MOLECULAR, CYTOGENETIC, ANATOMICAL, AND MORPHOLOGICAL VARIATIONS ASSOCIATED WITH POLYPLOIDS OF PRAIRIE CORDGRASS

BY
SU MIN KIM

DISSE TATION
Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Crop Sciences in the Graduate College of the University of Illinois at Urbana-Champaign, 2015

Urbana, Illinois

Doctoral Committee:
Professor A. Lane Rayburn, Chair, Director of Research
Associate Professor DoKyoung Lee, Co-Director of Research
Professor Thomas Voigt
Professor Germán Bollero
ABSTRACT

Climate change or global warming, gradually increase in average global temperature, is now well documented and accepted by scientists as fact. The main cause of climate change is increasing carbon dioxide level (CO$_2$) in the atmosphere. Bio-renewable energy sources can play a role in providing energy services to meet basic human needs in sustainable manner, in particular, in migrating climate change. However, the current bioenergy sources face both social and environmental challenges, mainly because they use food crops which lead to land-use competition between food and liquid biofuels. In order to minimize competition with food crops, the next generation bioenergy sources should be grown on marginal lands, where food crops cannot be grown. Prairie cordgrass (*Spartina pectinata* Link) has been recommended as a dedicate energy crop grown on marginal land. This species is tall (1-3 m), rhizomatous, C$_4$ perennial grass, native to North America, and commonly found in lower, poorly drained soils along roadside, ditches, stress, marshes, wet meadows, and potholes. Prairie cordgrass is well known as polyploid species, including tetra- (2n=40), hexa- (2n= 60), and octoploids (2n=80), and has high abiotic stress tolerances such as salinity, cold, and waterlogging. Prairie cordgrass is successfully adapted in wide geographic distribution throughout Canada to 60° N latitude and in the United States throughout the Northeast, Great Lakes, and Midwest states, as well as most other states. According to previous studies, U.S. geographic distribution of this species is closely related to ploidy levels. Only tetraploid populations are found in the New England region, while most octoploids are distributed in the West North Central regions. Populations of tetra- and octoploids were found in close proximity in the West North Central and the West South Central regions. Unlike tetra- and octoploids, neo-hexaploids have only been reported in a single location co-existing with tetraploids. Since the polyploid evolutionary process in prairie cordgrass is dynamic, it is critical to understand how the polyploidization events influence on genetic diversity, chromosome structural changes, and morphological variations within the species. Through chloroplast DNA (cpDNA) phylogenetic analyses, three distinct genetic haplotypes were identified, which are
relatively associated with geographic distribution and ploidy level. Genetic diversity in polyploids has resulted in variation in the number of silver stained NORs (AgNORs) on chromosomes at metaphase. The distribution patterns of AgNORs in metaphase cells of tetra- and octoploids appears to reflect changes in ploidy. Two populations of tetra- and octoploids have the number of AgNORs expected from their mitotic metaphase chromosome number. Four AgNORs were found in tetraploids, while octoploids have eight AgNORs. However, two hexaploids have differing numbers of AgNORs (3 and 6), indicating that chromosome structure may not be stabilized in early generations of neo-polyploidy. This recent polyploidization event also appears to have modified morphological characteristics such as stem height, thickness, and mass, which are strongly associated with lodging resistance. Hexaploid populations have higher lodging resistance than their putative tetraploid populations due to specific morphological characteristics such as shorter height or thicker and heavy stem. Overall, the past and new polyploidization events may develop novel molecular, cytogenetical, anatomical, and morphological characteristics in prairie cordgrass. Therefore, the results of this study plays an important role in understanding the creation of genetic diversity which is a critical factor for developing prairie cordgrass as a dedicated energy crop grown in stressful environmental conditions.
To my family and friends
ACKNOWLEDGEMENT

This project would not have been possible without the support of many people. Many thanks to my advisors, Dr. D.K. Lee and Dr. A. Lane Rayburn. They allowed me to work in their labs, and offered guidance to successfully finish my research projects. I would also like to thank my committee members, Dr. Thomas Voigt and Dr. Germán Bollero for their helpful advices on data analysis and their great experimental suggestions. For help in the field trials, I would like to thank to Allen Parrish, undergraduate helpers, and other graduate students. Without their help, I might not be haven able to maintain my research plot and collect field data. For help in the laboratory, I would like to thank to undergraduate students who help keep the laboratory clean. I additionally would like to thank to Dr. Ainouche Malika and Dr. Ainouche Abdelkader for their help with genomic analysis. I also thank Moonsub, Hannah, Jeff, Joe, Jia, Chris, and Santanu for helping me. And finally, thanks to my parents and my brother who endured this long process with me, always offering support and Love.
# TABLE OF CONTENTS

CHAPTER 1: GENERAL INTRODUCTION.................................................................................. 1  
CHAPTER 2: INTRASPECIFIC PHYLOGEOGRAPHY ANALYSIS............................................. 4  
CHAPTER 3: ANALYSIS OF ACTIVE NUCLEOLUS ORGANIZING REGIONS ......................... 21  
CHAPTER 4: EFFECTS OF POLYPLOIDY ON LODGING..................................................... 36  
CHAPTER 5: CONCLUDING REMARKS............................................................................. 66  
REFERENCES................................................................................................................. 71
CHAPTER 1
GENERAL INTRODUCTION

Climate change or global warming, a gradual increase in average global temperature, is now well documented and accepted by scientists as fact. The main cause of climate change is increasing carbon dioxide levels (CO₂) in the atmosphere. Atmospheric CO₂ levels increased markedly in industrial times, and have recently reached the highest recorded levels of 401.3 ppm over the last 650,000 years (Pieter and Ralph, 2015). Environmental Protection Agency (EPA) has announced the Clean Power Plan (EPA, 2015). The goal of this plan calls for a 32%, reduction of the 2005 atmospheric CO₂ levels by 2030 (EPA, 2015). To meet this goal, the EPA recommends using more renewable energy from solar, wind, and bioenergy sources, (EPA, 2015). These renewable sources can play a role in providing energy services in a sustainable manner, in particular, in mitigating climate change (Moomaw et al., 2011). Among the renewable energy sources, bioenergy is the largest renewable energy source today, providing 10.2% of world primary energy supplies (Moomaw et al., 2011). However, the current bioenergy sources face both social and environmental challenges. One main challenge is that most bioenergy comes from food crops, which could lead to a land-use competition between food production and the conversion of that food to liquid biofuels (WEC, 2013). In order to meet global 10% transportation biofuel target in 2050, 30% of the total energy in all the crops that people harvest today will be needed (Wirsenius, 2000; EIA, 2013; FAO, 2013). Thus, to avoid direct and indirect competition with food production, growing bioenergy crops on marginal land has become important (Domburg et al., 2010).

Stress tolerance provides an important suite of traits that allow dedicated energy crops to be grown on marginal lands where food crops cannot grow (Simmons et al., 2008). Prairie cordgrass (Spartina pectinata Link) is a species suitable for the production of bioenergy on marginal lands, and possesses the co-benefit of water/soil conservation (Skinner et al., 2009). Prairie cordgrass is a tall (1-3 m), rhizomatous, warm-season (C4) perennial grass, native to North America and is commonly found in lower, poorly drained soils along roadsides, ditches, steams, marshes, wet meadows, and potholes where
soils are too wet to grow grain, forage, and other biofuel crops (Hitchcock, 1950; Mobberley, 1956; Stubbendieck et al., 1982; USDA NRCS, 2002; Barkworth et al., 2007). Skinner et al. (2009) reported that under waterlogged conditions, prairie cordgrass had the greatest root growth and highest survival rate compared to the warm season species, switchgrass, Indiangrass, big bluestem, and eastern gamagrass. Prairie cordgrass is also well adapted in various climatic and environmental stresses including cold, salinity, drought, and waterlogged soils (Weaver, 1954; Mobberley, 1956; Long, 1975; Potter et al., 1995; Montemayor et al., 2008; Boe et al., 2009; Kim et al., 2011a). Under drought and saline conditions, prairie cordgrass has produced comparable biomass yields to switchgrass cultivars with a high frequency of reproductive tillers (Boe et al., 2009; Kim et al., 2011a). The combination of salt and drought tolerances within this species results in a wide geographic distribution throughout the eastern coast and inland marshes of the Middle West to Alberta in North America (Canada and United States) (Barkworth et al., 2007).

Despite these promising features, growing and processing prairie cordgrass for bioenergy production is still in its infancy. Breeding improvement programs may include wild populations of plants that occur in a wide range of geography, climate, and soils. Wild selections planted in our local nurseries were used to identify the best genetic material to establish improvement programs in order to test for the genetic control of economically important traits such as growth, form, and diseases resistances. Present-day prairie cordgrass consist of polyploid species that potentially have more genetic variation including increased cell size and gene expression and changes in physiology, morphology, and ecological tolerances that arise due to natural selection acting on random mutation than their parental ancestors (Levin, 2002; Ramsey and Schemske, 2002; Lumaret, 1988; Adams and Wendel, 2005). These characteristics may allow that polyploid prairie cordgrass to successfully adapt to new environments, and therefore have the potential to invade new regions (Levin, 1983; Felber, 1991; Thompson and Lumaret, 1992; Ranney, 2006). Polyploid prairie cordgrass is reported to have three ploidy levels: tetra- (2n=40), hexa- (2n=60), and octoploid (2n=80) (Kim et al., 2010 and 2012), with a basic number of x=10 (Church, 1940). Kim et al. (2012) has identified geographic distribution patterns for all three cytotypes across the U.S. In the New
England region of the U.S., only tetraploid populations are found, while the octoploid populations are mostly distributed in the West, North Central regions. Both tetraploids and octoploids are distributed in the Central regions of the U.S. with no distinct geographical patterns. Unlike other ploidy levels, hexaploids are found as a mixed population with tetraploids occurring in a single location (Kim et al., 2012a) and a single seed from a tetraploid population (Kim et al., 2010). The origin of the octoploids and hexaploids has not yet been ascertained, but it’s likely that the higher ploidy populations originated from lower ploidy populations (Krahulcovà and Krahulec, 2000; Levin, 2002). Not surprisingly, there is pronounced morphological variability in polyploid prairie cordgrass across its geographic range (Jia et al., 2015). The morphological variation among prairie cordgrass populations might be influenced by local environments, genetic changes during polyploidization, or both. These morphological variations may be associated with increased heterozygosity, genetic redundancy (e.g. extra copy of genes), genomic rearrangement, epigenetic reprograming, or variation in gene expression (Jackson and Chen, 2010; Balao et al., 2011). The morphological variations found among polyploid prairie cordgrass populations emphasize the need for further studies of the molecular, cytogenetic, anatomical, and morphological consequences of polyploidization events.

Molecular, cytogenetic, anatomical and morphological investigations of multiple cytotypes in prairie cordgrass are critical steps for understanding the evolutionary process of polyploids involved in its origin, as well as the evolution of polygenic adaptive traits selected during polyploidization. Moreover, polyploidy events in prairie cordgrass may result in increasing the genetic diversity, which may lead phenotype innovation and an increased tolerance to extreme environmental changes (van de Peer, 2009; Jiao et al., 2011, De Bodt et al., 2005; Fawcett et al., 2009). Therefore, having knowledge of the genetic diversity associated with polyploidy events can play an important role in developing prairie cordgrass as a model species suitable for bioenergy production on marginal and/or less productive lands resulting from conditions such as soil salinity, drought, and water logging.
CHAPTER 2
INTRASPECIFIC PHYLOGEOGRAPHY ANALYSIS

ABSTRACT

Chloroplast DNA (cpDNA) sequences are appropriate for studying intraspecific phylogeography. Comparing cpDNA phylogeny with different cytotypes provides insights into the origin and establishment of polyploid species. We have previously defined the geographic distribution of cytotypes in prairie cordgrass throughout the different regions of the United States. In this study chloroplast haplotype variation is explored in 54 populations of prairie cordgrass, using 9, non-coding chloroplast regions. These chloroplast analyses were combined with the cytotype surveys of prairie cordgrass to infer the phylogeography and to elucidate the origin of the different cytotypes. In this study, three haplotypes, Prairie Cordgrass Group 1 (PCG1), Prairie Cordgrass Group 2 (PCG2), and Prairie Cordgrass Group 3 (PCG3), were identified. In general, related haplotypes were strongly associated with geographic distribution. Useful insertions-deletions (indels) were also found among prairie cordgrass populations. The PCG1 haplotypes collected in the East North Central to the New England regions of the US, were polyploid (tetraploid, hexaploid, and octoploids), while the PCG2 haplotypes, found in southern South Dakota, Iowa, and Missouri, were primarily octoploids, but also included a small number of tetraploids. The PCG3 haplotypes were octoploids and were collected in North Dakota, South Dakota, and Minnesota.

INTRODUCTION

Prairie cordgrass (*Spartina pectinata* Bosc ex Link) is a warm-season (*C₄*) perennial grass native to North America that commonly grows in a wide range of environmental conditions that include low, poorly drained areas (e.g., road sides, ditches, streams, marshes, wet meadows, and potholes) in which the soil is too wet to produce other native forage or grass crops such as switchgrass and big bluestem (Hitchcock, 1950; Mobberley, 1956; Stubbendieck et al., 1982; USDA NRCS, 2002; Barkworth et al., 2007). Prairie cordgrass also has potential to be a highly productive biofuel feedstock grown in stressful environment conditions including saline soils (Boe et al., 2009; Kim et al., 2012c). It is envisioned that prairie cordgrass will become a source of biomass used for bioenergy production on marginal lands as it also conserves water and soils (Bonilla-Warford and Zedler, 2002; Boe and Lee, 2007; Boe et al., 2009; Gonzalez-Hernandez et al., 2009; Skinner et al., 2009).

Prairie cordgrass is a polyploid species comprised of three cytotypes, tetraploids (*2n = 4x = 40*), hexaploids (*2n = 6x = 60*), and octoploids (*2n = 8x = 80*), with a basic number of x= 10 (Church, 1940; Marchant, 1968 a and b; Kim et al., 2010). Early sampling of natural populations identified the geographic distribution of tetraploid and octoploid cytotypes of prairie cordgrass (Reeder, 1977). Recent studies have reported the occurrence of naturally growing hexaploid prairie cordgrass and have identified the geographic distribution patterns for all three cytotypes across the US. (Kim et al., 2012a and b). In the New England region of the US, only tetraploid populations were found, while the octoploid cytotypes were mostly distributed in the West North Central regions. Both tetraploids and octoploids were distributed in the Central regions of the US with no distinct geographical patterns. The origin of the octoploids and hexaploids has not yet been ascertained, but it’s likely that the higher ploidy populations originated from lower ploidy populations (Krahulcová and Krahulec, 2000; Levin, 2002). Hexaploid prairie cordgrass plants have been found in mixed populations with tetraploids only in IL, USA. These
cytogeographic studies are informative for analyzing the geographic pattern of ploidy and also for understanding the relationship between ploidy levels and geographic distribution of plant.

