MARKER-ASSISTED VERIFICATION OF HYBRIDS IN PEARL MILLET-NAPIERGRASS (*PENNISETUM GLAUCUM* [L.] R. BR. X *PENNISETUM PURPUREUM* SCHUMACH.)

A Thesis

by

CHARLIE D. DOWLING, III

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

MASTER OF SCIENCE

December 2011

Major Subject: Plant Breeding

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ABSTRACT

Marker-Assisted Verification of Hybrids in Pearl Millet-Napiergrass (*Pennisetum glaucum* [L.] R. Br. x *Pennisetum purpureum* Schumach.). (December 2011)

Charlie D. Dowling, III, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Russell W. Jessup

A high-biomass perennial grass that is directly seeded using existing farm equipment can reduce both planting and overall input costs. Three cytoplasmic male-sterile *cms* A-lines and four fertile genotypes of pearl millet (*Pennisetum glaucum* [L.] R. Br.) and one novel pearl millet selection from the Perennial Grass Breeding Program at Texas A&M University were selected to cross with napiergrass (*Pennisetum purpureum* Schumach.). The pearl millet parents were chosen based on characteristics such as basal tillering, plant height, and days to anthesis. Three napiergrass accessions from the Perennial Grass Breeding Program and the cultivar Merkeron were used as pollinators for these crosses.

The *cms* and fertile pearl millet accessions produced full heads of seed when pollinated with napiergrass. There was a large range of seed sizes and weights for each hybrid family, and the seed were separated into four size classes. The weight differences from the largest to smallest class of seed varied by more than 30%. All of the seed classes germinated, and seed size, in this case, was completely unrelated to the ability to germinate. 100% germination was observed in five seed size classes for both PMN

hybrids, and 90% germination was observed in three of the eight classes. Essentially all of the hybrid seed recovered from the original pearl millet x napiergrass crosses germinated, but all of the F_1 hybrids were sterile in that none of them produced viable seed. Flow cytometry could not be used to identify the hybrids because the DNA content of pearl millet and napiergrass were essentially the same, even though distinct 2C and 4C peaks were seen from the diploid pearl millet. From the 58 EST-SSRs surveyed in the bulked segregate analysis, several were heterozygous dominant and many were homozygous dominant and hemizygous at its particular loci. Seven hemizygous EST-SSRs were identified for Merkeron, seven for PEPU09FL01, eight for PEPU09FL02, and six for PEPU09FL03. These markers are extremely valuable to any pearl millet x napiergrass hybridization program because they provide a means whereby the hybrids can be easily identified. Identification of hemizygous pearl millet markers will also assist in future DNA sequencing and also in a marker-assisted breeding program.

DEDICATION

I would like to dedicate this work to my family: my parents, Grams Berit, and my Uncle Slim. Slim, you have been my best friend since I can remember, and I have always looked to you for advice, classy jokes, and good company. May you live out the rest of your life in joy. Lastly, I want to dedicate this achievement to my Papa Fred. For without your influence of hard work, proper ethics, and a good golf game, it is possible I would not be in the place I am today. R.I.P.

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Finally, I would like to acknowledge my parents who have supported me through school, encouraged me always, and told me the sky was the limit; I thank you and love you. To my Grams Berit, you are a rock to the family, and words cannot express how much I appreciate everything you have done.

NOMENCLATURE

PMN	Pearl Millet x Napiergrass
EST	Expressed Sequence Tags
SSR	Simple Sequence Repeat
PCR	Polymerase Chain Reaction
BSA	Bulk Segregant Analysis
DEC	Dedicated Energy Crop

