tips were stained with 1% carmine in 45% acetic acid following squash preparation. Photomicrographs were taken with an Olympus DP71 camera. For more details, see the protocol described at the Kansas State University Wheat Genetic and Genomic Resources Center website (http://www.k-state.edu/wgrc/Protocols/labbook.html [verified 6 Oct. 2010]).

Fluorescence in situ hybridization analysis was performed using clone pTa71 derived from common wheat, *Triticum aestivum* L. Clone pTa71 is a 9 kb *Eco*RI fragment of the 18S-25S rDNA containing the coding sequences of the 18S, 5.8S, and 25S rRNA genes and the intergenic spacer sequence (Gerlach and Bedbrook, 1979). To generate a FISH probe, pTa71 was labeled by nick translation with rhodamine-6 dUTP according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN). Hybridization conditions and post-hybridization washes were performed according to Zhang et al. (2001). Chromosomes were counterstained with 4', 6 diamidino-2-phenylindol (DAPI). Slides were analyzed with an epifluorescence Zeiss Axioplan 2 microscope. Images were captured with a SPOT 2.1 CCD (charge coupled device) camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and processed with Photoshop v5.5 software (Adobe Systems, San Jose, CA).

Image Analysis

Images of metaphase chromosome spreads were examined by three people (DEC, BF, MJS), using two methods: (i) manual counts from images and (ii) automated counts using ImageJ (NIH, Bethesda, MD) software. Images were processed with ImageJ as follows: Raw images were converted to grayscale, then converted to black and white threshold images. When necessary, cellular debris was eliminated from threshold images to allow accurate counting. In addition, chromosomes that laid over or touched other chromosomes were separated by clearing pixels between them to allow accurate counting. Automated chromosome counts were generated using the Analyze Particles function and tracings were outputted as numbered ROIs (regions of interest). For quality control, all automated counts were compared against raw images and reiterated if they did not agree. Final chromosome count outlines were then overlaid on raw images.

Two sets of counts were generated, and the cases where the counts were nonconcordant were re-examined by DEC and MJS to determine what the discrepancies were. If an image was deemed too ambiguous, for example, due to overlapping chromosomes or pre-metaphase stage, it was discarded. For this study, we applied a high level of quality control—a total of 180 images were examined, with only 136 used in the subsequent analysis.

Results

Three separate experiments were performed to answer the fundamental questions we had about the nature and extent of genome-size variation in our core collection of switchgrass accessions. The first experiment was a flow-cytometric survey of 11 cultivars, including two cultivars represented by two different seed sources (Carthage and Forestburg), with a sample size of 15 individuals from each cultivar or seed source. These were run on the flow cytometer in five blocks, with three randomly assigned individuals from each cultivar in each block. Thus, every plant was run once. Two additional randomly chosen plants, an octoploid 'Cave-in-Rock' and a tetraploid 'Kanlow', were run as controls in all five blocks. Although normally we would strongly advise replication, in this case we wanted to know if one measurement would accurately allow us to assign ploidy level (tetraploid, hexaploid, octoploid), given our future plan to survey hundreds of plants in our field experiments. The histogram plot of the data clearly shows two separate populations (Fig. 1). These represent the tetraploid and octoploid genome types. No hexaploids were present in our samples. Descriptive statistics are presented by cultivar in Table 2. In every cultivar, except one, all 15 individuals sampled were the same ploidy. In the one exception, Cave-in-Rock, 14 plants were in the octoploid range plus one was tetraploid (i.e., mixed ploidy). An analysis of variance was performed using the GLM procedure in SAS, and confirms the overwhelming effect of ploidy on genome size (Table 3). The block effect is significant, as is cultivar, but these are both substantially

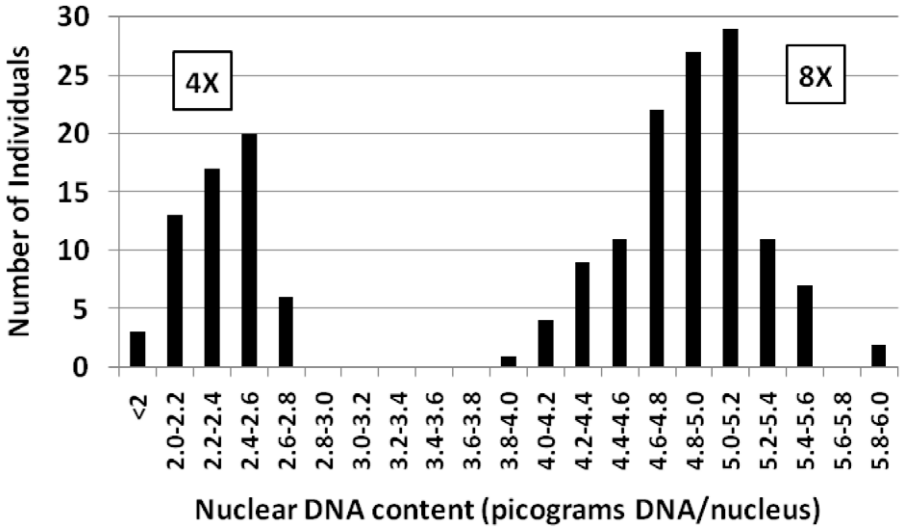

Figure 1. Nuclear DNA content (picograms of DNA/nucleus) measured by flow cytometry of 182 switchgrass plants. For tetraploids (4*X*), *n* = 59, mean = 2.35, SE = 0.027, range = 1.81–2.77. For octoploids (8*X*), *n* = 123, mean = 4.88, SE = 0.033, range = 3.89–5.85.

minimal, compared to ploidy, based on the size of the mean squares.

To further examine the differences among cultivars, we tested for differences among the best linear unbiased estimates of the cultivar means within ploidy, with the block effect removed. In both ploidy levels, the two cultivars with the smallest genomes significantly differed from their reference cultivar, Kanlow (tetraploid) or 'Pathfinder' (octoploid) (Fig. 2). The variation in genome size within cultivars (the percentage difference between the maximum and minimum individual genome sizes) ranged between 32.7 and 12.0% for tetraploid cultivars and 25.4 to 18.1% for octoploid cultivars. In comparison, the percentage differences between the maximum and minimum genome size measures for the two control plants in each of the blocks of the experiment were 13% for Kanlow (4*X*) and 11% for Cave-in-Rock (8*X*). This gives an estimate of variation attributable to random error plus block differences. By subtraction, that still leaves, on average, about an 11% difference in genome size between the largest and smallest genomes within a cultivar. We suspected that this difference could be attributed to the presence of aneuploids in our germplasm.