Noncoding sequences of chloroplast genomes may provide useful information in the perspective of intraspecific phylogeographic studies (Shaw et al., 2007; Scarcelli et al., 2011). Various studies have reported intraspecific variation in cpDNA in Spartina (Blum et al., 2007) and have investigated the maternal origin of hybrids and polyploids in several species (Ferris et al., 1998, Baumel et al., 2001, Fortune et al., 2007, Castillo et al., 2010). Furthermore, cpDNA variation may be geographically structured in several species such as beech (Demuesure et al., 1996), white oaks (Dumolin-Lapègue et al., 1997), European oaks (Ferris et al., 1998), and pitted stripeseed (Maskas and Cruzan, 2000). Therefore, understanding cpDNA variation in prairie cordgrass will be useful for documenting the evolutionary processes affecting this polyploid complex, and for studying intraspecific phylogeographical patterns, which are critical steps for enhancing breeding and germplasm conservation efforts.

In this study, we used 9, non-coding chloroplast DNA regions identified in Taberlet et al. (1991) and Shaw et al. (2007) to examine the intraspecific variation and phylogeography of 54 populations of prairie cordgrass collected from various eco-geographical areas of the US. The geographical distribution of the different ploidy levels (4x, 6x and 8x) was mapped on the cpDNA phylogeny in order to get information on the polyploidy formation events that occurred in prairie cordgrass throughout their establishment in a wide ecogeographic range. The main aims of this study were to (1) address chloroplast diversity within and among prairie cordgrass population cytotypes, (2) infer evolutionary forces such as polyploidization that may have shaped the observed prairie cordgrass population structure, and (3) associate prairie cordgrass phylogenetic analysis with the geographic origins of populations.
MATERIALS AND METHODS

Plant material

In 2009 and 2010, 49 natural populations of prairie cordgrass were sampled from five geographic regions of the US (U.S. Census Bureau 2011). The regions sampled were New England states (Maine, Massachusetts, Connecticut); Middle Atlantic (New Jersey, New York); East North Central (Wisconsin, Illinois); West North Central (Minnesota, Iowa, Missouri, North Dakota, South Dakota, Nebraska, Kansas); and West South Central state (Oklahoma) (Fig. 2.1). Rhizomes, or seeds when possible, were collected in each location to represent the regional population. In addition, a population originating in New York was obtained as fresh rhizomes from the Big Flats Plant Material Center of USDA-NRCS (New York, USA). In 2012, rhizomes of six more populations were collected from 6 sites representing distinct clusters of plants occurring along railroad embankments (~ 100 m) in Dixon, IL. Germlasm of ‘Red River’ prairie cordgrass, a type developed through open pollination among vegetative propagules in an isolated common garden nursery using populations from east-central Minnesota, northeastern South Dakota, and east-central North Dakota (Boe et al., 2009), was obtained as seeds from a commercial seed source (Millborn Seed Inc., Brookings, SD, USA). Rhizomes of 3 other Spartina species including *S. alterniflora* (2 populations), *S. cynosuroides* (1 population), and *S. patens* (2 populations) were obtained for outgroups of prairie cordgrass. *Spartina alterniflora* and *S. cynosuroides* were obtained as seeds from Louisiana State University Agricultural Center (Baton Rouge, LA, USA) and USDA-NRCS (Big Flats Plant Materials Center, NY, USA), respectively. Two populations of *S. patens* were obtained as fresh rhizomes provided by USDA-NRCS (Brooksville Plant Material Center, FL, USA). All prairie cordgrass samples and 5 from other *Spartina* species were planted in 11 cm x 11 cm pots filled with Sunshine SB300 Universal soil (American Plant Product & Service, OK, USA) and maintained under greenhouse conditions (16 h photoperiod at temperature of 24-26° C). Fresh young leaf tissues from each of 59 populations were sampled and stored at -80 °C until DNA extraction was performed.
Analysis of DNA content

The ploidy levels of several prairie cordgrass populations have been reported in Kim et al. (Kim et al. 2010, 2012a, and 2012b). In 2012, we collected six additional prairie cordgrass populations in Illinois, and the relative ploidy levels of these populations were determined by flow cytometry, according to methods described by Rayburn et al. (2009). According to Kim et al. (2010) who also used chromosome counts as references, populations with genome size of 3.16 pg were considered as octoploids, while populations having genome sizes ranging from 1.57 to 1.65 pg were considered tetraploids. Ploidy levels found in prairie cordgrass populations used in this study have been listed in Table 1.

DNA extraction, PCR, and sequencing

Total genomic DNA was extracted from frozen leaf tissue using the CTAB method described by Mikkilineni (1997) with slight modifications. The samples were extracted in a buffer containing 5% CTAB, 0.7 M NaCl, 10 mM EDTA pH 8.0, 50 mM Tris–HCl pH 8.0, and 0.1% 2-mercaptoethanol and incubated for 20 minutes at 70 °C with occasional gentle mixing.

For all sequences, each amplification reaction (25 µl final volume) contained 100 ng template DNA, 5 X GoTaq reaction buffer (Promega, Madison, WI, USA), 5 µL of 2 mM dNTP, 10 µM each primer, and 5 units of GoTaq polymerase (Promega, Madison, WI, USA). Nine noncoding regions of cpDNA were amplified using the oligonucleotide primers designed by Taberlet et al. (1991) and Shaw et al. (2007) (Table 2.2). The polymerase chain reaction (PCR) cycling conditions for trnT-trnL and trnL-trnF regions were denatured for 5 min at 94 °C followed by 30 cycles of denaturation at 94 °C for 1 min, 48 °C annealing for 1 min, and 72 °C extension for 2 min, whereas other 7 cpDNA regions (petL-psbE, rpl32-trnL(UAG), psbJ-petA, 3'trnV-ndhC, atpI-atpH, and 3'rps16-5'trnK) were denatured for 5 min at 80 °C followed by 30 cycles of denaturation at 95 °C for 1 min, 50 °C annealing for 1 min, and 65 °C extension for 4 min. The PCR products were purified using EZ-10 spin column PCR purification kit (Bio Basic Inc. Canada) after electrophoresis in 1.2% agarose gels using a 0.5 X TBE buffer. The purified
PCR products were sent to Biotechnology Center (University of Illinois, Urbana, IL, USA) for direct sequencing. Sequences were manually corrected with BioEdit (http://www.mbio.ncsu.edu/BioEdit/BioDoc.pdf) and aligned with Clustal Omega (Sievers et al., 2011). The phylogenetic trees of the cpDNA sequences were constructed using both maximum parsimony (MP) and neighbor-joining (NJ) methods as implemented in the MEGA5 (Tamura et al. 2007 and 2011). Bootstrap analyses (Felsenstein 1985) were performed, using 1000 replicates, to obtain reliable estimates for nodes. Five additional Spartina samples were included as outgroups in both MP and NJ analyses.

RESULTS

A total of 54 prairie cordgrass populations, including 33 tetraploids, 2 hexaploids, and 19 octoploids have been analyzed for cpDNA in this study (Table 2.1). Phylogenetic analyses based on an aligned data set of 9 non-coding chloroplast regions from 54 prairie cordgrass populations and the 5 additional Spartina samples, resulted in a 7205 character dataset with 280 (4.15 %) variable sites. Both MP and NJ trees were constructed independently based on the concatenated sequence data set. In this manner, three main clades, corresponding to Prairie Cordgrass Group 1 (PCG1) haplotype, Prairie Cordgrass Group 2 (PCG2), and Prairie Cordgrass Group 3 (PCG3), were distinguished (Fig. 2.2). The tree topology resulting from NJ and MP analyses was essentially the same, except for one small sub-clade in PCG1 haplotype (Fig. 2.2). The parsimony analysis yielded the 10 most parsimonious trees (tree length = 108, CI = 0.9615, RI = 0.979). In NJ analysis, the optimal tree with the sum of branch lengths was 0.014. Within the resulting strict consensus tree, the prairie cordgrass accessions form a monophyletic group separated from the outgroup (Fig. 2.2). Among the later, S. alterniflora, and to a lesser extent S. cynosuroides, is more distantly related to prairie cordgrass than S. patens representatives, which form a paraphyletic group to prairie cordgrass here.
Sequence variability in each chloroplast region is summarized in Table 4. When including outgroups, a total 66 informative variable sites and 16 indels (insertions and deletions), varying from 1 to 35 bp in length, were identified within the 9 chloroplast regions. A total of 9 informative variable sites and 15 indels, varying from 1 to 29 bp, were identified among prairie cordgrass populations. Only a few variable sites were detected within the prairie cordgrass populations, while 15 informative indels were identified, which makes some of them useful distinguishing haplotypes. Among all chloroplast sequences, the trnT-trnL sequence length within prairie cordgrass populations was highly variable (indels ranging from 2 to 27bp) (Table 2.4). The longest indel (27 bp) in the trnT-trnL region appeared in both haplotypes PCG2 (2 populations including 19-107 and 17-111-5) and PCG3 (all 4 populations). One indel (4 bp insertions) in the trnT-trnL region only appeared in haplotype PCG2.

Chloroplast DNA haplotypes were strongly associated with both geographic distribution and different cytotypes as shown in Figure 2.1. All three cytotypes, tetra-, hexa-, and octoploids, were found in the PCG1 haplotype. Moreover, the PCG1 haplotype occupied the greatest geographic area and was found in all five geographical regions, with the exception of North Dakota, South Dakota, and Minnesota (Fig. 2.1). In the PCG1 haplotype, four sub-clades were formed with bootstrap values of approximately 60% (Fig. 2.2). Among these sub-clades, populations from the New England and East North Central regions, except for two populations from Connecticut, were clustered together with weak support (64 % bootstrap in MP tree and 66 % bootstrap in NJ tree). The other three sub-clades were widely distributed in both tetra- and octoploids from the central regions of the US. The PCG2 haplotypes were mostly found in octoploids distributed in Western North Central regions, and appeared only in southern South Dakota, Iowa, and Missouri. Two tetraploids from Illinois and Missouri each were clustered in the PCG2 haplotype clade. One Illinois tetraploid from PCG2 haplotype, 17-111-5, was found on a railroad embankment in an area where octoploids also existed (Table 2.3). Populations exhibiting the PCG3 haplotype were all octoploids distributed in the Western North Central regions covering North Dakota,
South Dakota, and Minnesota. The PCG3 haplotype appears geographically distinct from the other two haplotypes.

DISCUSSION

The phylogenetic placement of S. pectinata accessions compared to 3 other Spartina species included as out groups, agree with the results of Baumel et al. (2002) who reported the phylogenetic relationships of 10 Spartina species analyzed from two, non-coding chloroplast regions of trnT-trnL and trnL-trnF. Unlike Baumel et al. (2002), our strict consensus trees show better phylogenetic resolution because more variable sites were used in this phylogenetic analysis. In addition, phylogenetic analysis of the Waxy gene reported by Baumel et al. (2002) shows that S. cynosuroides appeared to be a sister species of prairie cordgrass (with a moderate support of 81 % bootstrap value), while our trees instead support a common origin of the plastid genome for S. patens and prairie cordgrass (with 99 % bootstrap in MP tree and with 98 % bootstrap in NJ tree). However, in both cases the three latter taxa are closely related, and such nuclear versus cytoplasmic phylogenetic incongruence might result from reticulate evolutionary events (hybridization, allopolyploidization), which are known to have frequently occurred, and most probably still occur, in Spartina (Aï nouche et al., 2012). Hybrids between S. patens and S. pectinata (S. x ceaspitosa) have been reported and support the close phylogenetic relationships between these taxa (Mobberley, 1956). Our results indicate strong evidence for the existence of three cpDNA haplotypes in prairie cordgrass. The number of informative variable sites within the 9 surveyed plastid regions was very low, which is common in cpDNA sequences (Wolfe et al., 1987; Muse, 2000). However, we found a total of 15 useful informative indels that resolved the phylogenic relationship among natural accessions of prairie cordgrass into three clades. It has been shown from previous studies that, in the chloroplast genome, indels can be phylogenically informative and extremely useful for resolving relationships among closely related taxa (Golenberg et al., 1993; Kelchner and Clark, 1997;
Kelchner 2000) and may even increase the resolving power at the intraspecific level (Graham et al., 2000). In the trnT-trnL region, only 27 indels were observed in two haplotypes of PCG2 and PCG3, with none in PCG1, which suggests its diagnostic value (Fig. 2.1).

Based on the conservative information generated from this dataset, it appears that the ancestral plastid genome of prairie cordgrass has rapidly diverged into three main haplotypes: Prairie Cordgrass Group 1 (PCG1) haplotype, Prairie Cordgrass Group 2 (PCG2), and Prairie Cordgrass Group 3 (PCG3). The geographic distribution of the observed cpDNA haplotypes also revealed strong geographic structuring (Fig. 2). The distribution of the cpDNA haplotypes throughout the US clearly indicates that PCG1 haplotype is the most common haplotype occurring in central US regions with colonization extending to New England and the Middle Atlantic region. Prairie Cordgrass Group 1 haplotypes were detected from the most prairie cordgrass populations (44), were comprised of all three cytotypes of tetra-, hexa-, and octoploids, and provided information on the common origin of their maternal genome. Prairie Cordgrass Group 2 haplotypes consisted of both tetra- (2) and octoploids (4), which occupied a distinct region overlapping that of PCG1 haplotype in the Western part of Central US regions (SD, MO, IA, and IL). Two tetraploid populations exhibited the PCG2 haplotype, but were widely separated from octoploids and appeared to be the most probable maternal progenitors of the octoploids having the PCG2 haplotype. Further studies using nuclear markers are expected to provide more insight to best understand prairie cordgrass migration. Only a few isolated octoploids from the North Western part of Central US regions (ND, SD, and MN) shared the PCG3 haplotypes, which suggests that their progenitors either disappeared or were not collected and sampled in this study.