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CHAPTER I

INTRODUCTION

Competition for land resources is increasing and will continue to increase because the world population is expected to reach at least nine billion by 2050 (UN Dep. Social Economic Affairs, 2010). The production of bioenergy crops, therefore, is largely being targeted away from productive agricultural lands but towards marginal and abandoned grasslands. In order to reach the goal of producing 36 billion gallons of bioethanol production by 2022 and address the mandatory cap on corn ethanol production that was mandated by the U.S. federal government, such alternative sources of land must be utilized to grow biofuel crops (Sissine, 2007). These alternative sources exist and can be provided by marginal and currently fallow farmlands and grasslands. According to the Wisconsin Grasslands Bioenergy Network (WGBN, 2011), an estimated 20 - 27 million hectares of marginal land exist in the U.S. Similarly, the 2007 U.S. Census of Agriculture reported that approximately 14 million hectares of land including idle lands, land currently in cover crops for soil improvements, and fallow rotations are available. If perennial grasses were cultivated on these lands, the U.S. could produce and estimated 377 million tons of biomass yr^{-1} for chemical conversion to primary biofuel products (Perlack et al., 2006). This tonnage does not take into account biomass from annual crop residues, and assumptions were made that 22.25 million ha of cropland, idle cropland, and pastures were dedicated to the production of perennial

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This thesis follows the style of Crop Science.

biomass crops. It is important to conserve marginal grasslands that are native prairies, but a vast majority of marginal grasslands no longer contain native species and can be allocated towards improved agricultural productivity via low-input, perennial, biofuel systems. The utilization of perennial crops versus annuals, such as corn or grain sorghum, can further provide a sustainable system of producing lignocellulosic ethanol while simultaneously preventing soil erosion, reducing the amount of fertilizers applied, reducing herbicide applications, and satisfying EPA mandates of reducing carbon emissions by sequestering carbon in the soil (Costanza et al., 1997; Kort et al., 1998; McLaughlin and Walsh, 1998; Lewandowski et al., 2003; Khanna et al., 2010). There is a tremendous rate of return of energy production in perennial biomass systems with such low-input requirements. The incorporation of low-input management practices with biomass feedstocks such as napiergrass (*Pennisetum purpureum* Schumach.) or pearl millet (*Pennisetum glaucum* [L.] R. Br.) x napiergrass hybrids (PMN) could be used in these systems.

Widely adapted and productive perennial crops are best suited as alternative energy sources. Pearl millet and napiergrass possess such wide adaptation potential and produce vigorous interspecific hybrids (Burton, 1944). With the capacity of being seed propagated, these interspecific hybrids have the potential to greatly reduce labor costs and improved the economic feasibility in comparison to vegetatively propagated perennial biomass such as energycane (*Saccharum* L. spp.) or *Miscanthus* Andersson species. In addition, perennial biomass crops such as switchgrass (*Panicum virgatum* L.) that are typically broadcast seeded present crop establishment challenges because of

small seed size and seed dormancy issues. Switchgrass' slow seedling growth and negative response to high planting density hinder its establishment and reduces biomass production during the establishment year (Guretzky, 2007). According to a recent announcement from the USDA and the U.S. Department of Energy (DOE), the leading candidates for bioenergy crops are switchgrass, hybrid poplar (*Populus* L.), *Miscanthus giganteus* Keng., and *Brachypodium* P. Beauv. (USDA Press Release, 08/12/11). None of these crops, however, are capable of producing both a high biomass feedstock that have superior yield in the establishment year and can be direct seeded. Switchgrass can be direct seeded and *Miscanthus* can provide establishment year yields, but neither can provide both aspects. Pearl millet x napiergrass, in contrast, is a perennial dedicated energy crop (DEC) that may be direct seeded. Hybrid vigor and feedstock sterility in F_1 PMN progeny further add to the advantages of this interspecific hybrid in comparison to other DECs.

The objectives of this research were to: 1) evaluate the efficiency of interspecific hybridization between pearl millet and napiergrass using cytoplasmic male-sterile (cms) pearl millet lines as described by Powell and Burton (1966); 2) produce a diverse collection of novel PMN hybrids; and 3) screen and identify a number of DNA markers specific to napiergrass and that can be used to verify PMN hybrids.