For the second experiment, we kept the pair of plants with the smallest and largest genomes in each cultivar, and re-measured their genome sizes with increased replication. Each individual plant was measured three times in each of two blocks. A mean genome size was calculated for each member of the high-low pair in each block, and the assignment of "high" versus "low" was rechecked. Out of a total of 21 comparisons, the original high-low difference was confirmed nine times, it was reversed eight times, and in four cases the difference between the high and low plants was less than 0.1, and thus was classified as not different. Therefore, it is evident that, although flow cytometry is highly accurate in assigning ploidy level in switchgrass, it is not a reliable means of detecting finer scale genome-size variation within a ploidy level.

In the third experiment, we collected chromosome number and flow cytometry data on the same set of plants to (i) compare the incidence of aneuploidy in the tetraploids and octoploids, (ii) examine further the relationship between the variation in genome sizes as detected with flow cytometry and cytological counts, and (iii) validate the ploidy assignments made in the first two experiments, which were based solely on flow cytometry data and comparison to published genome sizes for switchgrass. A total of 136 counts from 26 plants (average of 5.2 counts per plant) were included in the final analysis. Sample sizes varied among plants, dependent on the quality and quantity of root tips produced and the frequency of mitotic cells in metaphase for accurate counts of maximally condensed chromosomes. Those plants that provided chromosome number data were each run once in four different blocks on the flow cytometer.

The distribution of the entire set of chromosome counts shows an unmistakable pattern: two distinct populations corresponding to tetraploids ($2n = 4X = 36$) and octoploids ($2n = 8X = 72$) (Fig. 3). However, the frequencies of chromosome number classes within the two ploidies are strikingly different, due to ploidy-level specific variation in the incidence of aneuploidy (Table 4A). Examining all counts, 23.2% of those from tetraploid individuals are aneuploid (greater or less than 36), whereas 86.3% of those from octoploid plants are

Table 3. Analysis of variance of switchgrass cultivar genome size, as measured by flow cytometry. Type III sums of squares used in analysis. R^2 for the model = 0.95. Significance levels are $p < 0.0001$ **; $p < 0.01$**.**

Source	df	MS	F
Block	4	0.9093	11.54 ****
Ploidy	1	249.9104	3171.15 ****
Cultivar (Ploidy)	11	0.2099	2.66 **
Error	165		

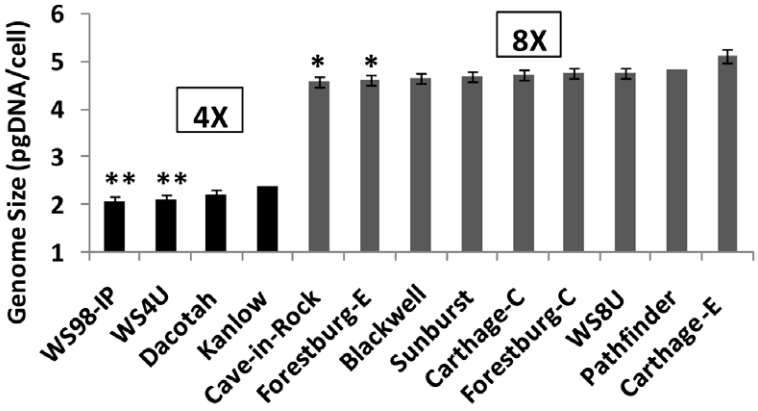

Figure 2. Variation among switchgrass accessions in survey (Experiment 1): Best linear unbiased estimates of genome sizes of cultivars within ploidy levels, with the significant block effect removed, as calculated by the statistical model in Table 3. Tetraploid (4*X*) cultivar estimates compared to Kanlow, octoploid (8*X*) cultivar estimates compared to Pathfinder (* = 0.05; ** = 0.01).

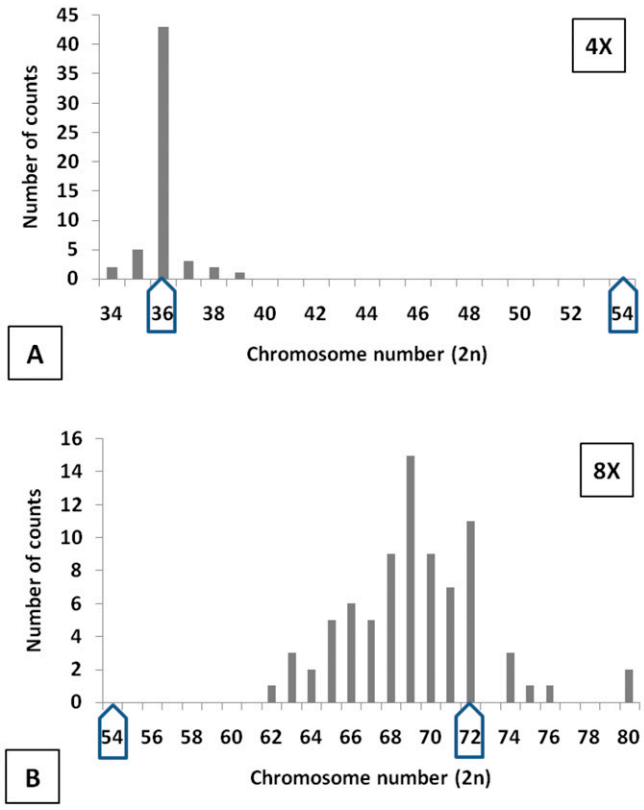

Figure 3. Mitotic chromosome counts in metaphase meristematic cells in root tips of switchgrass plants. Sample sizes vary for individual plants. Euploid numbers are indicated in the *x*-axes: 4*X* = 36 chromosomes; 6*X* = 54; 8*X* = 72. (A) Counts from tetraploid plants: mean ± se = 36.0 ± 0.1; *n* = 56. (B) Counts from octoploid plants: mean ± se = 69.2 ± 0.4; *n* = 80.

aneuploid (greater or less than 72). Furthermore, the incidence of low (less than the euploid complement) versus high (greater than the euploid complement) is about equivalent in tetraploids. This is clearly not the case in octoploids, where close to 90% of the aneuploid counts are lower than the euploid number.

On a within-plant basis, almost all (93.8%) of the octoploid plants had counts that varied from the euploid number of 72 chromosomes, whereas only half of the tetraploid plants had any counts that varied from the euploid number of 36 chromosomes. As a further indication that the stability of chromosome numbers varies according to ploidy level, in more than half of the octoploid plants, all of the cells counted were aneuploid, a condition that was not found in tetraploid plants. An example of chromosome-number variation within a plant is illustrated in Fig. 4: three mitotic cells from one Cave-in-Rock root tip possessed 63, 66, and 72 chromosomes. Two other cells counted for this same root tip (not pictured) had 71 and 63 chromosomes.