Unlike previous studies (Reeder, 1977; Kim et al., 2010 and 2012b), we found octoploids co-occurring with tetraploids in one small northern region of IL. All prairie cordgrass populations collected in this area were naturally growing on a railroad embankment, and the individual clumps were separated by short distances. In this area, 5 populations, including 2 octoploids and 3 tetraploids, were clustered in the PCG1 haplotype, whereas the remaining tetraploid population was clustered in the PCG2 haplotypes.
Moreover, the tetraploid PCG2 haplotype was morphologically different from other populations (data not shown). Due to the proximity of the railroad tracks, trains may have introduced the northern IL PCG2 haplotype into this area. The octoploid of PCG1 haplotype from this region could be a recent event of doubling of the tetraploids, or more likely, due to west-to-east train travel as well.

In our previous report (Kim et al. 2012a), another mixed ploidy population comprising tetraploids and hexaploids was found at a single location in IL. No cpDNA variation was found in mixed populations between tetraploids and hexaploids, which have also been observed in other plant species (e.g., *Plantago media*; Van Dijk and Backx-Schotoman, 1997). According to Kim et al. (2010), a hexaploid plant has developed from seeds collected from tetraploid plants, so this phylogeny suggests that hexaploids co-occurring with tetraploids may be a consequence of the emergence of a neopolyploid within a progenitor population (Petit et al., 1999).

**CONCLUSION**

This research reports the first wide-ranging cpDNA-based phylogeographic study of prairie cordgrass across several US regions. Our results showed that cpDNA haplotypes were strongly related to geographic distribution. The PCG1 haplotypes occurred in two regions, the New England/Middle Atlantic region in the east and the central US, while the PCG2 haplotypes were found in southern SD and northern IA, IL, and MO in the central USA. The PCG3 haplotypes were identified in a distinct region that includes portions of ND, SD, and MN. Our phylogenetic analyses involving different cytotypes provide insight into the origins and establishment of polyploids in natural populations of prairie cordgrass across the US and provide a springboard for further population genetic studies of *Spartina* spp.
FIGURES AND TABLES

Table 2.1 Ploidy levels found in prairie cordgrass natural populations in the US, according to Kim et al. (2010 and 2012ab) and present records.

<table>
<thead>
<tr>
<th>Ploidy level</th>
<th>Number of Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ME$^1$</td>
</tr>
<tr>
<td>Tetraploid</td>
<td>3</td>
</tr>
<tr>
<td>Hexaploid</td>
<td>2</td>
</tr>
<tr>
<td>Octoploid</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$ According to Boe et al. (2009), the Spartina pectinata ‘Red River’ (RR) is a natural germplasm developed from the open pollination of three natural populations from ND, SD, and MN.

$^1, 2, 3, 4, 5$ Based on the map of U.S. Census Bureau (2011), the study was divided into five regions: (1) New England; (2) Middle Atlantic; (3) East North Central; (4) West North Central; and (5) West South Central.
Table 2.2 Primer sequences used for the amplification of 9 non-coding chloroplast DNA regions in *Spartina pectinata*.

<table>
<thead>
<tr>
<th>Chloroplast Regions</th>
<th>Primer Pair Sequences (5’-3’)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward</td>
<td>Reverse</td>
</tr>
<tr>
<td><em>trnT-trnL</em>¹</td>
<td>CATTACAAATGCAGTAGCTCT</td>
<td>TCTACCGATTTCGCCATATC</td>
</tr>
<tr>
<td><em>trnL-trnF</em>¹</td>
<td>GTTCAAGTCCCTCTATCC</td>
<td>ATTTGAACCTGGTACACGAG</td>
</tr>
<tr>
<td><em>petL-psbE</em>²</td>
<td>AGTAGAACAACGAAATAACTAGTTA</td>
<td>TATCGAATACTGGTAATAATATCAGC</td>
</tr>
<tr>
<td><em>rpl32-trnL</em>²</td>
<td>CATGTTCAAAAAACGTACTTC</td>
<td>CTGCTTCTAAAGACAGAGC</td>
</tr>
<tr>
<td><em>rpl14-rpl36</em>²</td>
<td>AAGGAATCCAAAAGGAAGCTC</td>
<td>GGRGGGAACAAATTACTATAATTC</td>
</tr>
<tr>
<td><em>psbJ-petA</em>²</td>
<td>ATAGGTAATGTARCYGGTATT</td>
<td>AACARTTYGARAAGGTTCATT</td>
</tr>
<tr>
<td><em>3’trnv-ndhC</em>²</td>
<td>GTCTACGGTGARTCCGTA</td>
<td>TATTATTAGAAATGYCCARAAATATCATATTTC</td>
</tr>
<tr>
<td><em>atpL-atpH</em>²</td>
<td>TATTACAAAGYGGTATTCAAGCT</td>
<td>CCAASYCCAGCAAGAATAC</td>
</tr>
<tr>
<td><em>3’sps16-5’trnK</em>²</td>
<td>AAAGTGGGTTTTATGATCC</td>
<td>TTAAAGCCAGTACTCTACC</td>
</tr>
</tbody>
</table>

¹Taberlet et al. (1991), ²Shaw et al. (2007)
Table 2.3 Population ID, number of samples analyzed (n), mean 2C genome size (± SD), 1Cx genome size, and ploidy level of the tetra- and octoploid prairie cordgrass populations collected in IL.

<table>
<thead>
<tr>
<th>Population ID</th>
<th>Number (n)</th>
<th>2C Genome size (pg ± SD)</th>
<th>1Cx genome size (Mb*)</th>
<th>Ploidy level</th>
</tr>
</thead>
<tbody>
<tr>
<td>17-111-1</td>
<td>4</td>
<td>3.16 ± 0.03</td>
<td>386</td>
<td>8x</td>
</tr>
<tr>
<td>17-111-5</td>
<td>2</td>
<td>1.60 ± 0.02</td>
<td>391</td>
<td>4x</td>
</tr>
<tr>
<td>17-111-6</td>
<td>2</td>
<td>1.57 ± 0.01</td>
<td>383</td>
<td>4x</td>
</tr>
<tr>
<td>17-111-7</td>
<td>2</td>
<td>1.61 ± 0.40</td>
<td>393</td>
<td>4x</td>
</tr>
<tr>
<td>17-111-8</td>
<td>2</td>
<td>3.16 ± 0.06</td>
<td>385</td>
<td>8x</td>
</tr>
<tr>
<td>17-111-9</td>
<td>2</td>
<td>1.65 ± 0.01</td>
<td>403</td>
<td>4x</td>
</tr>
</tbody>
</table>

* Conversion: 978 Mb =1pg according to Dolezel et al. (2003)
Table 2.4 Summary of sequence variation within prairie cordgrass populations and with outgroups.

<table>
<thead>
<tr>
<th>Chloroplast Regions</th>
<th>Alignment length (bp)</th>
<th>Within prairie cordgrass (54 populations)</th>
<th>With outgroups (59 populations)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Informative</td>
<td>Uninformative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Variable sites</td>
<td>Indels</td>
</tr>
<tr>
<td>trnT-trnL</td>
<td>658-703</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>trnL-trnF</td>
<td>914-919</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>petL-psbE</td>
<td>1154-1198</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>rpl32-trnL</td>
<td>648-663</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>rplL14-rpL36</td>
<td>1108</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>psbJ-petA</td>
<td>811-844</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3'trnu-ndhC</td>
<td>294-298</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>atpL-atpH</td>
<td>416-420</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3' rps16 5'trnK</td>
<td>595-598</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Combined</td>
<td>6598-6750</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.1 Chloroplast DNA haplotypes and cytotype distributions in prairie cordgrass. Rectangle, cross, circle, and triangle represent tetraploid, mixed populations of tetra- and hexaploids, octoploids, and mixed population of tetra- and octoploids, respectively. The PCG1 haplotypes are colored green, the PCG2 haplotypes are colored pink and the PCG3 haplotypes are colored blue.
Figure 2.2 Bootstrap consensus tree based on the (a) MP and (b) NJ analyses of combined 9 cpDNA sequence data for 54 prairie cordgrass populations, with 3 other Spartina species as outgroups. Ploidy levels (4x, 6x, and 8x) and location are shown next to the population name. Bootstrap values from 1000 replicates are indicated on the nodes as percentages. Major clades were identified as PCG1 (green), PCG2 (pink), and PCG3 (blue).
Figure 2.2 (cont.)
CHAPTER 3

ANALYSIS OF ACTIVE NUCLEOLUS ORGANIZING REGIONS

ABSTRACT

Prairie cordgrass has recently gained attention as an important biotic component of stressed ecosystems. This polyploid species is distributed broadly across the U.S. More cytogenetic data are needed to investigate how increased ploidy levels influence chromosome changes and gene expression in order to understand the formation of stable cytotypes. Silver staining is the cytogenetic method commonly used to study the number and distribution of active nucleolar organizing regions (NORs) on chromosomes at metaphase, and the number and size of nucleoli in nuclei at interphase. Intensity and distribution patterns of silver stained NORs (AgNORs) reveal differential ribosomal gene expressions in plants and animals. The purpose of this study is to estimate the number of AgNORs, their locations, and their activities in metaphase chromosomes and to determine heteromorphic variation in size and number of nucleoli within interphase nuclei for tetra-, hexa-, and octoploid prairie cordgrass populations. Increases in mean numbers of AgNORs in each metaphase and interphase cell were observed as ploidy level increases. Although distribution patterns of silver stained NORs at metaphase cells of tetra-and octoploids reflect changes in ploidy, the neo-hexaploids did not follow the pattern, indicating that active NORs were not stable in early generation of formation of neo-hexaploidy. Collectively, these results suggest that distribution patterns of silver stained NORs in metaphase and number of silver stained nucleoli in interphase cells can be used as markers to detecting chromosome variations within and among ploidy levels and to determine when ploidy levels stabilize in prairie cordgrass.

INTRODUCTION

Prairie cordgrass (*Spartina pectinata* Link) is a tall, rhizomatous, perennial, and warm-season grass (*C₄*) native to a wide geographic range in North America. This species has recently gained attention as an important biotic component for soil and water conservation practice on agricultural landscapes along with biomass production (Long 1975, Potter et al., 1995, Boe and Lee, 2007, Montemayor et al., 2008, Skinner et al., 2009, Boe et al., 2009, Kim et al. 2012a). Due to its massive rhizome and deep root system (Weaver, 1968), prairie cordgrass is able to survive and even thrive under abiotic stress conditions such as waterlogging (Jensen, 2006), soil salinity (Montemayor et al., 2008, Kim et al., 2012c) and drought (Boe et al., 2009). In addition, the combination of genomic changes and increased genetic diversity induced by polyploidization (Parisod et al., 2010) may be a key factor in growth, performance, adaptability of polyploid prairie cordgrass in such marginal environments.

Prairie cordgrass has been reported as a multi-polyploid species consisting of three ploidy levels: tetraploid (2n=40), hexaploid (2n=60), and octoploid (2n=80) with a basic chromosome number of x=10 (Church 1940, Marchant 1968 a, b, Kim et al. 2010). Kim et al. (2012a) studied intraspecific ploidy level variations in 183 prairie cordgrass individuals collected across the U.S. to understand the potential relationship between ploidy levels and geographic distribution. According to cytogeographic distribution of prairie cordgrass, the tetraploids extended from the East North Central to the New England regions of the U.S., while the octoploids were mostly distributed in West North Central regions. Hexaploids were only found in one location in which hexaploids were co-occurring with tetraploids in central Illinois (Kim et al. 2012b). Our previous studies indicated strong associations of genetic (Kim et al., 2013) and morphological variation (Guo et al., 2013) with geographic distributions, meaning that genetic diversity found in polyploid prairie cordgrass can be exploited for phenotypic variation and wide geographical adaptation. However, increased genes and genome dosage in polyploids can also cause negative effects such as genome instabilities, chromosome imbalances, regulatory incompatibilities, and reproductive failures (Chen, 2007). We need more sufficient cytogenetic
and molecular data are needed to investigate how increased ploidy influences the structural changes of chromosome that maintain the stability of cytotypes within their respective distributed area.

Silver staining is the cytogenetic method commonly used to study the number, size, and distribution of silver stained nucleolar organizer regions (AgNORs) at metaphase or nucleoli at interphase. During metaphase, silver atoms deposit onto argyrophilic proteins associated with nucleolar organizer regions (NORs) which involved in high rate of production of ribosomal RNA genes and of ribosomes that are necessary for all protein synthesis in the cell (Olert et al., 1979, Buys and Osinga, 1980, Jordan, 1987, Hubbell, 1985). AgNORs are located in secondary constriction of chromosome satellites which is constant in shape and size for each particular chromosome (Mahabal, 2010), and the intensity of the silver reaction is reflective of the nucleolar transcriptional activity taking place during the interphase preceding metaphase (Ferraro and Lavia, 1983, Schwarzacher et al., 1978). Comparison of the distribution patterns and intensity of AgNORs at metaphase between and within ploidy levels, therefore, can help in evaluating alterations of chromosomal structures and the rate of production of ribosomal materials, which may be involving with growth and performance associated with the stabilization of polyploidy series. In interphase, silver atoms deposit onto the fibrillar centres and the fibrillar components of the nucleolus containing contain ribosomal DNA (rDNA) and acidic proteins associated with rDNA sites (Howell and Black, 1980, Pébusque and Seite, 1981, Paweletz and Risueño, 1982, Fernández-Gómez et al., 1982, Sumner 1982). The amount of silver staining in the nucleoli has been shown to correlate with ploidy level and/or proliferative activity of cells (Darvey and Driscoll, 1972, Crocker et al., 1998, Sumner, 2003).

In this study, six prairie cordgrass (Spartina Pectinata Link) populations were studied: two tetraploids (IL102 and 20-102), two hexaploids (17-116 and 103-6x), and two octoploids (PCG109 and 19-108). Their cytotypes and geographic distribution were already given in Kim et al. 2010 and 2012ab. The objectives of this study were to estimate the number of AgNORs, their locations, and their activities on metaphase chromosomes and to determine the heteromorphic variation in size and number of nucleoli at interphase cells for different ploidy levels in prairie cordgrass populations.
MATERIALS AND METHODS

Plant material

Fresh clones of 6 populations (2 tetra-, 2 hexa-, and 2 octoploids) of prairie cordgrass were used for this experiment (Table 3.1). The rhizomes were grown in an aeroponic system and maintained under greenhouse conditions (16-h photoperiod at temperature of 24-26 °C).

