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ABSTRACT

Panicum virgatum L. (switchgrass) is an ecologically important, native, warm-season, perennial grass in North America. Its distribution includes the coastal grasslands of the Northeastern United States including the Long Island Sound Coastal Lowland ecoregion. Because of its development as a model lignocellulosic bioenergy crop, its distribution, genetic resources, genomics, phenology, morphological traits, gene flow and reproduction have been closely studied. Switchgrass has been divided into two ecotypes, the Lowland ecotype which is tetraploid (2n= 4x= 36 chromosomes) and the Upland ecotype which is tetraploid or octaploid (2n= 8x= 72). Most research has focused on genetic lines from the Midwestern or Southeastern regions of North America. This is the first study to determine the ploidy number for wild switchgrass growing in the Long Island Sound Coastal Lowland ecoregion in Connecticut. Flow cytometry was performed on nuclei extracted from 37 leaf samples from 19 collected switchgrass plants and 8 well-known cultivars representing either Upland octoploid or Lowland tetraploid genotypes. Analysis of leaf tissue from wild plants showed that all individuals tested were tetraploid with a mean value of 2.6 pg DNA/nuclei. This result was consistent with a previous study in our lab using wild plants and microsatellite markers. Five Lowland tetraploid cultivars (control group) had a DNA concentration similar to the wild plants. In contrast, three Upland octoploid cultivars produced a mean value of 6.2 pg DNA/nuclei. This new knowledge could help identify coastal switchgrass populations worthy of protection, genetic resources that may be useful for breeding programs, and optimal seed stock for coastal habitat restoration projects.

INTRODUCTION

Panicum virgatum L., commonly known as switchgrass, is a warm-season perennial grass in the Poaceae family (Missaoui et al. 2006). It is an adaptable grass as demonstrated by its geographic distribution across much of the United States and Canada (Hitchcock 1971; USDA Plants Database http://plants.usda.gov/core/profile?symbol=PAVI2). Two ecotypes, Lowland and Upland, have been identified based on morphological traits, habitat, distribution, and ploidy levels (Hultquist et al. 1996; Vogel 2004; Parrish and Fike, 2005; Narasimhamoorthy et al. 2008; Zhang et al. 2011). Upland plants are adapted to middle to northern latitudes in North America while Lowland plants are adapted to warmer regions (Narasimhamoorthy et al. 2008; Zhang et al. 2011). Plants in the Upland ecotype are characterized as being shorter with finer stems and adaptation to drier habitats (Hultquist et al. 1996; Cortese et al. 2010; Zhang et al. 2011). Plants in the Lowland ecotype are characterized as taller and more robust with coarser stems and adapations for wetter habitats including those that are periodically flooded (Hultquist et al. 1996; Cortese et al. 2010; Zhang et al. 2011).

The ploidy number for any organism is the number of sets of chromosomes in the cell nucleus. Switchgrass is an outcrossing grass that is highly heterozygous, self-incompatible and has diverse ploidy levels (Hultquist et al. 1996; Parrish and Fike, 2005; Narasimhamoorthy et al. 2008; Jakob et al. 2009). Observed ploidy values range from $2n= 2x= 18$ to $2n= 12x= 108$ (Church 1940; Nielson 1944). Upland ecotype plants have been characterized as tetraploid ($2n=4x=36$), hexaploid ($2n=6x=54$) or octaploid ($2n=8x=72$). Lowland ecotype plants are generally tetraploid ($2n=4x=36$). Hybridization can occur between the two ecotypes as long as both parents have similar ploidy levels (Missaoui et al. 2005; Narasimhamoorthy et al. 2008). However, the two ecotypes are generally incompatible and, if fertilization occurs, seeds will not develop normally suggesting pre- and/or post-zygotic incompatibilities (Parrish and Fike, 2005). Previous studies of switchgrass populations on the East Coast (New York to Florida) suggest that native plants are typically Lowland tetraploids (Hultquist et al. 1996; Cortese et al. 2010; Zhang et al. 2011). However, switchgrass populations in the Northeastern coastal region (e.g. Long Island Sound Coastal Lowland ecoregion including Connecticut) have never been studied.