Next we confirmed the ploidy assignments using flow cytometry estimates of genome size in our earlier experiments by plotting mean genome size versus mean chromosome number for a set of 25 plants, 10 tetraploids plus 15 octoploids. As expected, these two variables are highly correlated ($R^2 = 0.98$; $P < 0.01$) (Fig. 5). However, within ploidy level, the correlations are low ($R^2_{4X} = 0.21$; $R^2_{8X} = 0.19$), confirming the results of our second experiment: flow cytometry data lack the resolution needed to consistently detect aneuploids in switchgrass.

The high degree of aneuploidy in octoploids could be an indicator of genome instability in these switchgrass cultivars. Genome instability can also affect individual chromosome constitutions, causing translocations, inversions, and chromosome derivatives to be formed. To begin to address the question of chromosomal constitution, we performed FISH with the rDNA clone pTa71 on selected tetraploid and octoploid cultivars. The 18S-25S rDNA multigene family is arranged as tandem repeats and is located at the nucleolus organizing regions (NORs). All of the tetraploids analyzed had two pairs of telomeric NOR FISH signals (Fig. 6, Supplemental Fig. 1, Table 5). In contrast, we observed a large amount of variation in number, location, and size of NOR FISH signals in the octoploids (Fig. 7, Supplemental Fig. 2, Table 5). For example, in addition to the expected telomeric NOR FISH signals, we also observed interstitial FISH signals. In all octoploid cultivars examined, we saw chromosomes with telomeric NOR FISH signals at the ends of both chromosomes arms (see

Table 4. Euploidy and aneuploidy in switchgrass (Experiment 3): Descriptive statistics for (A) all counts and (B) individual plants.

(A) All counts							
	Total *n*	Euploid *n*	Aneuploid *n*	% Aneuploid	Low aneuploid *n*	High aneuploid *n*	% Low Aneuploid
4X	56	43	13	23.2	7	6	53.8
8X	80	11	69	86.3	62	7	89.9
(B) By individual plants (with two or more counts)							
	Total *n*	100% Euploid	100% Aneuploid	Mixture of both	% with aneuploid cells		
4X	10	5	0	5	50.0		
8X	16	1	10	5	93.8		

Fig. 7 f,h; Table 5). These latter chromosomes appear to be isochromosomes, which varied in number from one to seven among the accessions analyzed (Table 5). Isochromosomes are derived from misalignment of the chromosomes relative to the spindle pole and are commonly found with aneuploidy. Surprisingly, the ancestral tetraploid state of two pairs of telomeric NOR signals is the rarest state detected in octoploids. These FISH data indicate that octoploid cultivars have accumulated chromosomal rearrangements in addition to aneuploidy, indicating that their genomes are unstable.

Discussion

Reports of variable ploidy levels in switchgrass germplasm accessions and registered cultivars have been relatively common in the literature (Table 1) and, indeed, provided the motivation for this study. In 10 of the 11 cultivars we surveyed initially, the ploidy level we found in our material, based solely on flow cytometry estimates, had been found previously (Forestburg was the exception) (Table 2). The one cultivar (Cave-in-Rock) where we found more than one ploidy is widely considered a "mixed" accession (Narasimhamoorthy et al., 2008). Since multiple ploidy levels are the trademark of upland switchgrass, our results basically confirmed what we already suspected: one cannot assume the ploidy of a switchgrass plant, especially in the case of an upland ecotype, without quantifying it. However, what was truly extraordinary about our results was the widespread occurrence of aneuploidy, which was only revealed by cytology. Thus, we need to amend our previous statement: one cannot assume that a ploidy assignment based on flow cytometry of a switchgrass plant means it possesses the euploid number of chromosomes, without cytological confirmation. A case in point is the "mixed" Cave-in-Rock seed

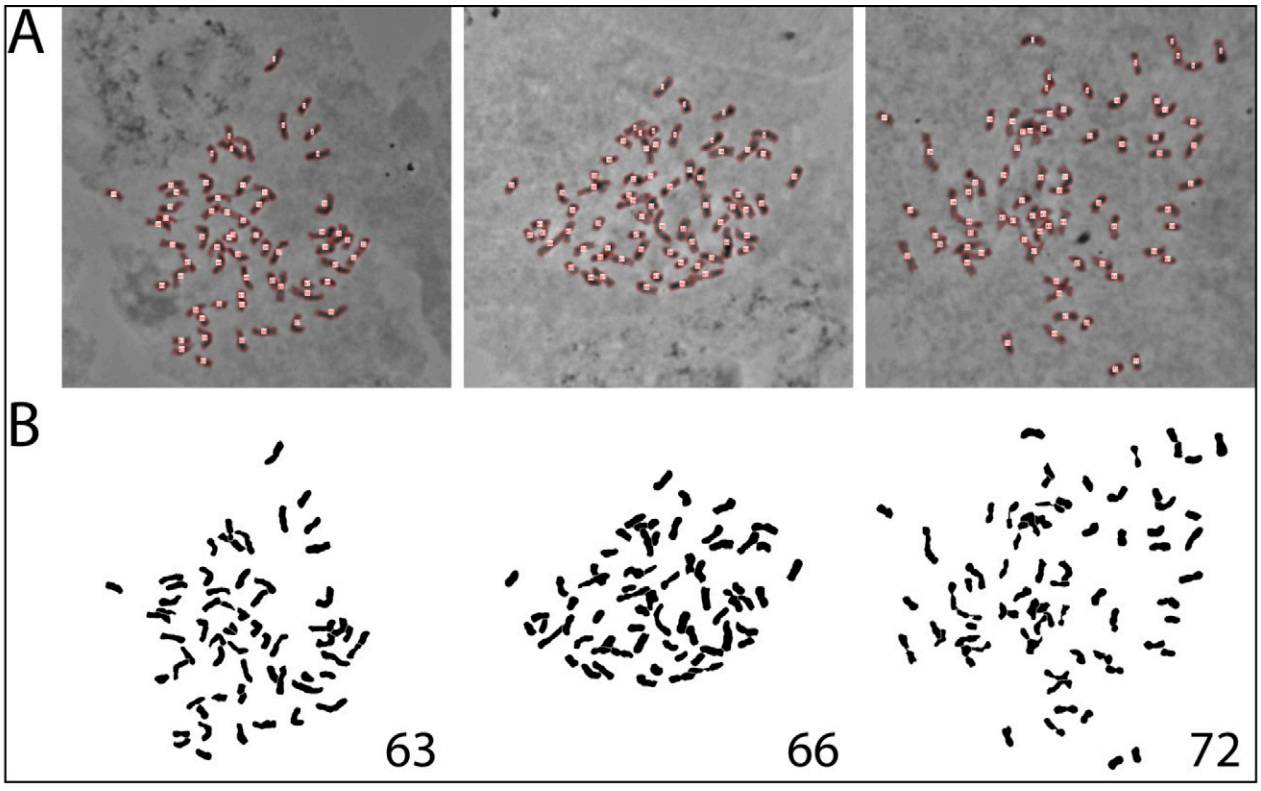

Figure 4. Variation in chromosome number counts within a single root tip of 'Cave-In-Rock'. (A) Raw images of acetocarmine-stained root tip chromosome spreads from three cells. Final chromosome counting traces have been overlaid (red). (B) Threshold images generated from raw images (in A) were used to automate counting with the Analyze Particle function of ImageJ (Rasband, 2009).