Silver staining

Fresh young root tips were collected and fixed in ethanol-acetic acid (3:1) after a 2-hour treatment with 0.005% of 8-hydorxyquinolin. The root tips were stored at 4 °C for 2 days. The fixed root tips were digested in an enzyme solution (0.2 g Cellulysin and 0.1 g Macerase in 10 mL of 10 mM EDTA, pH 6) for 2.5 h. The root tips were placed on a slide with a drop of 45 % acetic acid and were chopped with thin blade. A cover slip was placed on the tissue and gently tapped with a dissecting needle to disperse all of the tissue. Direct pressure was applied to the slide, and the slide was stored at -80 °C until use. The cover slip was removed after freezing and preparation was air-dried for 1.5 hr.

Slides were incubated in 2xSSC (300 mM NaCl and 30 mM Na3C6H5O7 in 1L of ddH2O, pH=7) with coverslips in a humidity chamber at 37 °C for 5 min, washed in ddH2O, and air-dried. Slides were covered with 50 µm nylon mesh after adding a few drops of freshly prepared 50 % silver nitrate solution in ddH2O. The slide was incubated in a humidity chamber at 60 °C for 7 hrs, washed in ddH2O, and air-dried in the dark for 1 day. The dried slide was soaked in Xylene for 5 min. A few drops of permount were added, extra permount was rinsed with xylene, and a coverslip was added. The silver staining was visualized by using Olympus BX61 microscope. Photographs were taken using an Olympus U-CMAD3 camera. Image analysis software (Image J) was used to estimate the number and size of Ag-positive dots and size of nucleus from 100 randomly selected nuclei per each population.
**Feulgen Staining**

Fresh young root tips of “IL102” were collected and stored in 3:1 ethanol/acetic acid for 24 hrs at 4°C. The fixed root tips were rinsed in ddH₂O, hydrolyzed in 5M HCl for 45 min, and placed in Feugen’s stain for 2hrs. Root tips were rinsed in ddH₂O and incubated in enzyme solution (0.2 Celluysin and 0.1 g Macerase in 10 mL of 10 mM EDTA) for 1hr at 37°C. The enzyme solution was washed with 45 % acetic acid. Root tips were squashed onto slides in a drop of 45 % acetic acid. The slides were viewed using an Olympus BX61 microscope. Photographs of nuclei were taken using an Olympus U-CMAD3 camera.

**RESULTS**

The six populations of prairie cordgrass analyzed in the present work showed the following chromosome numbers: 2n=40 (“IL102” and “20-102”), 2n=60 (“17-116” and “103-6x”), and 2n=80 (“PCG109” and “19-108”) (Table 3.1). The silver stained active nucleolar organizer regions (AgNORs) observed at metaphase are visualized as black spherical bodies on yellow-brown chromosome arms (Fig. 3.1). The number and position of AgNORs varies among six prairie cordgrass populations. In “IL102” three black silver precipitates were found near to one side of telomeric regions: two AgNORs located on both chromosome arms and the other located on only one chromosome arm (Fig. 3.1A). In “20-102” three chromosomes showed AgNORs located in telomeric regions on both their arms, and one chromosome has an AgNOR located on only one chromosome arm (Fig. 3.1B). “17-116” has six AgNORs bearing chromosomes which possess four AgNORs located in telomeric regions on both chromosome arms and two AgNORs located in the pericentromeric region on only one side of the chromosome arms (Fig. 3.1C). “103-6x” possesses two AgNORs located in telomeric regions on both chromosomes arms and one AgNOR located in the pericentromeric region on one side of chromosome arm (Fig. 3.1D). Eight AgNORs were found in “PCG109”, consisting of two AgNORs located in telomeric regions on both chromosome arms, one located in the pericentromeric region on both chromosome arms, one located in the pericentromeric
region on one chromosome arm, and four located in telomeric regions on one chromosome arm (Fig. 3.1E). “19-108” shows seven AgNOR bearing chromosomes showing four AgNORs located in telomeric regions on both chromosome arms, one located in the pericentromeric region on one chromosome arm, and two located in telomeric regions on one chromosome arm (Fig. 3.1F). Unlike tetra and hexaploids, several small AgNORs were observed on either of the end or pericentrometric region of a single chromosome arm for octoploid populations.

Two types of interphase cells were observed in root tips; one located in the meristematic region and the other located in the root cap region (Fig. 3.2). The nucleolus of the cap cell is much smaller than the nucleolus of the meristem cell, but a higher number of nucleoli per cell were observed in the cap cell (Fig. 3.2). In order to confirm the presence of nucleolus in interphase cell, a few squash preparations of “IL102” were stained according to the Feulgen technique (Fig. 3.3). The Feulgen staining is the highly specific for DNA, so the results show that the regions in the nucleolus mostly containing rRNA and proteins turn a pale pinkish color, reflecting a lack of DNA (Fig. 3.3). The distribution of cells with different numbers of nucleoli per interphase nucleus located in both meristem and cap cells and the percentage of interphase cells with heteromorphic nucleoli are shown in Table 3.2. The mean number of nucleoli varies among ploidy levels. In meristematic interphase cells, the tetraploid population mostly has one large nucleolus per cell, but higher ploidy populations have from 2 to 4 nucleoli found at low frequency (0.33% - 9.83%) (Table 3.2 and Fig. 3.2A, 3.2D, and 3.2G). In tetraploid cap cells, the mean of number of nucleoli per interphase cells of “IL102” and “20-102” are 2.26 and 2.04, respectively, and both populations have mostly 2 nucleoli per cell (Fig. 3.2B and 3.2C). The mean number of nucleoli per interphase cap cells of “17-116” and “103-6x” are 2.66 and 2.76, respectively. In most cases 2 and 3 nucleoli per nucleus in cap cells were present (Fig. 3.2E and 3.2F), although occasionally more than 3 nucleoli were also observed. A different distribution of nucleoli per interphase cap cells was observed within octoploids. “19-108” population has the highest mean number of nucleoli per cap cell, 3.95, and in most cases between 2 and 4 nucleoli per cap cell were present (Fig. 3.2I), with a maximum of 7 nucleoli per cap interphase cell, while “PCG109” population (Fig. 3.2H) has a similar mean number of nucleoli per cap cell as hexaploids.
because this population has mostly 2 nucleoli per cap interphase cell. The higher mean number of nucleoli has resulted in the higher frequency of cells with silver stained nucleoli heteromorphism. For example, “19-108” has the highest mean number of nucleoli observed at a higher frequency in meristem and cap cell as well was the highest percentage of heteromorphic silver stained nucleoli (8.19 % and 70.41 %).

**DISCUSSION**

In the present study, we have shown the successful use of silver staining to visualize active nucleolar organizer regions (NORs) on mitotic chromosomes and evaluate the number, size, and distribution pattern in six prairie cordgrass population with different ploidy levels. Moreover, the number of silver stained nucleoli were counted per interphase nucleus in both meristem and cap cells, and we have found that interphase nucleoli count correlates with ploidy.

According to our observation, variability of the metaphase AgNOR pattern and the number, locations (Fig.1), and size of silver-stained nucleoli during interphase (Fig. 3.2), were observed among and within ploidy levels. The size and intensity of AgNORs localized in secondary constrictions have been correlated with the activity of rRNA genes which are transcribed during the previous interphase (Hubbell, 1985, Guerra, 2000). The size of silver granule dots at metaphase is much smaller than silver granule dots at interphase because only 10 % of the silver stained nucleolar protein during interphase are retained on metaphase chromosomes (Sumner, 2003). The AgNOR pattern in the metaphase cell is specific and constant (Sozansky et al., 1985, Isakova, 1994), and thus can be used as the chromosomal marker to detect polymorphisms of Ag-stained NORs on prairie cordgrass chromosomes.

Although two populations within each tetra- and octoploidy have similar numbers of active NOR sites on metaphase chromosomes, two hexaploid populations have different numbers of AgNORs. Unlike “17-116” population, only 3 out of 6 NOR sites were normally stained in “103-6x” population. A plausible explanation for this result is that 3 AgNORs localized on chromosome arms that were normally active and visible while the other three additional sets of NORs which may also exist, were functionally inactive.
during the preceding interphase (Sumner, 1990, Wachtler and Stahl, 1993). Moscone et al. (1995) and Bustamante et al. (2014) reported that AgNOR site is correlated with the 45S rDNA site where breaks and gaps caused chromosome fragmentation, resulting in new chromosomal rearrangements. Thus, the loss of nucleolar activity of the NOR observed in “103-6x” population may be a result of rapid changes in chromosomal structures and gene expressions which may be due to the formation and establishment of neo-polyploidy in mixed ploidy populations co-occurring with tetraploids. This similar result has been observed in allotetraploid Arabidopsis suecica, reporting that some loci in newly formed ploidy are unstable to cause loss, gain, and rearrangement of NOR sites (Pontes et al., 2004). Silver-stained NORs on mitotic chromosomes are therefore useful in determining the stabilization of newly formed polyploidy of prairie cordgrass.

The application of staining technique to interphase nuclei results in deposition of silver atoms in nucleoli, corresponding to the fibrillar centres and fibrillar component which contains ribosomal DNA and acidic proteins associated at the rDNA sites (Hernandez et al., 1980, Fakan and Hernandez-Verdun, 1986, Hubbell, 1985). In the present study, two types of interphase cells were observed: one located in the meristem region and the other located in the cap cell. Various numbers of nucleoli in both cell types were detected within a population, which can be explained by the fact that the quantity of interphase nucleoli progressively increases from early G1 phases and reaches the maximum value at S and G2 phases (Mourrad et al., 1997). In addition, differences in the size and the maximum number of nucleoli were observed between meristem and cap cell. Due to the process of nucleolar fusion, most meristematic interphase cells were found to contain one large nucleolus, but some contained two, three, or four nucleoli at low frequencies. After late telophase the number of small silver stained granules decreases whereas the size of larger granules increases, so interphase nucleoli recover their elliptical shape (Anastassova, 1977, Ploton et al., 1987). In contrast, the size of the nucleolus in cap cells was much smaller than in the meristem cells, and higher maximum number of nucleoli involving with high heteromorphic variations of size of nucleoli was observed in cap interphase cells. The maximum number of nucleoli in the cap cells was equal to active AgNORs at metaphase in the samples analyzed, with some exceptions. This
observation can be explained by Jordan et al. (1982) who reported that smaller nucleoli have low possibility of fusing, which may increase the number of nucleoli per cell showing several patterns of nucleolar fusion associated with the various combinations of fused and unfused nucleoli in interphase cell.

In general, the mean number of nucleoli increased with increase in ploidy level. The mean number of nucleoli in cap cell of tetra-, hexa-, and octoploids were approximately 2.15, 2.72, and 3.36, respectively. According to previous studies (McClinton, 1934; Wilkinson, 1944; Sen and Bhadrui, 1968), a multiplication of chromosome sets is invariably associated with a proportionate increase in the number of nucleoli in interphase cell. Thus, the number of nucleoli in cap cell can be used as a reliable marker for ploidy level particularly where mitosis chromosome size is too small to obtain preparations in which most of the cells are in a dividing stage to enable count chromosomes of each cell. Moreover, the high heteromorphic variations in the size of nucleoli were observed in higher ploidy levels. This feature is common in polyploid plants such as wheat (Aegilops speltoides, Darvey and Driscoll, 1972) and ryegrass (Lolium multiflorum, Bustamante et al., 2014). Variation in number of nucleoli is also observed within ploidy level, but not in hexaploids. Unlike other ploidy populations, two hexaploids were found in mixed ploidy populations located in single location in IL (Kim et al., 2012b), so they showed similar nucleolar fusion patterns. Jordan et al. (1982) reported that the fusion depends on the length of cell cycle, so longer cell cycle increases the possibility of fusion resulting of lower mean number of nucleoli per cell. Based on our results, “19-108” has a higher mean number of nucleoli per cell, reflecting more frequent cell division, and therefore a lower possibility of nucleolar fusion than “PCG109” population.

CONCLUSION

Using a modified, highly reproducible silver staining procedure, we have studied the variation in the maximum number of AgNOR at metaphase and of nucleoli at interphase cells of tetra-, hexa-, and octoploid prairie cordgrass populations. All prairie cordgrass had from 3 to 8 AgNORs per metaphase cell, and a variation of AgNOR pattern was found among ploidy levels. All populations within each ploidy
level, except hexaploidy, have similar numbers of active NOR sites on metaphase chromosomes. Variation in number of active NORs was observed among two hexaploids, suggesting that the active NORs are unstable due to rapid changes in chromosome structure and gene expression caused by formation of neo-polyploidy in mixed ploidy populations. Since the AgNORs detected on mitotic chromosomes are constant and species-specific, they can be used as markers to detect chromosome variations within and among ploidy levels and may play a critical role in determining their chromosomal changes which may affect the gene expressions involving both formation and establishment of polyploidy. The mean number of silver stained nucleoli increases from tetra-to octoploids; therefore the quantitative analysis of silver stained nucleoli per interphase cell should be sufficient to determine the ploidy levels in prairie cordgrass. In addition, silver staining active NORs helps to determine the stabilization of neo-polyploidy in prairie cordgrass. However, further cytogenetic analysis such as fluorescence in situ hybridization (FISH) is needed to provide markers that allowed individual chromosome pairs to be distinguished, and then to trace the structural changes in chromosomes of newly formed polyploids of prairie cordgrass.
Table 3.1 Geographic origin, accession number, ploidy level, reference of prairie cordgrass populations used in this study.

<table>
<thead>
<tr>
<th>Line</th>
<th>Ploidy level (x=10)</th>
<th>Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL102</td>
<td>4x</td>
<td>IL</td>
<td>Kim et al., 2010</td>
</tr>
<tr>
<td>20-102</td>
<td>4x</td>
<td>KS</td>
<td>Kim et al., 2012b</td>
</tr>
<tr>
<td>1036x</td>
<td>6x</td>
<td>IL</td>
<td>Kim et al., 2012a</td>
</tr>
<tr>
<td>17-116</td>
<td>6x</td>
<td>IL</td>
<td>Kim et al., 2012b</td>
</tr>
<tr>
<td>PCG109</td>
<td>8x</td>
<td>SD</td>
<td>Kim et al., 2010</td>
</tr>
<tr>
<td>19-108</td>
<td>8x</td>
<td>IW</td>
<td>Kim et al., 2012b</td>
</tr>
</tbody>
</table>
Table 3.2 The frequency of silver stained nucleoli per cell and the percentage of cells with heteromorphic nucleoli in meristematic and cap cells at interphase measured in tetra- (IL102 and 20-102), hexa- (17-116 and 103-6x), and octoploid (PCG109 and 19-108) prairie cordgrass populations.