CHAPTER II

LITERATURE REVIEW

Taxonomy and General Overview of *Pennisetum* Species

Most members of the genus *Pennisetum* are widely adapted making the genus one of the most diverse in the *Poaceae* family. It consists of both tropical and subtropical species (Juahar, 1981). The genus consists of more than 140 species, and it is considered one of the most important genera in the *Paniceae* tribe (Jauhar, 1981). It contains a heterogeneous assortment of species and subspecies that have an array of ploidy levels (diploids to octoploids), life cycles (annual to perennial), modes of reproduction (sexual and apomictic), base chromosome numbers (x = 5, 7, 8, 9), morphological distinctions, and useful applications. The genus consists of five subgenera: *Gymnothrix*, *Eu-pennisetum*, *Penicillaria*, *Heterostachya,* and *Brevivalvula*. Pearl millet and napiergrass are both members of the subgenus *Penicillaria* (Stapf and Hubbard, 1934). Domestication of millet species began approximately 10,000 years ago in east Asia, and domestication of pearl millet occurred about 4,000 years ago (Lu et al., 2009). The species' primary and secondary centers of diversity include a diffuse belt stretching from the Saharan plateau region to western Sudan and east India, respectively (ICRISAT, 2011). The International Crop Research Institute for the Semi-Arid Tropics (ICRISAT) germplasm collection consists of 21,594 accessions of cultivated pearl millet from 50 different countries, and it is the largest collection of pearl millet germplasm in the world. More than 25 million ha of pearl millet are cultivated in Africa and East Asia

where it is a staple grain crop. Because of its wide range of adaptation, pearl millet has been labeled the world's hardiest crop in arid environments and on marginal soils (ICRISAT, 2011). There is a wide range of variability in the ICRISAT collection, and it includes collections from institutions (10,201), farmers' fields (6,537), commercial markets (1,681), farmer co-ops (1,357), and threshing floors (479) (ICRSAT, 2011). The USDA National Plant Germplasm System (NPGS) is an additional source and has more than 2,000 pearl millet accessions (GRIN, 2011).

Napiergrass, also commonly referred to as elephantgrass, is a C_4 perennial grass with creeping rhizomes. It is native to the tropical regions of Africa where cut and carry systems are popular for supplementation of livestock. Napiergrass was introduced into the United States by the USDA in 1913 (Thompson, 1919). After its introduction test plots and improvement programs were established. The labor requirement to establish napiergrass vegetatively by either crown sections or stem cuttings, along with susceptibility to the eyespot disease (*Helminthosporium sacchari* Butler); however, it caused farmers to lose interest in the crop (Burton, 1944). Overall, napiergrass has desirable characteristics such as insect resistance, rapid growth rate, and a high nutrient value (Rao et al., 2003). When the improved cv. 'Merkeron' was registered by Burton in 1984, several specific and generalized 'genes' of interested were noted (Burton, 1989). The two most valuable traits were resistance to the eyespot disease and a dwarfing gene that resulted in the release of 'Mott' (Burton, 1989). Napiergrass was documented to produce as much as 100 tons of fresh weight per ha of biomass and has one of the fastest growth rates of higher plants (Renard et al., 2011). *In vitro* testing further supports this

assertion. Karlsson and Vasil (1986) reported napiergrass cell cultures were the fastest growing of all C_4 species tested. Napiergrass is short-day photoperiod sensitive and typically does not flower during pearl millets' growing season in production zones across Texas and Arizona (Osgood et al., 1997). However, napiergrass flowering will occur under greenhouse conditions once the day length reaches its preferred minimum in regions where aboveground winter kill prevents such under field conditions. The combination of pearl millet's abiotic stress tolerance and integrated agronomic systems with napiergrass' perennial growth habit and biotic stress tolerance provides a unique possibility of developing large-seeded hybrid lines with superior perennial biomass production and forage potential.

Wide Hybridization in *Pennisetum*

Wide hybridization of *Pennisetum* species for the development of valuable forage crops is extensive (Dujardin and Hanna, 1983; Sotomayor-Rios et al., 1989; Sobrinho, 2005). Pearl millet has been successfully crossed with numerous other *Pennisetum* species such as *P. orientale* L., *P. squamulatum* Fresen, and *P. alopecuroides* (L.) Spreng., and introgression of genes from these wild progenitors could improve cold hardiness and produce germplasm that reproduced by apomixis (Hanna, 1982). In PMN hybrids pearl millet provides hybrid vigor and high quality forage while napiergrass contributes perenniality, biomass production, and photoperiodism (Juahar and Hanna, 1998). In nature, both species are predominately cross-pollinated. The resulting heterozygosity from outcrossing, as long as the proper mechanisms for pollen distribution are present, produces an array of allelic combinations available for selection