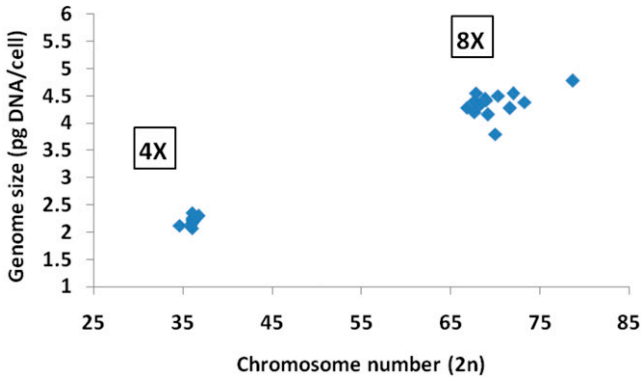

Figure 5. Mean genome size measured by flow cytometry versus mean mitotic chromosome number counts from root tips for individual plants (tetraploid *N* = 10; octoploid *N* = 15).

lot that we studied in detail: we detected two ploidy levels with flow cytometry (4*X* and 8*X*), but we subsequently found the four octoploid plants examined cytologically had mean chromosome numbers of 66.8, 67.7, 70, and 78.7, revealing two levels of variation in the switchgrass genome that have been virtually ignored in recent genetics studies.

Chromosome-number Variation among Plants: Aneuploidy

Early on in the grass cytology literature, switchgrass was already beginning to reveal its highly variable genome. In an oft-cited study that first documented the diploid to duodecaploid ploidy-level range in the species (Nielsen, 1944), four out of 34 mitotic chromosome counts in the $2n = 72–108$ range were labeled, "perhaps number indicated ± 1,2." In one of the first studies to examine the relationship between ecotype and chromosome number variability in switchgrass, it was found that 12 lowland plants were consistently $2n = 4X = 36$, while of 37 upland plants, 87% (32) were $2n = 8X = 72$, one plant was hexaploid, while the rest were aneuploids, with $2n = 68,70,76,78$ (Barnett and Carver, 1967). Furthermore, the authors admitted that they may have underestimated the frequency of aneuploidy, due to uncertainty in the counts of the octoploids.

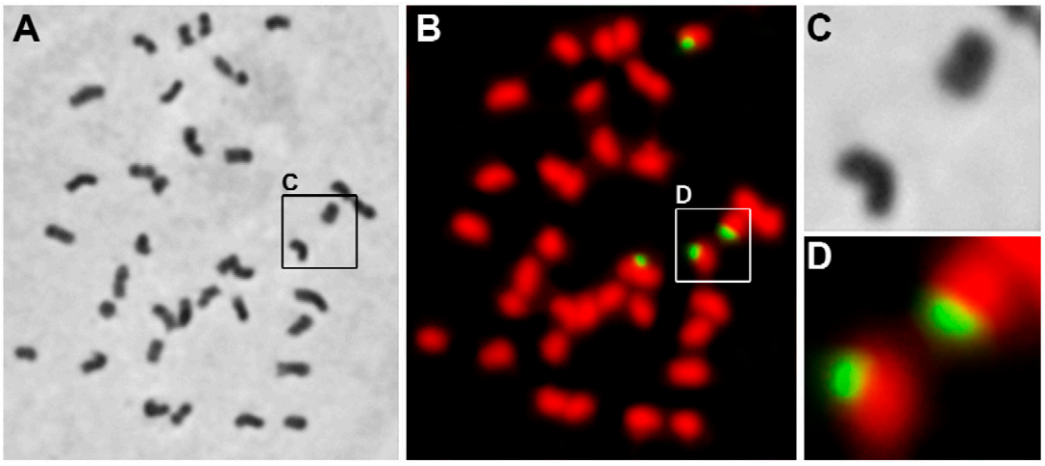

Figure 6. A representative fluorescent in situ hybridization (FISH) detection of nuclear organizing regions (NORs) in a tetraploid switchgrass cultivar. (A) Root tip spread prepared from 'Dacotah' ($2n = 4X = 36$) and stained with acetocarmine. (B) The same cell as in A with chromosomes stained with DAPI (pseudocolored in red) and subjected to FISH with a NOR probe (pseudocolored in green). There are two pairs of pTa71 FISH sites in tetraploids, and NORs are located at the telomeres (D) (see also Supplemental Fig. 1; Table 5). For comparison, (C) shows the acetocarmine-stained chromosomes from D.

Table 5. Nuclear organization region-fluorescent in situ hybridization (NOR-FISH) results reveal ploidy-specific variation in chromosomal constitution. See images in Fig. 6, 7; Suppl. Figures 1, 2.

Ploidy	2n	Cultivar	ID	Telomeric	Interstitial	Isotelomeric†	Images
4X	36	Dacotah	DCH-2	2 pairs	0	0	Fig. 6, Suppl. Figure 1A, B
	36	Dacotah	DCH-15	2 pairs	0	0	Suppl. Figure 1C, D
	36	WS4U	W4U-13.2	2 pairs	0	0	Suppl. Figure 1E, F
	36	WS4U	W4U-CL2	2 pairs	0	0	Suppl. Figure 1G, H
8X	72	WS8U	W8U-2.1	1	3	6	Suppl. Figure 2A, B
	70	WS8U	W8U-6.1	0	4	3	
	70	Cave-in-Rock	CIR-cl2.1	2	3	1	Suppl. Figure 2E, F
	70	Pathfinder	PFR-15.2	1	3	2	
	75	Sunburst	SBT-cl1.2	1	2	7	
	74	Carthage	CTE-19.1	0	4	7	
	72	Blackwell	BWL-2.2	2	3	5	Fig. 7, Suppl. Figure 2C, D

†Signals detected on the telomeres of both arms of isochromosomes.

More striking cases of aneuploidy in natural populations of switchgrass were described by Brunken and Estes (1975), who found 60.9% of an exclusively octoploid upland population was aneuploid, and in a mixed upland-lowland population, 26.7% of the octoploids were aneuploids. Tetraploids, whether in a monoploid lowland population or an upland mixed population, were invariably euploid. It is interesting to note that the highly aneuploid population in this later study is the same one that Porter (1966) reported to consist of hexaploids and octoploids. This disagreement could be attributable in part to the different sources of data in the two studies: meiotic counts from pollen mother cells in the Brunken and Estes (1975) study, as opposed to mitotic counts from root tips in the Porter (1966) study. We suspect that the pollen gametic cell population is more highly aneuploid than the mitotic cell population found in the root tips of established plants and will be testing this hypothesis in field populations.