<table>
<thead>
<tr>
<th>Line</th>
<th>Total no. of cells analyzed</th>
<th>Frequency of nucleoli per interphase cell (%)</th>
<th>Interphase cells with heteromorphic nucleoli (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Meristem cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL102</td>
<td>247</td>
<td>99.6, 0.45, 0, 0, 0, 0, 0</td>
<td>1.00, 0.45</td>
</tr>
<tr>
<td>20-102</td>
<td>287</td>
<td>100, 0, 0, 0, 0, 0</td>
<td>1.00, 0.00</td>
</tr>
<tr>
<td>17-116</td>
<td>114</td>
<td>93.9, 6.14, 0, 0, 0</td>
<td>1.06, 5.26</td>
</tr>
<tr>
<td>103-6x</td>
<td>171</td>
<td>93.6, 6.43, 0, 0, 0</td>
<td>1.06, 5.26</td>
</tr>
<tr>
<td>PCG109</td>
<td>166</td>
<td>94, 5.42, 0.6, 0, 0</td>
<td>1.06, 3.61</td>
</tr>
<tr>
<td>19-108</td>
<td>305</td>
<td>87.5, 9.83, 2.18, 0.33, 0, 0</td>
<td>1.15, 8.19</td>
</tr>
<tr>
<td><strong>Cap cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL102</td>
<td>100</td>
<td>7, 65, 23, 5, 0, 0</td>
<td>2.26, 40.40</td>
</tr>
<tr>
<td>20-102</td>
<td>100</td>
<td>15, 69, 13, 3, 0, 0</td>
<td>2.04, 27.00</td>
</tr>
<tr>
<td>17-116</td>
<td>100</td>
<td>16, 34, 26, 17, 6, 1</td>
<td>2.66, 63.00</td>
</tr>
<tr>
<td>103-6x</td>
<td>100</td>
<td>3, 45, 28, 18, 5, 1</td>
<td>2.76, 67.69</td>
</tr>
<tr>
<td>PCG109</td>
<td>100</td>
<td>13, 40, 21, 14, 8, 3</td>
<td>2.77, 54.00</td>
</tr>
<tr>
<td>19-108</td>
<td>100</td>
<td>0, 16, 27, 22, 19, 13</td>
<td>3.95, 70.41</td>
</tr>
</tbody>
</table>
Figure 3.1 The number of active NORs observed in mitotic metaphase chromosomes of prairie cordgrass: “IL102” (2n=40) with three active NOR sites (A); “20-102” (2n=40) with four active NOR sites (B); “17-116” (2n=60) with six active NOR sites (C); “103-6x” (2n=60) with three active NOR sites (D); “PCG109” (2n=80) with eight NOR sites (E); “19-108” (2n=80) with seven active NOR sites (F). Arrow indicates the AgNORs. The AgNOR bearing chromosome pairs are shown in the box.
Figure 3.2 The number of silver stained nucleoli of interphase nucleus located in meristem (A, D, and G) and root cap cells (B, C, E, F, H, and I) in prairie cordgrass. “IL102” (2n=40) with one nucleoli in meristem cell (A) and with two nucleoli in root cap cell (B); “20-102” (2n=40) with two nucleoli in root cap cell (C); “17-116” (2n=60) with two nucleoli in meristem cells (D) and with three and four nucleoli in root cap cells (E); “103-6x” (2n=60) with three nucleoli in meristem cell (F); “PCG109” (2n=80) with three nucleoli in root cap cells (H). “19-108” (2n=80) with three and four nucleoli in meristem cells (G) and with six nucleoli in root cap cell (I). The arrow indicates nuclear envelop (N) and nucleolus (NC).
Figure 3.3 Feulgen-staining of interphase nucleus in prairie cordgrass (IL102) root meristem (A) and cap cells (B). The arrow indicates nucleus (N) and nucleolus (NC).
CHAPTER 4

EFFECTS OF POLYPLOIDY ON LODGING

ABSTRACT

One of major effects of polyploidy is cell enlargement which can alter morphological features such as plant height and stem diameter related to lodging resistance in plants. Prairie cordgrass (*Spartina pectinata* Link) is a polyploid species that is considered as a perennial $C_4$ dedicated bioenergy crop grown on marginal land. In previous studies, two types of hexaploids were reported: type 1 was found as a seed from a tetraploid population and type 2 was found from a natural population coexisting with tetraploids. This is the first study reporting identification of differences in morphological, anatomical, and chemical characteristics associated with lodging resistance between tetra- and hexaploids found in the same origin. And thus the morphological differences between ploidy levels may be caused by polyploidization event in this species. Even though hexaploid populations were more resistant to lodging than tetraploids, different morphological, anatomical, and chemical characteristics related to lodging resistance were observed between the two types of hexaploids. Short plant height and lower center of gravity were critical factors related to lodging resistance for type 1 hexaploids, whereas the stem thickness and stem mass had the greatest effects on lodging in type 2 hexaploids. Unlike type 1 hexaploid, type 2 hexaploids had bigger inner vascular bundles and a greater number of outer vascular bundles, contributing to high fiber content in the stem. Compared to the type 1 hexaploids, type 2 hexaploids had more favorable traits related to lodging resistance which may enhance biomass yield. This may be why the type 2 hexaploids can successfully occupy the tetraploid’s territory in nature.

---

*Reprinted, with permission, from Kim S, Rayburn AL, Bishop J, Lee DK (Submitted) Effects of polyploidy on lodging: differences in morphological, anatomical, and chemical characteristics among polyploid prairie cordgrass (*Spartina pectinata* Link). GCB Bioenergy*
INTRODUCTION

Polyploids, defined as organisms containing more than two copies of a whole genome, are commonly found in angiosperms (Otto, 2007; Parisod et al., 2010). Polyploidization has been an ongoing occurrence in higher plants; with at least 70% (Goldblatt, 1980; Leitch and Bennett, 1997; Soltis et al., 2009) of all angiosperms having experienced a relatively recent polyploidy event. Polyploidization is believed to be an important mechanism of sympatric speciation in land plants (Otto and Whitton, 2000), due to the immediate reproductive isolation between newly formed polyploids and their parent (Bennett, 2004; Comai, 2005; Parisod et al., 2010). A number of factors, such as increased heterozygosity, genetic redundancy (e.g. extra copies of genes), genomic rearrangement, epigenetic reprogramming, or variation in gene expression (Jackson and Chen, 2010; Balao et al., 2011), may provide polyploids with evolutionary advantages over their diploid ancestors (Otto and Whitton, 2000; Comai, 2005; Parisod et al., 2010). These factors may cause morphological and physiological changes, which may allow neopolyploid species to outcompete with their parents for same habitat and to be successfully adapted to a new environment, and therefore potentially invade a new region (Levin, 1983; Felber, 1991; Thompson and Lumaret, 1992; Ranney, 2006; te Beest et al., 2012; Bennett and Leith, 2005; Chen, 2007).

A number of studies have demonstrated the relationship between genome size and phenotypic traits (Beaulieu et al., 2007, 2008; Knight and Beaulieu, 2008; Münzbergová, 2009). The major effect of polyploidy in plants is cell enlargement (Stebbins, 1971; Levin, 2002; Knight and Beaulieu, 2008) which is linked to a higher growth rate in plants with higher ploidy levels (Jibiki et al., 1993; Kim et al., 2012b; Yildiz, 2013). Higher ploidy plants also have thicker and therefore heavier stems (Kim et al., 2012b; Tucak et al., 2013), which are critical phenotypic characteristics for improving lodging resistance (Dunn and Brigg, 1988; Kong et al., 2013). Lodging resistance is a highly important trait in breeding programs aiming to increased yield, seed quality, and harvesting efficiency (Briggs, 1990; Pinthus, 1973; Easson et
al., 1993). Therefore, polyploidization can be used as a tool in breeding programs to improve lodging resistance, but only a few studies have determined if and how polyploidy or genome size can modify stem morphological traits associated with lodging resistance in different populations of a single species (Randolph, 1941; Gugsa and Loerz 2013). Prairie cordgrass (*Spartina pectinata* Link) offers a convenient model to study lodging response associated with polyploidy, and differences in adaptive capacity and morphology between ploidy levels occurring in natural conditions were recently found within this species (Kim et al., 2012b).

Prairie cordgrass has gained an attention as a dedicated energy crop that can be grown on marginal lands. This species is polyploid comprising of tetra- (2n=40), hexa- (2n=60), and octoploid (2n=80) cytotypes (Kim et al., 2010, 2012b) with a basic number of x=10 (Church 1940). Kim et al. (2012b) reported that tetra- and octoploid prairie cordgrass cytotypes were found in broad geographic regions ranging from the East North Central to West North Central regions in the US. Unlike other ploidy levels, hexaploids were found either as mixed populations with tetraploids occurring in a single location (Kim et al., 2012a) or as seed from a single tetraploid population (Kim et al., 2010). Not surprisingly, there is pronounced morphological variability in polyploid prairie cordgrass across its geographical origin (Guo et al., 2015). The morphological variation among prairie cordgrass populations might be influenced by local environment, genetic changes during polyploidization, or both. According to Guo et al. (2015), morphological traits such as plant height, stem mass, and phytomer number were significantly different among the populations, and phytomer number was positively correlated to biomass yield. Since a phytomer consists of a blade, sheath, node, internode, and axillary bud (Etter, 1951; Langer, 1972; Dahl and Hyder 1977), variation in the number of phytomers per stem would imply a variation in the number of internodes per stem. Moreover, the variation in these morphological traits (e.g. plant height, stem diameter, and internode length) found among prairie cordgrass populations (Guo et al., 2015), which are associated with lodging resistance (Crook and Ennos, 1993; Zuber et al., 1999; Kashiwagi and Ishimaru
2004; Sarker et al., 2007; Wu et al., 2011), can be affected by anatomical or chemical changes or both within the stem (Wang et al., 2006).

Certain anatomical features of the stem are correlated to lodging resistance (Dunn and Briggs 1988; Kokubo et al., 1989; Kelbert et al., 2004; Wang et al., 2006; Kong et al. 2013). A significant positive correlation between lodging resistance and the number and anatomical features (i.e. size and density) of vascular bundles was reported in previous studies (Brady, 1934; Wang, 2006; Wu et al., 2011). The size and density of vascular bundles are correlated to the densities of two types of cells, i.e. sclerenchyma (mechanical) and parenchyma cells. The sclerenchyma cells surround the vascular bundles and separate the phloem from the xylem (Esau 1997; Mati et al., 2012). These cells have thick, secondary wall layers that provide mechanical support that plays an important role in strengthening the stem (Mati et al., 2012). According to Wu et al. (2011), increased lodging resistance is observed in large culm rice cultivars which exhibited large vascular bundles, which may be resulting in high density of the sclerenchyma cells (Mallett and Ochard, 2002). Sclerenchyma cells are also distributed in a layer within cylindrical band under epidermis (Esau, 1997). The thickness of the sclerenchyma layer is highly correlated with lodging resistance (Kelbert et al., 2004; Kong et al., 2013). The parenchyma cells distributed between the vascular bundles and surround the sclerenchyma cells (Esau, 1997; Mallett and Ochard, 2002). The parenchyma cells may become lignified (Mallett and Ochard, 2002) and the thickness of this cell is highly associated with stem stability, which plays an important role in lodging resistance (Dunn and Briggs, 1989; Kong et al. 2013).

In addition, lodging response can vary depending on the stem strength, which is directly associated with chemical compositions in the cell wall (Forbes and Watson, 1992, Walter et al., 2009). Cell walls consist of a mixture of three polysaccharides: cellulose, hemicellulose, and lignin (Walter et al., 2009). The physical properties of the cell wall can vary by simply thickening the walls with additional depositions of cellulose and hemicellulose, which then increases the stiffness and strength in the cell wall (Forbes and Watson, 1992; Walter et al., 2009). Lignin is deposited around the cellulose fibrils and plays
a mechanical role to protect the hydrogen bonding between cellulose molecules from its interaction with water (Forbes and Watson, 1992). The strength of tissues, fibers, and stiffness of cell walls can be increased as more lignin is deposited in the secondary cell wall (Rubin, 2008). Depending on the species, the concentrations of chemical components vary, and the variation affects the stem strength and lodging resistance. Okuno et al. (2013) observed that increased lignin accumulation improved lodging resistance in rice, whereas Wang et al. (2012) reported that cellulose contributed more than lignin to the ability to resist lodging in wheat.

Based on these results, the variations in morphological and anatomical features and chemical composition of stems may result in different lodging responses among multiple prairie cordgrass populations. However, only a few studies have briefly reported stem lodging in a single population of this species (Boe and Lee, 2007; Boe et al., 2009; Gonzalez-Hernandez et al., 2009). For example, Boe et al. (2009) reported that some portions of an octoploid prairie cordgrass biomass in an experimental plot were completely lodged. This may have been linked to fertilizer application (about 75 kg N ha$^{-1}$) every other year. Fertilization of plots likely resulted in longer internode, taller plants, increasing their susceptibility to lodging, as prairie cordgrass grew extensively tall when fertilizer was abundant (Chauhan, 2012). Even though lodging has gained attention as an important consideration in breeding programs of prairie cordgrass for improvement in biomass yield potentials and seed production, there are no studies which have reported links between morphological, anatomical, and chemical characteristics of tiller and increased lodging resistance.