In the past decades, switchgrass seed lines have been selected from wild populations and cultivated as livestock forage, wildlife habitat, erosion control, and ornamental gardens (Bouton 2007; Casler et al. 2007*b*; USDA NRCS 2012). In recent years, it has become a model lignocellulosic bioenergy crop because, in theory, it is a more sustainable source of ethanol that maize (Missaoui et al. 2005; Heaton et al. 2008; Casler 2012). Recent research has produced various novel traits through biotechnology or traditional breeding (Zhang et al. 2011, Narasimhamoorthy et al. 2008; Cortese et al. 2010, Missaoui et al. 2006; Casler et al. 2007*a*). Indeed, several lines of genetically engineered (GE) switchgrass were approved for unconfined cultivation in the U.S. (Ledford, 2013), although information about the novel genes, traits, and adaptations in these GE lines has not been made public. There is concern that large-scale gene flow (by pollen or seed movement) will harm native switchgrass populations (Kausch et al, 2010). Some of the potential negative impacts of switchgrass feedstock plantations include: 1) environmental effects due to weedy or invasive switchgrass populations; 2) the reduction or extinction of native switchgrass populations due to gene flow or direct competition from GE switchgrass; 3) loss of genetic diversity or genetic integrity in wild populations; 4) changes in switchgrass distribution; 5) gene flow to native or non-native *Panicum* species with ecological impacts; 6) undesirable changes in natural or managed plant communities; 7) downstream negative effects on non-target organisms (Barney & DiTomaso, 2008; Kausch et al., 2010; Kwit & Stewart, 2012). Our previous pollen distribution modeling study predicted that viable, wind-blown switchgrass pollen could move up to 3.5-6 km under normal summer wind conditions in the Northeastern U.S. (Ecker et al, 2013). Thus, native switchgrass populations in the Connecticut Long Island Sound Coastal Lowland ecoregion could receive clouds of pollen from inland biofuels plantations. Indeed, recent research by our group has identified non-local, Upland switchgrass genotypes along Connecticut roadsides and in other human-impacted landscapes (Ecker et al, in preparation).

This project was initiated because of uncertainty about the ecotype and ploidy level of switchgrass plants observed in the Long Island Sound Coastal Lowland ecoregion. Native populations in this region are generally small, and often occur in fragmented or degraded coastal habitats. Thus, gene flow from large-scale bioenergy plantations could have a substantial impact. The primary goal of this study was to determine the ploidy level of wild switchgrass plants by comparison with well-known Upland and Lowland cultivars derived from seed collections made in other parts of its geographic range. This information could contribute to the development of appropriate risk assessments, conservation actions, and habitat restoration programs.

MATERIALS AND METHODS

Plant Materials

Eight well-known switchgrass cultivars were chosen to provide comparators based on ecotype, ploidy level, and geographic origin (Table 1). Seeds were obtained from USDA Germplasm Resources Information Network (USDA-GRIN), Ernst Conservation Seed (Meadville, PA, USA) and USDA Cape May Plant Materials Center (Cape May, NJ, USA). A total of 450 (~50 seeds/cultivar) seeds were sown in February, 2013 in the University of Connecticut's Floriculture greenhouse using Fafard's 3B soil mix (Anderson, SC, USA). Pots were warmed to 70°F and placed under a misting system. Twenty seedlings per cultivar were chosen for transplanting, moved from the propagation chamber to a misting room, held at 65°F-70°F and misted five times per day. As seedlings matured, plants were transferred to larger pots and fertilized based on company recommendations and pot size using Osmocote's 19-6-12 Smart Release Outdoor and Indoor Plant Food (The Scotts Company, Marysville, OH, USA).