Chromosome-number Variation within Plants

Fragile Chromosome Sites

Variation in chromosome number counts within plants could be explained by the existence of a fragile site. Fragile sites are genomic regions that have a tendency to cause breaks, constrictions or non-staining gaps within chromosomes. Fragile chromosome sites are very common in humans with over 120 documented sites within the genome (Buttel et al., 2004), but the existence of fragile sites in plants has only recently been discovered in *Lolium* spp. (Huang et al., 2008). In this study, the authors detected chromosome counts greater than the euploid number in 85% of root tip spreads, and furthermore that these increased counts were due to chromosome fragmentation occurring specifically at the 45S rDNA loci. The causative feature(s) of fragile sites is still speculative although there is some evidence to support that delayed or stalled DNA replication (Laird et al., 1987; Casper et al., 2002), chromatin modification (Coffee et al., 2002; Wang, 2006) and chromosome structure (Gericke, 1999; Huang et al., 2008) can contribute to fragile site establishment.

One interpretation of some of our data presented here is that fragile sites are present in switchgrass chromosomes, causing aneuploid counts. While there is some circumstantial support for this, we believe it is unlikely that our aneuploid chromosome counts in switchgrass are due to fragile sites as documented in *Lolium* spp. (Huang et al., 2008) for several reasons. First, fragile sites produce only increased chromosome counts, and although we do see counts greater than euploid in our data set, most of our counts are lower than the euploid count. Second, isochromosomes are a common feature of octoploid switchgrass, yet fragile sites are not known to generate them in vitro. Third, among clones of the same cultivar, we see variable numbers of isochromosomes (see WS8U in Table 5), suggesting that non-identical errors in segregation are still occurring in vivo. Fourth, it is unclear if fragile sites are indeed fragile in vivo as well as in vitro. Lastly, the 45S rDNA has never been reported to be a fragile site in other plants, suggesting that the 45S rDNA regions in *Lolium* spp. have unique differences that produce the fragile sites in these species. In conclusion, although we cannot definitively discount the existence of fragile chromosome sites in switchgrass, we believe that our data do not support this hypothesis.

Mosaicism

Although mosaicism is usually associated with plant cell cultures (Larkin and Scowcroft, 1981), there is ample evidence that it is a common phenomenon in grasses (Huskins and Smith, 1932; Church, 1940; Sachs, 1952; Watanabe, 1962; Thomas and Peregrine, 1964; Nielsen, 1968; Lavania, 1987; Burner and Legendre, 1993). Based on reports of

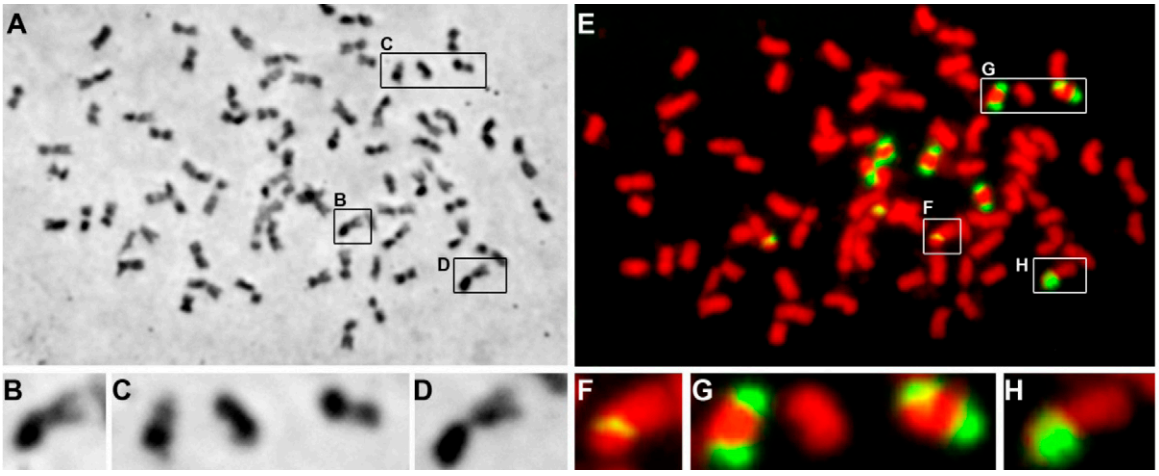

Figure 7. A representative fluorescent in situ hybridization (FISH) detection of nuclear organizing regions (NORs) in an octoploid switchgrass cultivar. (A) A root tip spread prepared from 'Blackwell' ($2n = 8X = 72$) and stained with acetocarmine. (B) The same cell as in (A) with chromosomes stained with DAPI (pseudocolored in red) and subjected to FISH with a NOR probe (pseudocolored in green). There are three types of pTa71 FISH sites in octoploids (see also Supplemental Fig. 2; Table 5): (F) interstitial (located between the centromere and telomere), (G) at both telomeres on an isochromosome, and (H) telomeric. For comparison, (B), (C), and (D) show the acetocarmine-stained chromosomes from (F), (G), and (H), respectively.

mosaicism in root tips available at the time, Church (1940) proposed that through this mechanism, autopolyploids could be produced vegetatively. The first report of mosaics in upland switchgrass roots (cells with chromosome counts of 54, 72, 90 in two root tips and 54, 90, 108 in one root tip) came much later (Porter, 1966). The chromosome-count variation we observed could be attributed to mosaicism; however, more data are needed to unequivocally support its occurrence in switchgrass. Mosaicism is still a largely unexplored, but potentially widespread, condition in polyploids in natural populations. The phenomenon of mitotic fidelity in allopolyploids has been examined in detail only recently, in the model genus *Arabidopsis*, where mosaicism was found to be prevalent in two tetraploids, *A. suecica* and *A. arenosa*. However, stable and novel cytotypes with chromosome numbers other than euploid have yet to be discovered (Wright et al., 2009).

The Road to Genome Stability

Speciation in plants often occurs after global changes to the genome, such as polyploidization or hybridization (as reviewed by Hegarty and Hiscock [2007] and Sobel et al. [2010]). Both types of global genomic change induce instability into a genome, thereby causing changes in gene expression, meiotic pairing disorders, and altered developmental patterns (Hegarty et al., 2008). Survival of the plant requires some measure of genome stabilization (Li and Ge, 2007; Sipiczki, 2008). For example, modern maize would not exist today had it not been for the dramatic diploidization event(s) that reorganized the allotetraploid genome, thereby allowing it to undergo regular, error-free meiosis (Gaut, 2001). This has been the case for many crop species.