The objective of this study was to help our understanding of whether and how the recent events of polyploidization can modify aboveground morphological, anatomical, and chemical characteristics associated with lodging resistance in different accessions of prairie cordgrass. This information will help breeders to select for useful traits related to lodging resistance for improvement in breeding programs and to understand how neopolyploidy can modify morphological and anatomical traits, allowing the neopolyploids to outcompete their putative ancestors in nature.
MATERIALS AND METHODS

Plant materials and plot arrangement

A total of eight different prairie cordgrass accessions were used in this study including the two types of hexaploids reported in previous studies: type 1, i.e. 99C, was found as seed from a tetraploid accession, i.e. 99A (Kim et al., 2010) and type 2, i.e. 1036x, MBB6x, and MID, was found from a natural, mixed population coexisting with tetraploid accessions, i.e. 1034x, MBB4x, and 1094x (Kim et al., 2012). Putative tetraploid progenitors of each type of hexaploid population, i.e. 99A, 1034x, MBB4x, and 1094x, were used in this study as well. Since hexaploids and their putative tetraploid progenitors inhabit in same area, the results of morphological variability associated with lodging will largely reflect consequences of recent events of polyploidization in prairie cordgrass. Both type 1 hexaploid, 99C, and its putative tetraploid progenitor, 99A, were kept in a field nursery at Urbana, IL prior to the experiment since 2009. In spring 2012, fresh rhizomes of type 2 hexaploids and their putative tetraploid progenitors were collected from four random spots of the above identified locations having both tetra- and hexaploid clusters within 1 m distance between ploidy levels: 1034x and 1036x, MBB4x and MBB6x, and 1094x and MID. The fresh rhizomes of all eight populations were propagated in 2 L pots and kept in a greenhouse until transplanting to the field. In May 2012, the plants of four tetra- (99A, 1034x, MBB4x, and 1094x) and hexaploids (99C, 1036x, MBB6x, and MID) were transplanted to a field nursery at Urbana, IL (Table 1). The experiment was a randomized complete block design with three replications. Individual plot size was 0.6 m x 2.4 m - 3 m x 2.4 m. Due to a limited number of plants, each plot consists of 4 to 20 plants spaced on 0.9 m centers to minimize inter-plant competition. No data was collected in 2012.
**Lodging scores**

In 2013 and 2014, lodging at the stage when the stem reached its maximum height, with the inflorescence completely emerged from the flag leaf, were scored from all plants from three replicate plots per population. The lodging score of individual plant was visually determined as follows: 1, plant that is fully erect; 2, plant that is slightly lodged from upright position, $< 45^\circ$ angle; 3, plant that is lodged at $45^\circ$ angle; 4, plant that are severely lodged, $> 45^\circ$ angle from upright, and 5, plant that lodged flat. This lodging scale is similar that used by Hoggard et al. (1978) and Silva and Gordon (1986).

**Aboveground morphological characterization**

At the end of November in 2013 and 2014, the replicate plots of each population were harvested by excising all plants at the surface of the soil with a mechanized cutter, and the plot fresh weight was measured. Two random subsamples, approximately 3 kg (dry matter, DM), from each plot were set aside at harvest for analysis. The subsamples were dried at 60 °C for a week for later determination of stem morphological traits including total dry weight per plant, stem density per plant, stem mass, plant height, and leaf length. Three reproductive stems were randomly selected from each subsample for measurement of plant height and leaf lengths of 1st and 5th leaves below the inflorescent peduncle. Plant height was measured from the base of the plant to the tip of the 1st leaf using a ruler.

The height of center of gravity, number of leaves per stem, and ratio of total leaf weight to stem mass were only determined on three random dry reproductive stems per subsample harvested in 2014. The height of center of gravity was determined by placing a stem between the index finger and thumb and moving the stem along the fingers until the balance point was reached. The height of center of gravity was recorded as the distance from the base of the stem to the balance point (Crook and Ennos, 1993). The ratio of total leaf weight to stem mass was determined from the weight of total leaves per stem divided by stem mass.
The stem internode morphological analysis was only conducted in 2014. The internodes on stems were numbered consecutively from the base (proximal) to the top (distal) of the stem. The maximum number of internodes was ranged between 5 and 10 without counting the peduncle. The 2nd, 3rd, 4th, and 5th internodes from the proximal end of each stem were used in this analysis. Three reproductive stems were randomly selected from each plot, so a total 9 reproductive stems per population were used. Leaves, blades and sheaths, were removed from each stem, so only culm (main axis) was used to measure length, diameter, and weight of 2nd, 3rd, 4th, and 5th internodes of each stem. Diameter was measured at the center of each internode.

Moreover, the internode cross section was examined to measure variation in the proportion of hollowness (empty space) within the stem cross section among ploidy levels. The hollowness of the 2nd and 3rd internodes of all eight populations was examined. In order to measure the proportion of hollowness, central regions of the 2nd and 3rd internodes were sectioned by hand. Hand-cut sections (~ 2 cm thick) were analyzed under a zoom stereo microscope (SZ-CTV, Japan), and pictures were taken using an Olympus DP11 camera system. Image analysis software (Image J) was used to estimate the area of empty space within the stem internode section. The proportion of empty space was determined from the empty space inside the stem cross section divided by total area of the cross-section.

Stem strength may also be affected by other anatomical characteristics, such as the number and size of vascular bundles, which as a result may be potentially related to lodging resistance in plant. This study also describes the relationship between stem anatomical features and distribution of cellulose and lignin within internodes of stems, which may be related to lodging resistance in prairie cordgrass. In September 2014, three random reproductive stems per population were used to measure the densities of outer and inner vascular bundles and size of inner vascular bundles, and to visualize the lignin and cellulose content in cross sections of the central sections of 2nd (basal) and 3rd internodes per stem. For lignin staining, fresh hand-cut sections (~20 µm thick) were incubated in phloroglucinol (sigma) in 20 % HCl for 2 minutes and examined under a light microscope (Olympus BX61, Japan). Under a microscope,
the lignin appears red-violet. For cellulose staining, fresh hand-cut sections (~20 µm thick) were incubated in calcofluor white staining solution (sigma) and one drop of 10 % potassium hydroxide. The stained slides were examined under UV light (Olympus BX61, Japan). The cellulose appears green. All photographs were taken using Olympus U-CMAD3 camera. Image analysis software (Image J) was used to estimate the number and size of the vascular bundles. Three micrographs with 1 mm² areas from each replicate internode cross sections were made for counting the number of outer and inner vascular bundles. Three inner vascular bundles per micrographs were randomly selected to measure the size of inner vascular bundles.

Acid detergent fiber (ADF), neutral detergent fiber (NDF), and acid detergent lignin (ADL) were analyzed with an Ankom 200 Fiber Analyzer (ANKOM Technology, Fairport, NY, USA) (Anonymous, 2002, 2003a,b) using the manufacture’s recommended procedures (http://www.ankom.com/media/documents/Method_5_ADF_4-13-11_A200.A200L.pdf). In September 2014, three random reproductive stems per replicate were collected, and the leaves, blades and sheaths, were removed from each stem. Only internodes were dried at room temperature for two weeks to its constant weight. From the bottom of the plant upwards, four internodes (2nd, 3rd, 4th, and 5th internodes) were collected from each stem, bulked together and ground to a fine powder using a coffee grinder (Hamilton Beach, NC, USA). The length of each internode was measured before grinding samples. The cellulose concentration (g kg⁻¹) was estimated by calculating the difference between ADF and ADL, while hemicellulose concentration (g kg⁻¹) was estimated by calculating the difference between NDF and ADF. The content of each chemical component, i.e. cellulose, hemicellulose, and ADL, per unit length of stem (mg cm⁻¹) were calculated by dividing total amount (mg) of each component in each internode with length of same internode (cm).

**Statistical analysis**

All statistical analyses were carried out using SAS 9.3 (SAS Institute Inc., Cary, NY, USA). Analysis of variance (ANOVA) with 95 % confidence limits was used to test the significant difference
among the populations in lodging score, total dry weight, stem density, stem mass, plant height, and leaf length. The year and replications were considered as random effects, while the population was considered as a fixed effect. The height of center of gravity, number of leaves per stem, proportion of leaf weight per stem section, and the proportion of empty space cross section were only collected in one year. Thus, they were analyzed in ANOVA with 95% confidence limits, in which replication and population were considered as random and fixed effects, respectively. Comparison of population means was calculated using Tukey’s HSD test for the traits with a significant difference \( (P = 0.05) \) with different letters. In both internode morphological traits, i.e. internode length, diameter, and mass, and chemical composition, i.e. both concentration per dry weight (g kg\(^{-1}\)) and content per unit length (mg cm\(^{-1}\)) of cellulose, hemicellulose, and lignin, ANOVA with 95% confidence limits was conducted within a population, where the number of internode was considered as fixed effect. Also, ANOVA with 95% confidence limits was conducted within each internode to test significant difference among populations for each variable, where population was considered as fixed effect. Tukey’s HSD test was used to compare the means of each of the stem’s internodes within a population in each variable and denoted significant differences by using different letters at \( \alpha=0.05 \). In the anatomical traits including outer and inner vascular number and size of inner vascular number, ANOVA with 95% confidence limits was conducted within each internode, where population was considered as a fixed effect. And comparison of the means of population of each variable within each internode were calculated using Tukey’s HSD test for the traits with a significant difference \( (P = 0.05) \) with different letters.

RESULTS

Lodging scores and all aboveground morphological traits vary significantly among eight prairie cordgrass populations (Table 4.1). Overall, hexaploids showed higher lodging resistance than tetraploids (Table 4.1). The lodging score was the highest in tetraploid 1034x (3.3), while hexaploids, MID (1.0) and
99C (1.0), had the lowest lodging score. The two hexaploid types had different morphological characteristics, which may be related to lodging. Plant height, stem density per plant, and stem mass were lower in 99C (type 1) than its putative tetraploid progenitor. In contrast, the type 2 hexaploids such as 1036x and MID had higher values for stem mass, plant height, and leaf length than their putative tetraploid progenitors. MBB6x and MBB4x showed similar morphological characteristics such as stem mass, plant height, leaf length, and number of leaves, but were different in stem density. MBB6x had greater stem density, which may contribute to higher total dry weight than MBB4x. Overall, the length of the 1st leaf was approximately half the length of the 5th leaf with the exception of 1034x, which has similar lengths for the 1st and 5th leaves. The ratio of leaf weight to stem mass for the hexaploids was higher than their putative tetraploid progenitors with the exception of 1036x, which has lower ratio of leaf weight to stem mass than that of its putative tetraploid progenitor, 1034x (Table 4.1).

The stem internode morphological characteristics are shown in Fig. 2. When internode length was measured, only 99C and 1094x showed significant differences among 2nd, 3rd, 4th, and 5th internodes. In 99C, the internode length increased gradually from the 2nd to 5th internode, whereas the internode length for 1094x increased only up to the 4th internode and then decreased in 5th internode. The internode diameter of 99C and MID significantly decreased from 2nd to 5th internode, whereas no significant differences among internodes were observed in other populations. Significant differences among 99A, 99C, and MID were observed in internode mass. The 2nd internode in 99C and MID had the highest mass, while the 3rd internode in 99A had the highest internode mass. Overall, 1036x had the highest internode mass in each internode among all populations, while 99C had the lowest internode mass (Table 4.2).

The hollowness (empty space) of the 2nd and 3rd internode for all populations was measured (Fig. 4.3; Fig. 4.4). The proportions of hollowness inside the 2nd and 3rd internodes were significantly different among all populations. In both the 2nd and 3rd internodes, 99C had the smallest hollowness among all populations. The putative tetraploid progenitors of type 2 hexaploids, i.e. 1034x, MBB4x, and 1094x, had 30% of hollowness inside both 2nd and 3rd internode.
In Fig. 3.5, we describe the anatomical features of the internode in prairie cordgrass. The xylems and phloems were paired in bundles, and these bundles were scattered throughout the ground cell tissue (Fig. 4.5a). The vascular bundles were surrounded by vascular bundle sheath cells, which separated the xylem and phloem (Fig. 4.5b). There were two types of vascular bundles: inner and outer vascular bundles (Fig. 4.5). The inner vascular bundles were scattered within the inner region of the cross section, while the outer vascular bundles were present as one layer within the cylindrical band of fiber cells near the outer region. The outer vascular bundles were smaller in size compared to the inner vascular bundles, and more of the bundles are found toward the stem periphery than in the center of the stem (Fig. 4.5a). The center of stem was empty where the ground cells were dried out.

In Fig. 6 and 7, the variations among populations in the amount of cellulose and lignin deposited on in the cell walls were determined visually by staining the stem’s cross section by calcofluor and phloroglucinol, respectively. The cellulose walls were stained bright green (Fig. 4.6), while the lignified walls were stained red (Fig. 4.7). These color intensities approximately reflected either the total cellulose or lignin contents. The lignin and cellulose contents were rich around vascular bundles and cylindrical band presented below the epidermis (Fig. 4.6; Fig. 4.7). The thickness of the cell wall differs between the two types of hexaploids. The type 2 hexaploid, 1036x, appeared to have dense, thick sclerenchyma cell walls within the cylindrical band and around vascular bundles. In comparison to its putative tetraploid progenitor, 1034x, it reflected the highest level of cellulose and lignin contents within the cell walls (Fig. 4.6c,d,g,h; Fig. 4.7c,d,g,h). Unlike the type 2 hexaploid, the type 1 hexaploid (99C) showed both less density of green brightness and more yellow color in the cell walls, which was presented within the cylindrical band under epidermis and around vascular bundles (Fig. 4.6b,f; Fig. 4.7b,f).

The densities of outer and inner vascular bundles and size of inner vascular bundles were investigated in the 2nd and 3rd internodes for all eight populations (Table 4.3). The number of outer vascular bundles per unit area was significantly different in the 2nd internode, while no significant difference was observed in 3rd internode. 99A had the fewest number of outer vascular bundles, while
MBB4x had the most outer vascular bundles within the cylindrical band under the epidermis. In general, hexaploids appeared to have more outer vascular bundles in 2nd internode than their tetraploid progenitors, except for MBB4x and MBB6x. Variation in the density and size of inner vascular bundles were observed between two types of hexaploids. The Type2 hexaploids such as 1036x and MID had fewer and larger inner vascular bundles in 2nd and 3rd stem internodes then their putative tetraploid progenitors, 1034x and 1094x, respectively (Table 4.3). Unlike type 2 hexaploids, 99C had smaller and more inner vascular bundles in the 2nd and 3rd stem internodes than its putative tetraploid progenitor, 99A.

Figure 9 showed the patterns in chemical composition of three fiber components, i.e. cellulose, hemicellulose, and lignin, along 2nd (bottom) through 5th (top) stem internodes of prairie cordgrass. Overall, the chemical composition of stem internode was composed of mostly cellulose (40-45 g kg\textsuperscript{-1} per dry weight, Fig. 4.8c) and hemicellulose (30-35 g kg\textsuperscript{-1} per dry weight, Fig. 4.8a). Acid detergent lignin (ADL) constituted less than 10 % of the dry weight of the stem internode (Fig. 4.8e). The ADL and cellulose concentration (g kg\textsuperscript{-1}) of four internodes was more varied than that of hemicellulose. For example, a significant decrease from 2nd to 5th internode of 99C was observed in ADL and cellulose concentration (g kg\textsuperscript{-1}) (Fig. 4.8e).