(Burton and Powell, 1968). Protogyny, the phenomena in which the stigmas are exerted prior to anther exertion, occurs in both pearl millet and napiergrass and facilitates outcrossing. This trait reduces inbreeding depression and increases heterozygosity in nature in both species. The use of cytoplasmic male-sterile (*cms*) lines of pearl millet in commercial seed production scenarios further maximizes the potential for hybrid seed production upon hybridization with napiergrass in frost-free (ie. greenhouse) or tropical environments (Osgood et al., 1997). Previous reports of interspecific hybridization between pearl millet (2n = 2x = 14) and napiergrass (2n = 4x = 28) confirmed the presence of 21 chromosomes in the resulting hybrids (Burton, 1944; Barbosa et al., 2003). The triploid PMN hybrids are sterile, providing a mechanism to alleviate concerns of invasiveness from seed. The aggressive rhizomatous growth habit and fertile seed of napiergrass, by comparison, have resulted in it being classified as an invasive species by the Florida Exotic Pest Plant Council (FEPPC) in 1999 (GRIN, 2011).

Extensive literature documents the cytogenetics of pearl millet, napiergrass, and their interspecific hybrids. Pearl millet and napiergrass are in the primary and secondary gene pools of *Pennisetum*, respectively (Harlan and De Wet, 1971; Martel et al., 1997). Napiergrass is considered an allotetraploid with a genome complement of A'A'BB. The A' genome is considered homologous to the A genome of pearl millet (Dujardin, M. and W.W. Hanna, 1985). These two parental species have good genetic combining ability, and the interspecific hybrids usually have greater resemblance to napiergrass (Gonzalez and Hanna, 1984). This is also generally attributed to the apparent dominance of

napiergrass' B subgenome over pearl millet's A subgenome. Napiergrass' A' chromosomes are smaller than the A chromosomes of pearl millet making chromosome pairing difficult during meiosis in PMN triploid hybrids, but they pair at a much higher frequency than the A and B genomes which are incomparable (Jahuar, 1981; Pantulu and Rao, 1982; Jahuar & Hanna, 1998). The univalents of the B genome in the triploid lags behind during anaphase as whole or portions of the chromosomes are not included leading to abberations and thus sterility. As reported by Juahar (1981), pearl millet's karyotype is considered symmetrical because of metacentric and submetacentric chromosomes. According to Stebbins (1958), in contrast, the fact that pearl millet's largest chromosome is 1.5 times larger than the smallest categorizes it in the 1a class for asymmetry using the classification he proposed. Stebbin (1958) also described the karyotype of napiergrass containing metacentric and submetacentric chromosomes as well as one acrocentric pair (Stebbins, 1958). Stebbins (1958) categorized napiergrass as asymmetrical in general, and scored it in the 2b class of symmetry.

Polymerase Chain Reaction Amplification Utilizing EST-SSRs

Cytological analysis of PMN and its parents is well documented in the literature (Burton, 1942; Jauhar, 1981; Jauhar and Hanna, 1998; and Barbosa et. al, 2003). Although this very useful in determining chromosome number and meiotic behavior, new techniques for hybrid verification would be highly useful towards marker assisted breeding and/or molecular mapping programs. The genetic relatedness of a number of napiergrass accessions was studied at Tifton, GA. using AFLPs (amplified fragment length polymorphism) (Harris et al., 2009). This was the first attempt to determine the

phylogeny of this germplasm collection. Isozymes were used to classify napiergrass lines in India (Bhandari et al., 2006), and RADPs (random amplified polymorphic DNA) were used to categorize germplasm at the International Livestock Research Institute (Lowe et al., 2003). Finding from this research revealed possible heterotic groups that could be useful in future breeding efforts (Harris, et al. 2009). Wide arrays of molecular resources are available for pearl millet because of its importance as a grain crop, but very limited molecular data is available for napiergrass. Implementation of comparative genomic approaches of extensive publicly available DNA sequences in the *Poaceae*, PCR amplification utilizing expressed sequence tags (ESTs) of microsatellites, or known otherwise as Simple Sequence Repeats (SSRs), provides unique marker sequences for the assistance in verification of the hybrid nature of putative PMN hybrids.