Samples of native plants were collected in natural or disturbed habitats near the Long Island Sound as reported in Ahrens et al. (2011) and Ecker et al. (in preparation). The collection zone was in the Level III Ecoregion called the Northeast Coastal Zone, and the Level IV ecoregion 59g Long Island Sound Coastal Lowland (LISCL). A total of 37 plants were used in flow cytometry with 1-3 individuals for each cultivar and study site location. The study was designed to provide a broad assessment of wild switchgrass plants rather than a detailed examination of the variation within each coastal population or cultivar.

Flow Cytometry

Flow cytometry measures the relative nuclear DNA content and ploidy level of single cell events through excitement by lasers and analysis of fluorescence emissions. Relative nuclear DNA content is then compared to a reference or standard of known ploidy and genome size. The technique requires an internal or external standard that is used to classify the unknown DNA content and infer ploidy level (Arumuganathan and Earle 1991; Hultquist et al. 1996, 1997; Lu et al. 1998; Casler et al. 2006; Doležel et al. 2007; Costich et al. 2010; Zalapa et al. 2011). This study used a Lowland tetraploid cultivar ('Alamo') and an Upland octoploid cultivar ('Shawnee') as external standards.

The protocol for flow cytometry in this study followed the procedure of Arumuganathan and Earle (1991) with modifications for switchgrass leaf tissue. Young leaves were harvested on the morning of the experiment (150 mg) and placed on ice. Extraction buffer (1 mL Solution A) was added to the Petri dish for ~1 min. Solution A consisted of: 39 ml $MgSO_4$ stock buffer (0.246 g $MgSO_4$, J.T. Baker, Phillipsburg, NJ, USA), 0.370 g KCl (Fisher Scientific, Pittsburgh, PA, USA), 0.120 g HEPES Buffer (Fisher Scientific, Pittsburgh, PA, USA), 2 g PVP-10 in 100 ml water adjusted to pH 8.0 (Sigma-Aldrich, Allentown, PA, USA), 40 mg dithiothreitol – DTT (PhytoTechnologyLabs, Shawnee Mission, KS, USA), 1 mL Triton-X 100 stock in 10 ml DI H_2O (1 g Triton-X 100, Fisher Scientific, Pittsburgh, PA, USA). Leaf tissue was finely chopped in a parallel pattern using a sharp scalpel. The sample was expelled through 30 μm mesh capped cuvettes (BD Falcon, San Jose, CA, USA) and then centrifuged at 3000 rpm for 5 minutes. Supernatant was discarded and 200 μL of the staining solution (Solution B) was added to the pellet and resuspended. Staining Solution B consisted of: 8 ml Solution A, 20 μL RNAse (Sigma-Aldrich, Allentown, PA, USA), and 160 μL propidium iodide stock (5 mg/mL PI stock powder (Sigma-Aldrich, Allentown, PA, USA) in 1 ml DI H_2O).

Flow cytometry on the switchgrass samples was done at the University of Connecticut's Flow Cytometry and Confocal Microscopy Facility using a BD FACSCaliber Flow Cytometer (BD Biosciences, San Jose, CA, USA) with two lasers (488 and 633 nm). DNA content per cell was analyzed using CellQuest Pro software (BD Biosciences, San Jose, CA, USA). Acquisition and storage events were set to 100,000 nuclei counts and mean DNA content per sample was based on >1,000 nuclei per sample (Hultquist et al. 1996; 1997; Lu et al. 1998). Ploidy levels of cultivars and wild plants were manually estimated by comparing the relative DNA content to two well-known switchgrass cultivars (external standards): ‘Alamo’ (tetraploid) and ‘Trailblazer’ (octaploid). Ploidy levels were determined from the ratio of unknown DNA content compared to a known DNA content from one of the external standards, the equation used: (mean peak value of the unknown individual/ mean peak value of ‘Alamo’ (standard)) x 3.0 (averaged mean DNA content of ‘Alamo’ as reported in this study) (Hultquist et al. 1996; Doležel et al. 2007; Narasimhamoorthy et al. 2008; Zalapa et al. 2011). Individuals with mean DNA content ranging from 2.3 – 4.3 pg DNA per nucleus were classified as tetraploid and individuals with mean DNA content of 5.6 – 6.6 pg DNA per nucleus were classified as octaploid (Hultquist et al. 1996, 1997; Lu et al. 1998; Costich et al. 2010; Zalapa et al. 2011).