The content per unit length of three stem chemical components, i.e. hemicellulose, cellulose, and lignin, were significantly varied among the populations for each internode (Table 4.2; Fig. 4.8b, d, f). The type 2 hexaploids such as 1036x and MID had the high cellulose, hemicellulose, and lignin contents (mg cm\textsuperscript{-1}) in 2nd (basal) stem internode among all eight populations. MBB6x had similar distribution of contents of stem chemical components with its putative tetraploid progenitor, MBB4x. Unlike the type 2 hexaploids, the overall lignin content in internodes of 99C (type 1)were significantly low (Fig. 4.8f) and a significant reduction in the contents of all hemicellulose , cellulose, and lignin were observed from 2nd (basal) to 5th (upper) internodes in 99C (Fig. 4.8b,d,f).
DISCUSSION

Even though lodging, defined as the permanent displacement of the stem of a free-standing plant from the vertical (Pinthus, 1973; Berry et al., 2004), has been commonly found in prairie cordgrass, lodging resistance has not been emphasized and developed in its breeding programs. In previous studies with prairie cordgrass, only non-lodging stems were used for the final data analysis (Boe et al., 2009) or lodging has been briefly described in results (Boe and Lee, 2007; Gonzalez-Hernandez et al., 2009). Since lodging can reduce crop yield, seed production, and harvest efficiency (Briggs, 1990; Pinthus, 1973; Easson et al., 1993), lodging should be an important consideration for improvement in prairie cordgrass breeding programs. For the first time, we have reported the effects of lodging in polyploid prairie cordgrass and potential relationships among morphological, anatomical, and chemical characteristic and lodging resistance in this study.

Aboveground morphological characteristics appeared to be associated with differences in lodging resistance. In general, hexaploids had higher lodging resistance than their tetraploid progenitors, but we had also found variations in morphological characteristics related to lodging resistance, i.e. total dry weight, stem density, and stem mass, between two types of hexaploids. The type1 hexaploid, 99C, had shorter plant height and lower center of gravity than its putative tetraploid progenitor, 99A. Also, total dry weight of 99C was much lower than 99A. According to Huang et al. (1988) and Okuno et al. (2014), decreasing plant height significantly lowers the height of the center of gravity of the stem and also reduces the stem weight, resulting in the decreased “self-weight” moment per main stem. The “self-weight” is defined as the estimation of the weight of the aerial parts of the stem which help to pull the plant when the plant leans due to wind-induced forces (Crook and Ennos, 1993). Decreased “self-weight” moment may be why 99C had higher lodging resistance. The importance of short plant height and lower center of gravity for lodging resistance is emphasized in several other studies (Dalrymple, 1986; Yoshida, 1981; Cheverton et al., 1992; Setter et al., 1995; Takayuki and Ishimaru, 2004; Okuno et al., 2014).
In addition to plant height, physical strength components of the stem are also important morphological traits related to lodging resistance (Zuber et al., 1999; Ma, 2009). Stem weight and diameter are closely associated with physical strength (Ma, 2009). For example, plants with thicker and heavier stems had better lodging resistance (Zuber et al., 1999). A similar result has been observed in the type 2 hexaploids such as 1036x, MBB6x, and MID. Even though they were taller and also had higher dry weight than their putative tetraploid progenitors, they had higher lodging resistance as a result of their heavier stems.

Lodging resistance can be associated with the weight distribution, length, and diameter of stem’s internodes (Jezowski, 1981; Xiao et al., 2002). The thickness of internodes for most lodging-tolerant plants such as 99C and MID significantly decreased from the 2nd (bottom) to 5th (top), whereas no significant differences were found among four internodes for lodging-susceptible plants. The thickness of basal internodes such as the 2nd internode is an important morphological trait, while the thickness of upper internode is not correlated with lodging resistance (Xiao et al., 2002). Neenan and Spencer-Smith (1975) also reported that large diameter of the basal internode plays an important role in lodging resistance. Unlike internode thickness, the length of basal internode significantly increased from the 2nd to 5th internodes for lodging tolerant plants. According to Dunn and Briggs (1988), plants with shorter basal internodes have higher lodging resistance because the greatest bending stress causing lodging occurs at the base of stem. Based on the results, the weight of the basal internode was negatively correlated to the length of internode in lodging-resistant plants. For example, the internode length of 99C increased from the 2nd to 5th internodes, while the internode mass decreased from the 2nd to 5th internodes. Sarker et al. (2007) reported that the weight of the second internode positively affected the second internode breaking strengthen, as a result contributed directly to the traits closely associated with stem lodging resistance.

Prairie cordgrass has round stems and the stems are essentially hollow except for a layer of cells that is 5-6 cells in thickness. The thickness of this region may vary depending on the cell size, which is highly associated with ploidy level (Kim et al., 2010, Kim et al., 2012b). The thickness of these regions
in the stem may be closely related to the number of outer vascular bundles. Hexaploids appeared to have more outer vascular bundles than their putative tetraploid progenitors. The smaller, surrounding outer vascular bundles may increase the layered tissues in stem as a result of smaller, hollowed center of the stem in hexaploids. According to Kong et al. (2013), the amount of pith tissue is positively correlated to stem strength, and greater stem strength results in greater lodging resistance. The hollowness of lodging-resistant plants such as 99C and MID is relatively small, which might increase the stem strength (stiffness) near the base of the stem. In contrast, lodging-susceptible plants, mostly tetraploids, had a large proportion of hollowness within the stem. Similar result was observed in Kong et al. (2013) who reported that solid-stemmed wheat was more resistant to lodging than hollow-stemmed.

Prairie cordgrass is a monocot species, and it has a unique vascular tissue. Outer vascular bundles are present within the cylindrical band of fiber cells near the stem’s outer region, and inner vascular bundles are scattered within the inner region of the stem. Lignin and cellulose contents were dense around vascular bundles and cylindrical band presented below the epidermis, where mostly mechanical cells such as sclerenchyma cells are embedded (Esau, 1997; Mati et al., 2012). In these regions, the plant cell wall functions as the skeletal frame work of plants (Hirano et al., 2010), and also provides mechanical support for the entire plant (Li et al., 2009; Cosgrove, 2005). Therefore, the thickness of cell walls affects the mechanical strength of the stem (Li et al., 2003; Zhong et al., 2002), which is strongly associated with lodging resistance (Wang et al. 2012). Compared to its putative tetraploid progenitor, 1034x, type 2 hexaploid, 1036x, had a high level of cellulose and lignin content within the cell walls, shown as dense, thicker sclerenchyma cell walls within the cylindrical band and around vascular bundles. This may be why 1036x had higher lodging resistance than 1034x. Unlike type 2 hexaploids, the type 1 hexaploid, 99C, showed lower density of green brightness and yellow color in the cell walls, which reflects a lower amount of mechanical support tissues in cell walls. This may be related to abnormal cell division at the lateral meristem when secondary growth begins (Rajput et al., 2010). The secondary growth provides a plant added stability which allows plants to grow taller. The limitation of secondary growth may result in
shorter plant height with smaller stem diameter in 99C, but further studies are necessary to elucidate the relation between plant growth and formation of secondary cells comprised mostly of mechanical cells. Unlike the type 1 hexaploid, the type 2 hexaploids had larger and fewer inner vascular bundles per unit area. In type 2 hexaploids, the large vascular bundles may contribute to the strength and mechanical support of the plant’s stem because most mechanical tissues were embedded around vascular bundles (Esau, 1997; Mati et al., 2012). This may have resulted in high chemical composition content (g cm\(^{-1}\)) within the stem section. The type 2 hexaploids, i.e. 1036x and MID, had the highest cellulose, hemicellulose, and lignin content in basal stem internode among all eight populations. Also, the thicker and denser cell walls in mechanical cells may reflect the high content of cellulose and lignin in 1036x. The high content of three stem chemical components in 1036x and MID contributed to higher lodging resistance than their putative tetraploid progenitors, 1034x and 1094x, respectively. According to Pagan and Geor (2005) and Kong et al. (2013), plants with high content of lignin and cellulose tend to have more rigid stems, resulting in higher lodging resistance.

The type 1 hexaploid, 99C, had smaller inner vascular bundles, resulting in fewer mechanical tissues linked to lower lignin content within the stem cross section. Based on this result, lignin content may not be significantly involved in resistance of crops to lodging, which is in agreement with many previous studies (Travis et al., 1996; Wang et al., 2012; Sattler et al., 2010, and Ching et al., 2006). However, distribution of the content of stem chemical components may play critical roles for lodging resistance. Ookawa and Ishihara (1993) reported that distribution and density of lignin contents were more important for lodging resistance than its concentration in rice stems. Significant reductions in the content per unit length for all cellulose, hemicellulose, and lignin were observed from 2\(^{nd}\) (basal) to 5\(^{th}\) (upper) internodes in 99C. Although no significant differences were statistically observed in type 2 hexaploids, their basal internodes had higher contents for stem chemical components. Since the basal internode provides stability of a plant’s height, higher contents for stem chemical components in basal
Internodes will improve the stem’s physical strength, which positively related to stem lodging resistance in plants.

**CONCLUSION**

The results of this study suggest that the recent events of polyploidization influenced stem morphological, anatomical, and chemical characteristics, resulting in improving lodging resistance. The two types of hexaploids had different morphological, anatomical, and chemical characteristics related to lodging resistance. The type 1 hexaploid, 99C was mostly influenced by shorter height and a significant reduction in polymer contents from basal to upper internodes. The basal internode was much stiffer than upper internodes, resulting in high lodging resistance in short plants. The type 2 hexaploids such as 1036x and MID had thicker and heavier stems, which may be critical factors for lodging resistance. The results of this study suggested that their thicker and heavier stems consisted of high polymer contents in the stem, strongly associated with increases in both the thickness and amount of mechanical tissues in outer rings and around large inner vascular bundles in basal internode. The combination of the morphological, anatomical, and chemical characteristics of their stems allowed the type 2 hexaploids to have higher lodging tolerance although they were taller than other populations. Overall, the two hexaploid types possessed different morphological traits that were closely related to lodging resistance, and are useful and valuable traits in terms of the development of breeding programs to create improved prairie cordgrass varieties.
Table 4.1 Means for traits of aboveground morphological characteristics including lodging score, total dry weight, tiller density, tiller mass, plant height, leaf length of 1<sup>st</sup> and 5<sup>th</sup> leaves, height of center of gravity, number of leaf per tiller, and proportion of leaf weight to stem weight of eight prairie cordgrass populations used in this study. Populations with same letters are not significantly different ($P<0.05$) within each variable (ANOVA, Tukey’s HSD). The data results are average over two years, except for variables denoted by symbol *.

<table>
<thead>
<tr>
<th>Line</th>
<th>Ploidy</th>
<th>Lodging Scores&lt;sup&gt;$-$&lt;/sup&gt;</th>
<th>Total Dry Weight (kg/plant)</th>
<th>Tiller Density (no./plant)</th>
<th>Tiller Mass (g/tiller)</th>
<th>Plant Height (m)</th>
<th>Leaf Length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>99A</td>
<td>4x&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.85&lt;sup&gt;dc&lt;/sup&gt;</td>
<td>63&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>13.90&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>2.28&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>51.80&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>99C</td>
<td>6x&lt;sup&gt;1&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.40&lt;sup&gt;d&lt;/sup&gt;</td>
<td>45&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.91&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.64&lt;sup&gt;c&lt;/sup&gt;</td>
<td>48.64&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>1034x</td>
<td>4x&lt;sup&gt;2&lt;/sup&gt;</td>
<td>3.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.06&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>96&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>11.09&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.07&lt;sup&gt;d&lt;/sup&gt;</td>
<td>81.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1036x</td>
<td>6x&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.31&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>57&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>23.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>60.60&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>MBB4x</td>
<td>4x&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.09&lt;sup&gt;bcd&lt;/sup&gt;</td>
<td>96&lt;sup&gt;ba&lt;/sup&gt;</td>
<td>11.64&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.19&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>47.13&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MBB6x</td>
<td>6x&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.66&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>134&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.57&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.18&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>50.59&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>1094x</td>
<td>4x&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.27&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>99&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>17.71&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>2.15&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>47.39&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MID</td>
<td>6x&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.71&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.42&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>65.75&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Kim et al. (2010)<br/><sup>2</sup>Kim et al. (2012a)<br/><sup>3</sup>Scale of 1-5 (1 = no lodging; 5 = total lodging)
Table 4.1 (cont.)

<table>
<thead>
<tr>
<th>Line</th>
<th>Ploidy</th>
<th>Central Gravity (cm)*</th>
<th>No. Leaf per Plant*</th>
<th>Leaf(g)/Stem (g) (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>99A</td>
<td>4x</td>
<td>93.70^a</td>
<td>8.7^ab</td>
<td>70.20^b</td>
</tr>
<tr>
<td>99C</td>
<td>6x</td>
<td>48.63^d</td>
<td>7.8^cd</td>
<td>80.65^a</td>
</tr>
<tr>
<td>1034x</td>
<td>4x^2</td>
<td>77.93^c</td>
<td>8.2^bc</td>
<td>79.05^a</td>
</tr>
<tr>
<td>1036x</td>
<td>6x^2</td>
<td>97.03^a</td>
<td>9.1^a</td>
<td>68.00^b</td>
</tr>
<tr>
<td>MBB4x</td>
<td>4x^2</td>
<td>84.53^bc</td>
<td>8.2^bc</td>
<td>74.39^ab</td>
</tr>
<tr>
<td>MBB6x</td>
<td>6x^2</td>
<td>79.38^c</td>
<td>8.8^ab</td>
<td>80.46^a</td>
</tr>
<tr>
<td>1094x</td>
<td>4x^2</td>
<td>94.35^a</td>
<td>7.7^cd</td>
<td>56.88^c</td>
</tr>
<tr>
<td>MID</td>
<td>6x^2</td>
<td>91.30^ab</td>
<td>7.4^d</td>
<td>67.05^b</td>
</tr>
</tbody>
</table>
Table 4.2 Significant of levels for F tests from analyses of variance for internode morphological and chemical traits within each internode of 8 prairie cordgrass populations. IL, internode length (cm); ID, internode diameter (mm); IM, internode mass (g); CL, cellulose; HC, Hemicellulose; L, acid detergent lignin.