Commercialization of Pearl Millet x Napiergrass

In addition to reducing the invasiveness compared to napiergrass, PMN hybrids also have several agronomic and economic advantages over previously mentioned DECs (dedicated energy crops). Seed of PMN (0.214g -- 0.78 g 100 seed $^{-1}$) is much larger than switchgrass (0.13 – 0. 21 g 100 seed $^{-1}$) or miscanthus (0.07 - 0.12 g 100 seed $^{-1}$) which allows for longer persistence in the field and the potential to close the crop canopy more rapidly (Smart and Moser, 1999). While *Miscanthus x giganteus* has comparable biomass yield to sugarcane (*Saccharum* [L.] spp.) and napiergrass, establishment of this crop requires a minimum of three years as well as greater inputs of water and fertilizer (Lewandowski et al., 2000). Woodard and Prine (1993) demonstrated that the intermediate phenotype of PMN can compete well with energycane and napiergrass,

with all of these C_4 species producing more than 35 Mg ha^{-1} yr^{-1}. Pearl millet x napiergrass hybrids are similar to *Miscanthus x giganteus* in that they are sterile triploids, but commercial scale seed production, and high establishment year biomass potential provide PMN agronomic advantages. Pearl millet x napiergrass and its reciprocal cross, referred to as kinggrass, have the potential to be use in traditional planting and cultural methods. Implementation of a system described by Osgood et.al (1997) in which F_1 seed can be mass produced from superior isogenic lines of pearl millet and napiergrass is such an improved scenario. Also, if a *cms* pearl millet line developed for biomass production becomes commercially or publicly available, the method described by Powell and Burton (1966) will become the most efficient system for commercial scale production of large seeded, perennial biomass feedstocks. Vegetative reproduction of PMN is a viable option in the tropics due to the lack of interest in commercial seed production and comparatively low labor costs, but its usefulness in the southern U.S. is limited because of higher labor costs (Boddorff and Ocumpaugh, 1986, and Osgood et. al, 2007). Thus, there is a need for a tropical winter nursery for PMN seed production. For example, in 1986 Osgood et al. (1997) produced 1145 kg ha^{-1} PMN seed in Kunia, Hawaii. This level of seed production parallels that of commercial forage sorghum and translates to economic feasibility for farmers seeking a biofuel crop that is 'seeded-yet-sterile'. Recent improvement efforts of biofuel crops emphasize the importance of including feedstock sterility in a product developed for the commercial bioenergy industry. *Sorghum* species hybrids are a well-documented example of biomass crops with the potential to create novel weeds (Cox et al., 1984).

Any diploid or tetraploid *Sorghum* species hybrid has the ability to cross-pollinate, and it can become an improved pest for farmers and ranchers. However, non-flowering crops eliminate the possibility of pollen flow to an undesirable wild-type or even cultivated *Sorghum*. Also, commercialization of transgenic species hybrids may lead to even a larger problem if they are not completely sterile. From a review of current options on the list of dedicated energy crops, one can acknowledge that a 'seeded-yet-sterile' system like PMN provides significant reduction in such risks.

Sustainability and Remediation

Another desirable characteristic of napiergrass in sustainable, low-input systems is its ability to not only persist but be productive on marginal lands. Utilizing marginal and abandoned lands removes potential land use impacts on corn, wheat, soybean and other major food crops offering farmers and investors significant acreages available for biofuel feedstock production. Cost effective DECs are critical towards the feasibility and practicality of developing sustainable bioenergy crops. One additional benefit of using such a crop is potential remediation of depleted or contaminated soils. Napiergrass may have the ability to remove high levels of nickel, cadmium, copper, zinc, and lead from the soil (Holm, 2010). According to research done by the Cooperative Research Center (CRC) and Contamination Assessment and Remediation of the Environment (CARE) in collaboration with Hong Kong-based HLM Asia Group and Shaoguan University in China's Guangdong Province, napiergrass has the ability to degrade hydrocarbons in contaminated soils (Holm, 2010). Pearl millet, however, not only can persist in sandy, acidic soils, but performs remarkably well in hot environments