Aneuploidy causes greater genome instability than polyploidy for organisms by adding or subtracting the gene dosage of a single chromosome to or from the euploid genome (Birchler et al., 2001; Fodde and Smits, 2002; Birchler, 2010). Aneuploidy is caused by segregation errors and nondisjunction during cell division via meiosis and/or mitosis. Some types of segregation errors generate chromosomal breaks and rearrangements (Griffin, 1996), which can lead to speciation (Ramsey and Schemske, 1998). Here we use rDNA as a FISH probe to show that, not only are there structural rearrangements in octoploids, but the nature and number of the rearrangements differ by individual examined. This is in stark contrast to the lack of chromosome rearrangements in tetraploids, where aneuploidy is low. Unlike reciprocal translocation that results in no net loss of genic material between daughter cells, aneuploidy will always present an unbalanced genic distribution. The presence of extra or missing chromosomes, or segments, as in the case of isochromosomes, has been shown to cause changes in DNA methylation in cancer cells (Matzke et al., 1999; Matzke et al., 2003).

Switchgrass octoploids demonstrate a large amount of genome instability, as evidenced by the swarm of aneuploidy surrounding the euploid 72-chromosome count. Furthermore, we found that the range of chromosome counts surrounding the octoploids did not extend past one haploid level. For instance, the lowest counts were close or at the septaploid ($7X = 63$) level and the highest counts were at the nonaploid ($9X = 81$) level. It is not known if these odd-numbered ploidy levels represent some form of genome stability or a genome still in flux. The fact that the swarm is biased toward the ~$7x$ end of the curve suggests that hexaploids could be produced through octoploid reduction, thereby improving the stability of the genome. Theoretically, hexaploids could be produced via an intermating between tetraploid and octoploid, but a postfertilization incompatibility system prevents this (Martinez-Reyna and Vogel, 2002). It remains to be seen whether hexaploids can be produced through an alternative mechanism, such as genome reduction.

Combining Flow Cytometry with Chromosome Counts

Although this research focused exclusively on switchgrass, it is likely that these findings will not be unique. Many perennial grasses have multiple and high ploidy levels, for example, *Phalaris arundinacea* L., reed canarygrass, is 4*X* to 6*X*, and *Andropogon gerardii*, big bluestem, has ploidy levels ranging from 6*X* to 9*X* (see Supplemental Table 1). In *Pennisetum* species, not only does the ploidy vary from 2*X* to 8*X*, but the base chromosome number varies from $x = 5$, to 7, 8, and 9 (Martel et al., 1997). The genome of modern sugarcane cultivars is characterized by both high ploidy levels and aneuploidy (Grivet and Arruda, 2001), two features it shares with switchgrass. In addition, the high ploidy states may provide a "genetic buffer" for aneuploidy. Based on the results presented here, we recommend that flow cytometry always be coupled with a method to determine chromosome counts in a sample of individuals from the entire range of genome sizes found in the population under study, as the standard practice for characterizing switchgrass and other polyploid perennial grass cultivars in future germplasm characterization and breeding projects.

Phenotypic Consequences of Aneuploidy

Polyploidy and aneuploidy are usually deleterious to animals, causing the kind of genomic instability associated with infertility, tumor formation, disease susceptibility and lethality (Matzke et al., 2003; Huettel et al., 2008; Torres et al., 2008). However, in plants, polyploidy is present in nearly 80% of all living plants, and nearly 100% of all plant lineages have undergone a paleopolyploidy event in their history (Otto and Whitton, 2000; Blanc and Wolfe, 2004). Recent studies on aneuploidy in plants have shown that it can occur without serious detriment to the plant, and can sometimes even enhance phenotypes (Henry et al., 2005; Henry et al., 2007; Huang et al., 2008). Detection and quantification of the effects of these phenomena are clearly critical to any studies of the genetic basis of phenotypic traits in switchgrass, as well as its future as the primary biofeedstock for the biofuels industry. In a prescient comment in the germplasm registration of KY 1625 switchgrass, Henry and Taylor (1989) noted, "Apparently, both polyploidy and aneuploidy …

may be present in this germplasm. This variability in chromosome number may account in part for the poor viable seed set." All aspects of the life cycle of switchgrass are likely to be affected by genome size and structure.

The sugarcane species and hybrid complex (genus *Saccharum*) are perhaps the most genetically complex crop taxa in the world. Interspecific hybridization, meiotic irregularities, multiple, high ploidy levels, aneuploidy, and chromosomal mosaicism are characteristics of this group (Burner and Legendre, 1993; Aljanabi, 1998; Cuadrado et al., 2004). The wild species, *Saccharum spontaneum*, shows the most similarities to switchgrass, with an extensive range of 2*n* chromosome numbers (40–128) and rampant aneuploidy. In a study of six cytotypes of this species, with 2*n* = 40, 42, 44, 54, 56, 72, considerable morphological variation was detected among them, which was attributed to both numerical and structural differences among the chromosomes (Mehra and Sood, 1974). This will likely be the case with switchgrass cytotypes. Cuadrado et al. (2004) point out that the accumulation of numerical and structural chromosome changes in sugarcane is caused by the reliance on vegetative propagation, which allows for a bypass of the "meiotic filter." Aneuploid switchgrass clones could likewise persist in prairies via vigorous vegetative growth and eventually be selected for breeding programs. The sugarcane genetics/genomics community has developed a diverse set of resources and complementary approaches that switchgrass workers should emulate in molecular cytogenetics (D'Hont, 2005), breeding (Burner and Legendre, 1994), tissue culture (Rajeswari et al., 2009), and mapping (Le Cunff et al., 2008). Rapid advances in the breeding of polyploid biofuel feedstocks, based on the molecular-genetic dissection of biomass characteristics and yield, will be predicated on the continual improvement of our understanding of the cytogenetics of these species.

Acknowledgments

Flow cytometry support was provided by R.G. Getchell, Jim Smith and Lavanya Gowri Sayam. Z. Zhang and F. Vermeylen gave statistical advice. C. Thomas and K. Goodwin provided greenhouse support. M.A. Gore provided comments on the manuscript. We thank them for their contributions to this research. This research was funded by a United States Department of Energy-Department of Agriculture Plant Feedstock Genomics for Bioenergy Program grant (DE-A102-07ER64454).

References

Aljanabi, S.M. 1998. Genetics, phylogenetics, and comparative genetics of *Saccharum* L., a polysomic polyploid Poales. Andropogoneae. Biotechnol. Annu. Rev. 4:285–319.

Arumuganathan, K., and E. Earle. 1991. Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol. Biol. Rep. 9:217–229.