<table>
<thead>
<tr>
<th>Internode</th>
<th>Morphological traits</th>
<th>Concentration (g kg⁻¹)</th>
<th>Content (mg cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL</td>
<td>ID</td>
<td>IM</td>
</tr>
<tr>
<td>2nd</td>
<td>0.0006</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3rd</td>
<td>&lt;0.0001</td>
<td>0.0006</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>4th</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>5th</td>
<td>0.0144</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 4.3 Means for number of outer vascular bundle and inner vascular bundle and size of inner vascular bundle at stem cross sections of 2nd and 3rd internodes of eight prairie cordgrass populations. Populations with same letters are not significantly different ($P < 0.05$) within each variable (ANOVA, Tukey’s HSD).

<table>
<thead>
<tr>
<th>Line</th>
<th>Outer Vascular Bundle</th>
<th>Inner Vascular Bundle</th>
<th>Inner Vascular Bundle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2nd</td>
<td>3rd</td>
<td>2nd</td>
</tr>
<tr>
<td>99A</td>
<td>1.25c</td>
<td>2.17a</td>
<td>8.04c</td>
</tr>
<tr>
<td>99C</td>
<td>2.84ab</td>
<td>2.78a</td>
<td>12.04a</td>
</tr>
<tr>
<td>1034x</td>
<td>1.95abc</td>
<td>2.52a</td>
<td>8.57bc</td>
</tr>
<tr>
<td>1036x</td>
<td>2.53abc</td>
<td>2.70a</td>
<td>8.12c</td>
</tr>
<tr>
<td>MBB4x</td>
<td>2.92abc</td>
<td>2.72a</td>
<td>9.39abc</td>
</tr>
<tr>
<td>MBB6x</td>
<td>2.44abc</td>
<td>2.50a</td>
<td>9.57abc</td>
</tr>
<tr>
<td>1094x</td>
<td>1.35bc</td>
<td>2.49a</td>
<td>11.43ab</td>
</tr>
<tr>
<td>MID</td>
<td>2.53abc</td>
<td>2.87a</td>
<td>7.26c</td>
</tr>
</tbody>
</table>
Figure 4.1 Shoot growth morphology of four different populations: 99A (a), MID (b), 1034x (c), and 99C (d). The bar size is 50 cm.
Figure 4.2 Length (a), diameter (b), and mass (c) of 2nd, 3rd, 4th, and 5th stem internode of eight prairie cordgrass populations. Populations with different letters are significantly different ($P < 0.05$) within each variable (ANOVA, Tukey’s HSD), while n.s. indicates no significant difference. The bar indicates standard error.
Figure 4.3 Means for proportion of empty space of stem cross section area at 2nd (a) and 3rd (b) internodes of eight prairie cordgrass populations. Populations with different letters are significantly different ($P < 0.05$) within each variable (ANOVA, Tukey’s HSD), while n.s. indicates no significant difference. The bar indicates standard error.
Figure 4.4 Picture of 2nd internode of hollow stem cross sections of 99A (a), 99C (b), 1034x (c), 1036x (d), MBB4x (e), MBB6x (f), 1094x (g), and MID (h). The features of the stem cross sections were distinguished by following criteria; large or small area of whole stem cross section, more or less proportion of empty space over stem cross section. Cs: area of whole stem cross section; H: area of hollowness (empty space) inside stem cross section.
Figure 4.5 Stem anatomy of stem cross sections at the second internode of prairie cordgrass. (a) Cross section of whole stem. Gray, epidermal cell; light green, ground cell composed of sclerenchyma and parenchyma cells; red, thickwalled fiber cell in cylindrical band near outer region of stem; orange, outer vascular bundle defined as small vascular bundle within cylindrical band of fiber cells; blue, inner vascular bundle defined as large vascular bundle in inner region of stem; white, hollow space (empty space). (b) Florescent detections of lignin and (c) indirect immunodetection of cellulose of stem cross section. Inner vascular bundle (IB), outer vascular bundle (OB), epidermis (EP), sclerenchyma sheath (SFCS), Thick-walled fiber cell (TFC), hollowness (empty space) (HS), phloem (P), xylem vessel (X), and air space (A).
Figure 4.6 Calcofluor staining of stem cross sections (a, b, c, and d) and higher magnification (e, f, g, and h) at second internodes of 99A (a and e), 99C (b and f), 1034x (c and g), and 1036x (d and h) showing different cellulose contents in the cell wall of epidermis, sclerenchyma cells, parenchyma cells, and bundle vascular cells.
Figure 4.7 Phlorogulcinol staining of stem cross sections (a, b, c, and d) and higher magnification (e, f, g, and h) at second internodes of 99A (a and e), 99C (b and f), 1034x (c and g), and 1036x (d and h) showing different lignin contents in the cell wall of epidermis, sclerenchyma cells, parenchyma cells, and bundle vascular cells.
Figure 4.8 Hemicellulose (a and b), cellulose (c and d), and lignin (e and f) contents per dry weight (g kg\(^{-1}\)) and contents per unit length (mg/cm) of 2\(^{nd}\), 3\(^{rd}\), 4\(^{th}\), and 5\(^{th}\) internodes of eight prairie cordgrass populations. Populations with different letters are significantly different (\(P<0.05\)) within each variable of each population (ANOVA, Tukey’s HSD), while n.s. indicates no significant difference. The bar indicates standard error.
Prairie cordgrass (*Spartina pectinata* Link) is suited to produce bioenergy on marginal land, and possesses the co-benefit of water/soil conservation. It is a tall (1-3 m), rhizomatous, warm-season (C₄) perennial native of North America, and is commonly found in lower, poorly drained soils along roadsides, ditches, streams, marshes, wet meadows, and potholes where soils are too wet for other grain, forage, and other biofuel crops. Prairie cordgrass is also well adapted to various climatic and environmental stresses caused by low temperatures and waterlogged and saline soils. Under drought and saline conditions, prairie cordgrass has produced comparable biomass yields to switchgrass cultivars with a high frequency of reproductive tillers. The combination of waterlogging, cold, and salinity tolerances within this species results in a wide geographic distribution throughout the eastern coast and inland marshes of Middle West to Alberta in North America. Prairie cordgrass is well known as a polyploid species consisting of tetra- (2n = 40), hexa- (2n=60), and octoploids (2n = 80), with a basic chromosome number of x=10. Ploidy distribution of prairie cordgrass is strongly associated with geographic distribution in the U.S. (Kim et al., 2012a). In New England, only tetraploid populations were found, while the octoploid populations were mostly distributed in the western North Central region. Both tetraploids and octoploids were distributed in the Central U.S., with no distinct geographical patterns. Unlike other ploidy levels, hexaploids were found either as a mixed population with tetraploids occurring in a single location (Kim et al., 2012ab), or as seed from a tetraploid population (Kim et al. 2010). According to previous studies, polyploidization events have played important roles in geographic adaptation and plant growth development of prairie cordgrass. Polyploidy, therefore, may enable development of prairie cordgrass as a dedicated energy crop for production on marginal land and may also increase the geographical adaptation, extensive genetic diversity, and high stress tolerance of this species. However, little is known about the development and importance of polyploidy in prairie cordgrass.
Using genetic diversity analyses, this thesis reports on cytogenetic analyses and morphological evaluations of multiple, widely distributed polyploid prairie cordgrass populations. Outcomes of this study will assist prairie cordgrass breeding programs, interested in polyploidy, develop improved biomass yields. This dissertation investigated evolutionary origins of polyploid prairie cordgrass populations distributed in wide geographic locations using chloroplast DNA (cpDNA) sequencing. The wide-ranging cpDNA-based phylogeographic analysis helps understand the origin and establishment of polyploid prairie cordgrass, which enables avoidance of genetic erosion caused by loss of germplasm due to loss of ecotype and landrace. This information provided in this thesis will also allow breeders to make appropriate decisions about plant genotypes that show high value within their locality.

Based on the information generated from the cpDNA phylogeographic analysis, it appears that the ancestral plastid genome of prairie cordgrass has rapidly diverged into three major haplotypes, PCG1, PCG2, and PCG3, which are strongly associated with geographic location. The PCG1 haplotype, collected from the New England/Middle Atlantic region in the east and the central U.S., were a mix of all three cytotypes (tetra-, hexa-, and octoploids), while the PCG2 haplotype, collected in southern SD and northern IA, IL, and MO in the central U.S., were primarily octoploids, but also included a small number of tetraploids. The PCG3 haplotype consisted of octoploids identified in a distinct region that includes portions of ND, SD, and MN. Our results suggest that the multiple origins can expand ecological amplitude of prairie cordgrass in its home region, as well as facilitate the reproductive isolation into an entirely novel habitat. The octoploids grouped into the PCG3 haplotype originate from greater cold regions, which have allowed prairie cordgrass to adapt into the northern Great Plains. These octoploid accessions may be useful for investigating cold tolerance in prairie cordgrass breeding program.

This cpDNA haplotype diversity may increase genetic diversity in polyploid prairie cordgrass, which can be exploited for morphological variability, is also highly associated with geographic distribution (Guo et al., 2015). The differences in morphological characteristics could be due to gene dosage effects, mostly caused by gene and genome duplications through polyploidization events. More
sufficient cytogenetic data, however, is needed to understand how polyploidy may generate novel genomes with novel phenotypes in traits that play important roles in wide geographic adaptation of polyploid prairie cordgrass. The silver staining technique was used in this study to detect the number, size, and distribution of silver-stained nucleolar regions (AgNORs) in metaphase and interphase cells. AgNORs are involved in the high-rate production of ribosomal RNA genes and ribosomes that are necessary for all protein synthesis in the cell. Thus, the number and size of AgNORs can help evaluate the alteration of chromosomal structures and the rate of production of ribosomal materials. This may be involved with morphological differences, as well as both stabilization and adaptation of polyploid series.

Variation in the number of AgNORs in metaphase and interphase cells was observed among the polyploid populations. The distribution pattern of AgNORs in metaphase cells of tetra- and octoploids reflect changes in ploidy level. This suggests that tetra- and octoploids have different transcriptional rRNA gene activity in root meristem cells, which may result in various stress responses between ploidy levels. In addition, various stress responses to abiotic stress play a role in the wide geographic adaptation of tetra- and octoploid prairie cordgrass across U.S. Using silver staining, we were able to detect chromosome instability of neopolyploidy in prairie cordgrass. Unlike tetra- and octoploids, neohexaploids have a different number of AgNORs in metaphase cells. This might be due to rapid changes in chromosome structure and gene expression caused by neoploidy formation in a mixed ploidy population. According to cpDNA phylogeny analysis, the mixed ploidy populations comprising tetraploids and neohexaploids have the same cpDNA haplotypes, indicating that neohexaploids and tetraploids share the same evolutionary origin. Thus, this phylogeny suggests that recent polyploidization in prairie cordgrass may have allowed the immediate reproductive isolation between newly formed polyploids and their parents. While no cpDNA variation within and between neohexaploids and tetraploids occurred in same location, chromosome instability of neohexaploids may increase heterozygosity that can result in substantial variation to phenotypic characteristics such as plant height and stem diameter.
characteristics are related to the lodging resistance, especially between neohexaploids and their putative tetraploid progenitors.

Prairie cordgrass lodging can reduce biomass yields and seed production and is an important consideration in breeding programs. In this study, we compared a range of morphological, anatomical, and chemical characteristics associated with lodging resistance between neohexaploids and their tetraploid progenitors. Since neohexaploids and their tetraploid progenitors inhabit the same area, morphological variability associated with lodging will largely reflect the consequences of recent events of polyploidization in prairie cordgrass. Overall, the neohexaploids have higher lodging resistance than their tetraploid progenitors due to thicker, heavier stems and a smaller stem hollowness percentage. This study first described the anatomical and chemical features of stem cross sections, and reported that higher polymer contents directly associated with more, as well as, bigger vascular bundles and thicker cell walls were observed in lodging-resistant neohexaploids. This will help prairie cordgrass breeders select useful traits (e.g., thicker cell walls and stems) related to lodging resistance to create improved types.

This information revealed on genetic, cytogenetic, anatomical, and morphological variation, associated with polyploids of prairie cordgrass, can be used to select and develop desired traits in the improvement of polyploid breeding programs of this species. Moreover, these analyses will aid in the development of a molecular marker system for polyploid prairie cordgrass, which may identify heterozygous genotypes. It is important to realize that heterozygous genotypes would be useful in developing this species to succeed in various stressful environmental conditions and effectively colonize new habitats and, thus, extend their geographic distribution. As a potential energy crop grown on marginal land, our study displayed the genetic and phenotypic evolutionary significances of polyploid prairie cordgrass which will guide current breeding programs.
In conclusion, polyploidy plays an important role in prairie cordgrass evolution, as well as possibly being an important factor in why prairie cordgrass succeeds in adapting and establishing in such wide geographic locations. Having greater environmental adaptabilities can possibly extend the range in which plants could grow, which will play a key role for improving prairie cordgrass biomass yield potential on marginal lands. In addition, polyploidy increases morphological complexity and genetic diversity over long-periods of evolutionary time. Understanding how polyploidy effects plants will provide important information to plant breeders who use polyploidy in their breeding programs. Using aspects of polyploidy will provide plant breeders more options for developing novel prairie cordgrass genotypes and improving existing genotypes. This thesis fills a void in the lack of understanding of polyploid effects on geographic adaptation and plant growth and development of prairie cordgrass. Polyploidy may be not in itself a solution for creating the best prairie cordgrass genotypes, but it can be a useful tool to incorporate into a breeding program.
REFERENCES


Fawcett JA, Maere S, Van de Peer Y (2009) Plants with double genomes might have had a better chance to survive the Cretaceous-Tertiary extinction event. PNAS 106:5737–5742.


Food and Agriculture Organization of the United Nations (FAO) (2013) "FAOSTAT." Rome:FAO.


77


Sen R and Bhadrui PN (1968) Ploidy level of nodular cells as determined by their nucleolar number and volume. Cytologia 34:202-212.


Weaver JE (1968) Prairie plants and their environment: a fifty-year study in the Midwest. Lincoln, University of Nebraska Press.