(Thomas Jefferson Institute, 2007). It has better drought tolerance and can grow on lower pH soils than milo *Sorghum bicolor* L. Moench. (Jefferson Institute, 2007). Both pearl millet and napiergrass possess advantageous traits for multiple uses on marginal lands, and PMN has a similar potential for use in perennial biofuel cropping systems.

CHAPTER III

MATERIALS AND METHODS

Pearl Millet x Napiergrass Hybridization

Three cytoplasmic male sterile A-lines and four fertile genotypes of pearl millet that varied in their area of collection from the USDA NPGS collection as well as one novel selection of pearl millet from the Perennial Grass Breeding Program at Texas A&M University were selected for hybridization with napiergrass based on their growth descriptions, morphological descriptions, and phenological data. Three novel napiergrass genotypes from the Perennial Grass Breeding Program and the cultivar, Merkeron, were selected as pollinators for these crosses (Table 1). All controlled crosses were made in a greenhouse during the fall and winter of 2010. A summary of all pollinations made is in Table 2. After the pollinations were made, all pollinated stigmas of the fertile and *cms* pearl millet lines were closely examined to ensure that napiergrass pollen was on them. This reassured complete pollination across the whole inflorescence no matter if it was a fertile or *cms* line. Since pearl millet is protogynous, emasculation of the fertile florets was not necessary which greatly simplifies this process. Prior to all pollinations, pearl millet inflorescences were enclosed in glycine prior to the exertion of the stigmas in order to ensure that no foreign pollen was transferred to the maternal parents. All pollinations were made when the temperature and humidity were suitable for induction of anther dehiscence of pollen. This was observed to occur prior to noon or very late in the day no matter the conditions of the greenhouse. Careful consideration

of the temperature in the greenhouse was taken as most anthers began dehiscence around 27 °C. To collect the pollen inflorescences were held directly over a large Petri dish and gently tapped. A lid was then placed on the Petri dish to ensure containment of the pollen. The glycine bag was then removed from the inflorescence of the female pearl millet parent and the inflorescences were gently rolled over the pollen in the Petri dish so that it came in contact with the receptive stigmas extending from the spikelets on the inflorescence. The glycine bags were then replaced onto the inflorescences of pearl millet for at least 5 to 7 days. Bags were removed once stigmas were no longer receptive. Seed were allowed to mature for 5 to 6 weeks, and hard seed was confirmed prior to harvest. Harvested inflorescences were stored at 10 °C at 30% relative humidity. Threshing was done by hand using a ribbed rubber mat and a block covered in the same ribbed rubber material. After the seeds were threshed, they were returned to the cold room for permanent storage.

Those crosses that yielded large quantities of seed made it possible to separate and bulk the seed based on size. Seed bulks were categorized by pouring all the threshed seed through layers of Seedburo® sieves. The sieve sizes were 1/13" Round, 1/14" Round, 1/15" Round and 1/17" Round. A select number of seed from each size class were germinated and the seedlings were transplanted into pots and eventually taken to the field in the spring of 2011 to observe any correlations between seed size and plant phenotypes.

Pearl Millet x Napiergrass Field Observations

PMN genotypes were planted into a nursery at College Station, TX on May 10, 2011. These plots were maintained with manual weed control and minimal herbicides. Occasional spot spraying of 2,4-Dichlorophenoxyacetic acid (2,4-D) was used to control moderate morning glory (*Ipomoea* [L.] spp.) and pigweed (*Amaranthus* [L.] spp.). The plants were observed and selected for flowering late in the growing season or photoperiod sensitive phenotypes. Sterility in the PMN hybrids was determined by collecting inflorescences from six of the genotypes. The total numbers of inflorescences collected from each hybrid genotype varied depending on how many were flowering. Inflorescences were allowed to mature in the field for at least 4 wk post anthesis, and timely collection ensued to prevent loss to seed shattering. At least two completely intact heads were collected and analyzed for each plant to ensure the results. The total number of florets were counted from each inflorescence for each genotype and threshed on ribbed rubber mats as described above. The total number of seed produced was then counted, and the percent seed set was determined.