Barker, R.E., R.J. Haas, E.T. Jacobson, and J.D. Berdahl. 1988. Registration of "Forestburg" switchgrass. Crop Sci. 28:192–193.

Barker, R.E., R.J. Haas, J.D. Berdahl, and E.T. Jacobson. 1990. Registration of "Dacotah" switchgrass. Crop Sci. 30:1158.

Barkworth, M.E., K.M. Capels, S. Long, and M.B. Piep (ed.) 2003. Magnoliophyta: Commelinidae (in part): Poaceae, part 2. Oxford Univ. Press, New York.

Barnett, F.L., and R.F. Carver. 1967. Meiosis and pollen stainability in switchgrass, *Panicum virgatum* L. Crop Sci. 7:301–304.

Biosure. 2010. Product Data Sheet. Available at https://biosure-best.securesites.com/rpages/controls.htm (verified 6 Oct. 2010). Biosure, Grass Valley, CA.

Birchler, J.A. 2010. Reflections on studies of gene expression in aneuploids. Biochem. J. 426:119–123.

Birchler, J.A., U. Bhadra, M.P. Bhadra, and D.L. Auger. 2001. Dosage-dependent gene regulation in multicellular eukaryotes: Implications for dosage compensation, aneuploid syndromes, and quantitative traits. Dev. Biol. 234:275–288.

Blanc, G., and K.H. Wolfe. 2004. Widespread paleopolyploidy in model plant species inferred from age distributions of duplicate genes. Plant Cell 16:1667–1678.

Brown, W.V. 1948. A cytological study in the Gramineae. Am. J. Bot. 35:382–395.

Brunken, J.N., and J.R. Estes. 1975. Cytological and morphological variation in *Panicum virgatum* L. Southwest. Nat. 19:379–385.

Burner, D.M., and B.L. Legendre. 1993. Chromosome transmission and meiotic stability of sugarcane (*Saccharum* spp.) hybrid derivatives. Crop Sci. 33:600–606.

Burner, D.M., and B.L. Legendre. 1994. Cytogenetic and fertility characteristics of elite sugarcane. Sugar Cane 1:6–10.

Buttel, I., A. Fechter, and M. Schwab. 2004. Common fragile sites and cancer: Targeted cloning by insertional mutagenesis. Ann. N. Y. Acad. Sci. 1028:14–27.

Casler, M.D., K.P. Vogel, and A.C. Beal. 2006. Registration of WS4U and WS8U switchgrass germplasms. Crop Sci. 46:998–999.

Casler, M.D., K.P. Vogel, C.M. Taliaferro, N.J. Ehlke, J.D. Berdahl, E.C. Brummer, R.L. Kallenbach, C.P. West, and R.B. Mitchell. 2007. Latitudinal and longitudinal adaptation of switchgrass populations. Crop Sci. 47:2249–2260.

Casper, A.M., P. Nghiem, M.F. Arlt, and T.W. Glover. 2002. ATR regulates fragile site stability. Cell 111:779–789.

Church, G.L. 1940. Cytotaxonomic studies in the Gramineae Spartina, Andropogon, and Panicum. Am. J. Bot. 27:263–271.

Coffee, B., F. Zhang, S. Ceman, S.T. Warren, and D. Reines. 2002. Histone modifications depict an aberrantly heterochromatinized FMR1 gene in fragile x syndrome. Am. J. Hum. Genet. 71:923–932.

Cuadrado, A., R. Acevedo, S. Moreno Diaz de la Espina, N. Jouve, and C. de la Torre. 2004. Genome remodelling in three modern *S. officinarum* x *S. spontaneum* sugarcane cultivars. J. Exp. Bot. 55:847–854.

D'Hont, A. 2005. Unraveling the genome structure of polyploids using FISH and GISH; examples of sugarcane and banana. Cytogenet. Genome Res. 109:27–33.

Darlington, C.D. 1956. Chromosome botany. George Allen & Unwin Ltd, London.

Fodde, R., and R. Smits. 2002. Cancer biology. A matter of dosage. Science 298:761–763.

Gaut, B. 2001. Patterns of chromosomal duplication in maize and their implications for comparative maps of the grasses. Genome Res. 11:55.

Gericke, G.S. 1999. Chromosomal fragility may be indicative of altered higher-order DNA organization as the underlying genetic diathesis in complex neurobehavioural disorders. Med. Hypoth. 52:201–208.

Gerlach, W.L., and J.R. Bedbrook. 1979. Cloning and chracterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. 7:1869–1885.

Griffin, D.K. 1996. The incidence, origin, and etiology of aneuploidy. Int. Rev. Cytol. 167:263–296.

Grivet, L., and P. Arruda. 2001. Sugarcane genomics: Depicting the complex genome of an important tropical crop. Curr. Opin. Plant Biol. 5:122–127.

Hegarty, M.J., G.L. Barker, A.C. Brennan, K.J. Edwards, R.J. Abbott, and S.J. Hiscock. 2008. Changes to gene expression associated with hybrid speciation in plants: Further insights from transcriptomic studies in Senecio. Philos. Trans. R. Soc. Lond. B Biol. Sci. 363:3055–3069.

Hegarty, M., and S. Hiscock. 2007. Polyploidy: Doubling up for evolutionary success. Curr. Biol. 17:R927–R929.

Henry, I.M., B.P. Dilkes, and L. Comai. 2007. Genetic basis for dosage sensitivity in *Arabidopsis thaliana*. PLoS Genet. 3:593–602.