RESULTS

Flow cytometry (Table 1) showed that all of the wild plants collected in the Long Island Sound Coastal Lowland ecoregion were tetraploid. The wild plants had a mean DNA content of 2.6 pg/nuclei (range 2.3 – 2.8 pg) (Table 1). When the wild collected plants were combined with the Lowland cultivar samples, the mean increased slightly to 2.79 pg DNA/nuclei (range = 2.3 – 4.3 pg). In contrast, the octaploid cultivars in this study had a mean of 6.2 pg DNA / nuclei (range = 5.6 – 6.6 pg), nearly double the mean for the wild tetraploid plants. A graph showing the distribution of all individual plants relative to their nuclear DNA content (Figure 1) showed two distinct groups. Plants clustered toward the lower values (2.0- 4.4 pg) included the wild plants and five Lowland tetraploid cultivars (external standards). These plants were labeled tetraploids. The smaller number of individuals clustered towards the higher values (5.5 – 6.9 pg) included only individuals from the three Upland octoploid cultivars used as standards. As expected, the process of flow cytometry was able to clearly separate the tetraploid and octoploid plants.

In total, five Lowland cultivars and three Upland cultivars were analyzed as comparators for the wild plants (Table 1). The switchgrass cultivar ‘Alamo’ was chosen as the primary standard because it is an older, well-characterized Lowland tetraploid originating in Texas (Zalapa et al. 2011; Casler 2012). A second standard was developed from the octoploid cultivar ‘Trailblazer’ from Nebraska (Casler et al. 2007*a*; Zalapa et al. 2011; Jefferson and McCaughey et al. 2012). These two standards provided DNA concentrations and ploidy numbers consistent with previous reports. All of the Lowland cultivars were classified as tetraploid with a mean DNA value of 3.4 pg DNA/nuclei. Lowland cultivars originating from the Eastern states of Florida and North Carolina had somewhat higher values than the wild plants in the study, but still clearly grouped as tetraploids. As expected, the three Upland cultivars selected for the study (originated in Illinois, Nebraska or West Virginia) were found to be octoploid (mean = 6.2 pg DNA/nuclei) (Table 1).

DISCUSSION

The ploidy number for an organism is defined as the number of sets of chromosomes in the cell nucleus. Ploidy number and genetics, along with environmental cues, determine critical biological processes such as flowering phenology, biomass growth rates, species distribution, adaptation and, in some cases, invasiveness (Costich et al. 2010). Switchgrass has a high degree of diversity in ploidy levels ranging from diploid (2n=2x=18) to dodecaploid (2n=12x=108) (Church 1940; Nielson 1944). In our study, flow cytometry clearly separated tetraploid plants from octoploid plants (Figure 1). All wild plants collected from natural or disturbed sites in the Long Island Sound Coastal Lowland ecoregion were tetraploid. This result corresponded nicely with the results from our other study using microsatellite (SSR) markers and many more cultivar comparators (Ecker et al, in preparation). This verification of ploidy level using two unrelated analytical methods strengthens the conclusion that the native coastal switchgrass populations in this coastal region are Lowland tetraploid. In addition, our results were consistent with a project that used over 88,000 single nucleotide polymorphisms (SNPs) and switchgrass plants collected in New York (Lu et al. 2013).

In the Northeastern US, the pre-settlement distribution of switchgrass is thought to have been a narrow zone restricted to the upland edge of the coastal salt marsh (Niering and Warren, 1980). While some studies have examined ecological function, these wild populations have not been included in national germplasm collections or extensively surveyed for traits such as cold hardiness. We have submitted one wild switchgrass plant from our study site named 'Hammonasset' to a sequencing project conducted by the Joint Genome Institute (Joint Genome Institute, 328 Project #1030572, http://genome.jgipsf.org/Panvirsequencing_24/Panvirsequencing_24.info). Future analysis of this dataset could reveal unique traits and genetic resources.