Henry, I.M., B.P. Dilkes, K. Young, B. Watson, H. Wu, and L. Comai. 2005. Aneuploidy and genetic variation in the *Arabidopsis thaliana* triploid response. Genetics 170:1979–1988.
Henry, D.S., and T.H. Taylor. 1989. Registration of KY 1625 switchgrass germplasm. Crop Sci. 29:1096.
Hopkins, A.A., C.M. Taliaferro, C.D. Murphy, and D.A. Christian. 1996. Chromosome number and nuclear DNA content of several switchgrass populations. Crop Sci. 36:1192–1195.
Huang, J., L. Ma, F. Yang, S.Z. Fei, and L. Li. 2008. 45S rDNA regions are chromosome fragile sites expressed as gaps in vitro on metaphase chromosomes of root-tip meristematic cells in Lolium spp. PLoS One 3:e2167.
Huettel, B., D.P. Kreil, M. Matzke, and A.J.M. Matzke. 2008. Effects of aneuploidy on genome structure, expression, and interphase organization in *Arabidopsis thaliana*. PLoS Genet. 4:1–13.
Hultquist, S.J., K.P. Vogel, D.J. Lee, K. Arumuganathan, and S. Kaeppler. 1996. Chloroplast DNA and nuclear DNA content variations among cultivars of switchgrass, *Panicum virgatum* L. Crop Sci. 36:1049–1052.
Huskins, C.L., and S.G. Smith. 1932. A cytological study of the genus *Sorghum* Pers. I. The somatic chromosomes. J. Genet. 25:241–249.
Laird, C., E. Jaffe, G. Karpen, M. Lamb, and R. Nelson. 1987. Fragile sites in human chromosomes as regions of late-replicating DNA. Trends Genet. 3:274–281.
Larkin, P.J., and W.R. Scowcroft. 1981. Somaclonal variation–a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60:197–214.
Lavania, U.C. 1987. Chromosomal instability in lemon grass, *Cymbopogon flexuosus* (Steudel) Wats. Genetica (The Hague) 72:211–215.
Le Cunff, L., O. Garsmeur, L.M. Raboin, J. Pauquet, H. Telismart, A. Selvi, L. Grivet, R. Philippe, D. Begum, M. Deu, L. Costet, R. Wing, J.C. Glaszmann, and A. D'Hont. 2008. Diploid/polyploid syntenic shuttle mapping and haplotype-specific chromosome walking toward a rust resistance gene (Bru1) in highly polyploid sugarcane (2n~12x~115). Genetics 180:649–660.
Li, Z.-y., and X.-h. Ge. 2007. Unique chromosome behavior and genetic control in Brassica x Orychophragmus wide hybrids: A review. Plant Cell Rep. 26:701–710.
Lu, K., S.W. Kaeppler, K. Vogel, K. Arumuganathan, and D.J. Lee. 1998. Nuclear DNA content and chromosome numbers in switchgrass. Great Plains Res. 8:269–280.
Martel, E., D. De Nay, S. Siljak-Yakovlev, S. Brown, and A. Sarr. 1997. Genome size variation and basic chromosome number in Pearl millet and fourteen related *Pennisetum* species. J. Hered. 88:139–143.
Martinez-Reyna, J.M., and K.P. Vogel. 2002. Incompatibility systems in switchgrass. Crop Sci. 42:1800–1805.
Matzke, M., M. Florian Mette, T. Kanno, and A. Matzke. 2003. Does the intrinsic instability of aneuploid genomes have a causal role in cancer? Trends Genet. 19:253–256.
Matzke, M., O. Scheid, and A. Matzke. 1999. Rapid structural and epigenetic changes in polyploid and aneuploid genomes. Bioessays 21:761–767.
McLaughlin, S.B., and L.A. Kszos. 2005. Development of switchgrass (*Panicum virgatum*) as a bioenergy feedstock in the United States. Biomass Bioenergy 28:515–535.
McMillan, C., and J. Weiler. 1959. Cytogeography of *Panicum virgatum* in central North America. Am. J. Bot. 46:590–593.
Mehra, P.N., and O.P. Sood. 1974. Floating chromosomal populations in *Saccharum spontaneum* L. Cytologia (Tokyo) 39:681–696.
Myers, W.M., and H.D. Hill. 1940. Studies of chromosomal association and behavior and occurrence of aneuploidy in autotetraploid grass species, orchard grass, tall oat grass, and crested wheatgrass. Bot. Gaz. 102:236–255.
Narasimhamoorthy, B., M.C. Saha, T. Swaller, and J.H. Bouton. 2008. Genetic diversity in switchgrass collections assessed by EST-SSR markers. Bioenergy Res. 1:136–146.
Nielsen, E.L. 1944. Analysis of variation in *Panicum virgatum*. J. Agric. Res. 69:327–353.
Nielsen, E.L. 1968. Intraplant morphological variations in grasses. Am. J. Bot. 55:116–122.
Otto, S.P., and J. Whitton. 2000. Polyploid incidence and evolution. Annu. Rev. Genet. 34:401–437.
Porter, C.L. 1966. An analysis of variation between upland and lowland switchgrass, *Panicum virgatum* L., in central Oklahoma. Ecology 47:980–992.
Rajeswari, S., S. Thirugnanakumar, A. Anandan, and M. Krishnamurthi. 2009. Somaclonal variation in sugarcane through tissue culture and evaluation for quantitative and quality traits. Euphytica 168:71–80.
Ramsey, J., and D. Schemske. 1998. Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annu. Rev. Ecol. Syst. 29:467–501.
Rasband, W.S. 2009. ImageJ. Available at http://rsb.info.nih.gov/ij/ (verified 13 Oct. 2010). U.S. National Institutes of Health, Bethesda, MD.
Riley, R.D., and K.P. Vogel. 1982. Chromosome numbers of released cultivars of switchgrass, indiangrass, big bluestem, and sand bluestem. Crop Sci. 22:1082–1083.
Sachs, L. 1952. Chromosome mosaics in experimental amphiploids in the *Triticinae*. Heredity 6:157–170.
SAS Institute. 2002. SAS for Windows. SAS Inst., Cary NC.
Sipiczki, M. 2008. Interspecies hybridization and recombination in Saccharomyces wine yeasts. FEMS Yeast Res. 8:996–1007.
Sobel, J.M., G.F. Chen, L.R. Watt, and D.W. Schemske. 2010. The biology of speciation. Evolution Int. J. Org. Evolution 64:295–315.
Thomas, H., and W.T.H. Peregrine. 1964. Chromosome mosaics in synthetic amphiploids in the Avenae. Chromosoma 15:123–131.
Torres, E.M., B.R. Williams, and A. Amon. 2008. Aneuploidy: Cells losing their balance. Genetics 179:737–746.
Turhollow, A.F., J.H. Cushman, and J.W. Johnston. 1988. Herbaceous Energy Crops Program: Annual progress report for FY 1988. Oak Ridge National Laboratory–6639. Oak Ridge National Lab., Oak Ridge, TN.
Wang, Y.H. 2006. Chromatin structure of human chromosomal fragile sites. Cancer Lett. 232:70–78.
Watanabe, Y. 1962. Chromosome-mosaics observed in a variety of wheat, "Shirahada." Jpn. J. Genet. 37:194–206.
Wright, K.M., J.C. Pires, and A. Madlung. 2009. Mitotic instability in resynthesized and natural polyploids of the genus *Arabidopsis* (Brassicaceae). Am. J. Bot. 96:1656–1664.
Wullschleger, S.D., M.A. Sanderson, S.B. McLaughlin, D.P. Biradar, and A.L. Rayburn. 1996. Photosynthetic rates and ploidy levels among populations of switchgrass. Crop Sci. 36:306–312.
Zhang, P., B. Friebe, A.J. Lukaszewski, and B.S. Gill. 2001. The centromere structure in Robertsonian wheat-rye translocation chromosomes indicates that centric breakage-fusion can occur at different positions within the primary constriction. Chromosoma 110:335–344.