Overall, the flow cytometry values in this study were very consistent with previous reports. For example, Lu et al (1998) reported that two tetraploids ('Summer', 'Kanlow') had 3.1 pg DNA/nuclei, while four octaploids ('Cave-in-rock', 'Trailblazer', 'Blackwell', 'Shawnee') had 6.1 pg DNA/nuclei. These values correspond closely to our own measurements for the octoploids 'Trailblazer' and 'Shawnee' of 6.2 pg DNA (Table 1). A study by Hultquist et al (1996) reported that 'Alamo' had a mean DNA content of 3.32 pg/cell, close to our value of 3.0 pg. However, they reported that 'Miami' had 3.23 pg DNA/nuclei which was lower than our measurement of 4.2 pg DNA/nuclei. Given the genetic heterogeneity in switchgrass, it is not surprising that seed populations could produce slightly different results in studies conducted more than 15 years apart.

Crop-to-wild gene flow and hybridization in switchgrass can occur as long as the parents have similar ploidy levels (Missaoui et al. 2005; Narasimhamoorthy et al. 2008). Thus, understanding ploidy number in wild switchgrass populations could help identify and protect small, fragmented populations that might be at risk from pollen-mediated gene flow from large biofuels feedstock plantations. Biocontainment strategies for biofuel fields could mitigate the risk of crop-to-wild gene flow. Examples of biocontainment strategies include large isolation distances between fields and wild populations (e.g. separation of more than 6 km), barriers to pollen dispersal (e.g. forest windbreaks), or the deployment of sterile switchgrass plants for biofuels production. Further studies on switchgrass genetics, adaptation, and ploidy number will support risk assessments and policies to conserve unique, native switchgrass populations throughout North America.

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Table 1. DNA content and ploidy number for *Panicum virgatum* leaf samples. Sample name, genotype (cultivar or native plant), geographic origin, ecotype, mean DNA content (pg DNA/nucleus) and ploidy levels (tetraploid, 4x; octoploid, 8x) are shown.

Sample	Cultivar/Wild	Origin	Ecotype	Mean DNA Content	Ploidy
Alamo	Cultivar	Texas	Lowland	3.0	4x
Miami	Cultivar	Florida	Lowland	4.2	4x
Performer	Cultivar	North Carolina	Lowland	3.3	4x
Stuart	Cultivar	Florida	Lowland	2.5	4x
Wabasso	Cultivar	Florida	Lowland	4.1	4x
Shelter	Cultivar	West Virginia	Upland	6.2	8x
Shawnee	Cultivar	Illinois	Upland	6.2	8x
Trailblazer	Cultivar	Nebraska	Upland	6.2	8x
CNA 15C	Native	Connecticut	-	2.8	4x
CDT 20B	Native	Connecticut	-	2.7	4x
CDT 18C	Native	Connecticut	-	2.7	4x
CDT 6C	Native	Connecticut	-	2.7	4x
CDT 2C	Native	Connecticut	-	2.7	4x
CNA 19B	Native	Connecticut	-	2.8	4x
CDT 17B	Native	Connecticut	-	2.5	4x
CDT 6A	Native	Connecticut	-	2.5	4x
CDT 20A	Native	Connecticut	-	2.4	4x
CDT 17C	Native	Connecticut	-	2.7	4x
CDT 2B	Native	Connecticut	-	2.8	4x
CDT 2A	Native	Connecticut	-	2.4	4x
CDT 18B	Native	Connecticut	-	2.3	4x
CDT 6B	Native	Connecticut	-	2.4	4x
CNA 19C	Native	Connecticut	-	2.5	4x
HAM 1C	Native	Connecticut	-	2.6	4x
HAM 1B	Native	Connecticut	-	2.4	4x
CDT 20C	Native	Connecticut	-	2.5	4x
CDT 18A	Native	Connecticut	-	2.5	4x

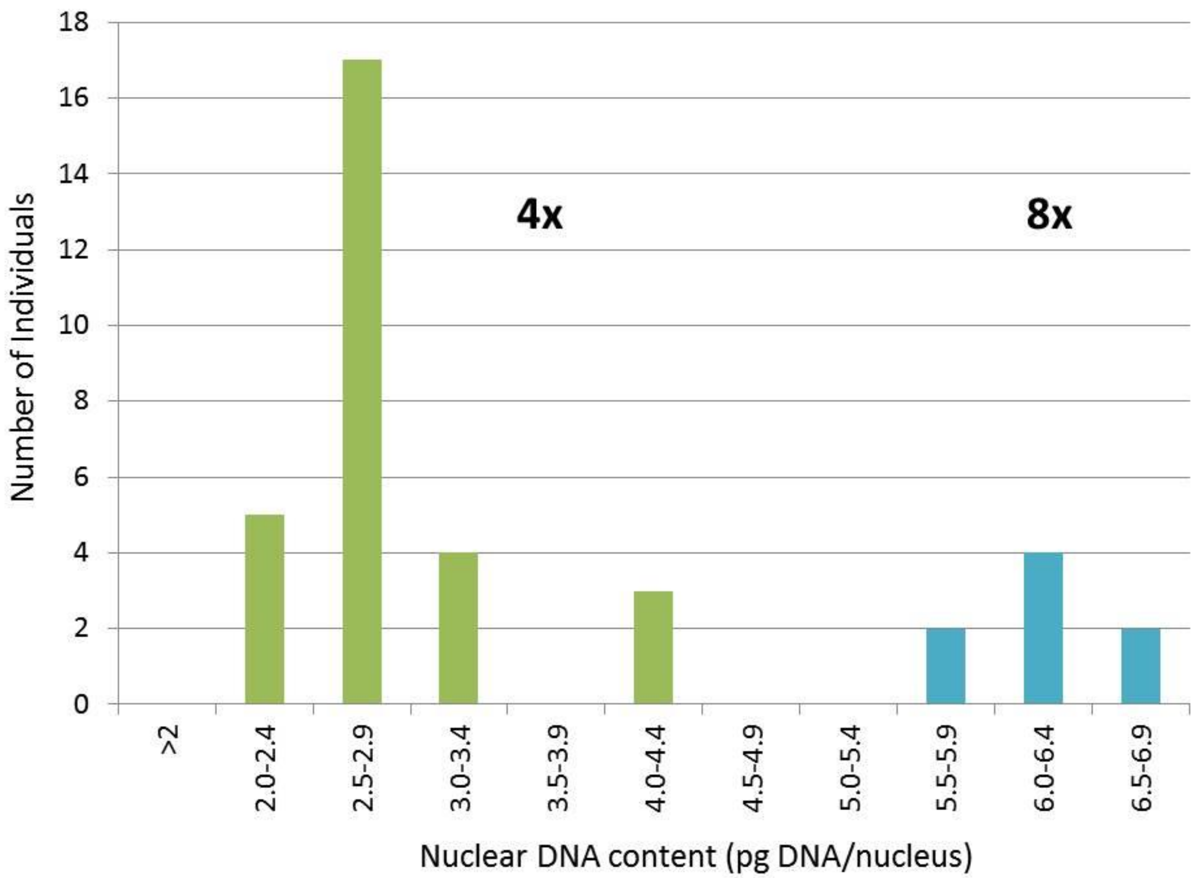

Figure 1. Summary of flow cytometry on 37 switchgrass plants. Tetraploids (4x) were n = 29, mean = 2.79 pg DNA/nuclei, SE = 0.085 pg, range = 2.3 – 4.3 pg. Octaploids (8x) were n = 8, mean = 6.20 pg DNA/nuclei, SE = 0.114 pg, range = 5.6 – 6.6 pg.