Seed viability was determined by germination. PMN seed of each of the four seed classes from two hybrid genotypes were germinated a commercial soil mixture in 72 cell trays (Figure 4). One seed was planted in each cell and a total of 10 seeds per seed size class were planted, and each class was replicated three times. Germination data was recorded after 7 days.

Flow Cytometry

Flow cytometry was used to measure the DNA content of each putative hybrid, a female pearl millet parent and a male napiergrass parent. If the chromosome number and DNA content of the parents are known, the ploidy level and possibly the chromosome number of the putative hybrids can be predicted from their DNA content. Leaves of a diploid female parent and each putative PMN hybrid were collected, placed on ice, and brought into the laboratory for analyses. Pieces approximately $1cm^2$ was cut from a leaf of an individual hybrid and the pearl millet female parent and these were placed into a Petri dish and the leaf material was chopped with a razor blade in 0.25 mL of Galbraith's buffer. After the tissue was properly macerated, 1.0 mL of Galbraith's buffer was added to the leaf tissue and strained through a 30 mμ filter into a 2.0 mL microtube. Leaf material was kept on ice before and after maceration. Fifty μL of propidium iodide was then added to each microtube and allowed to set for 15 minutes in a covered ice chest. The sample solutions were then analyzed for DNA content using a Partec CyFlow Counter (Partec GmbH, Münster, Germany). At least 3,000 particles were analyzed of each sample. This protocol was repeated for each hybrid analyzed.

DNA Isolation

Genomic DNA was isolated using a modified rapid salt extraction protocol described by Aljanabi and Martinez (1997). Four hundred microliters of homogenizing buffer (0.4 M NaCl, 10 mM Tris–HCl, pH 8.0, 2 mM EDTA, pH 8.0) and 100 mg of fresh leaf tissue were added to 1.7 mL microtubes. The plant tissue was pulverized for one to two minutes or until adequately pulped. Forty microliters of 20% sodium dodecyl

sulfate and 8 µL of 20 mg/mL proteinase K were added and vortexed for 5 seconds. Following incubation in a water bath at 65 °C for a minimum of 1 hour, 300 µL of NaCl saturated H_2O was added and the samples were vortexed for 30 seconds. Samples were spun at 12,000 rpm for 10 minutes, the supernatant was transferred to new tubes, samples were spun at 12,000 rpm for 20 minutes, and supernatant was transferred to new tubes without disturbing any of the remaining pellets. Following the addition of 800 µL of cold isopropanol and 20 gentle inversions, samples were incubated at −20°C for 1 hour. Samples were spun at 10,000 rpm for 5 min, and the supernatant was removed. Next was the addition of 500 µL of cold 70% ethanol. The samples were spun at 10,000 rpm for 5 min, and the supernatant was removed. Microtubes containing DNA were inverted until dry, and the DNA was re-suspended in 100 µL of sterile deionized H_2O. Re-suspended DNA was then quantified prior to its use in PCR amplifications. DNA quantification was completed with a spectrophotometer (Eppendorf, Hamburg, Germany). Dilution of the DNA solution was carried out by first filling a cuvette with 50µL of deionized H_2O and using this sample as a blank to zero the machine. Next, using a clean cuvette 49µL of deionized and autoclaved H_2O (PCR H_2O) was added followed by 1µL of the DNA solution. After the conclusion of quantifications, appropriate quantities of DNA and PCR H_2O were added to yield a final concentration of 50 ng μL^{-1} for each genotype included in the survey.

EST-SSRs Analysis

In the absence of publicly available sequence data for napiergrass, a diverse collection of SSRs were surveyed across napiergrass and PMN plant materials. A total of 21,745 full-length complementary DNA (cDNA) sequences from apomictic buffelgrass pistils [*Pennisetum ciliare* (L.) Link syn. *Cenchrus ciliaris* L.] were downloaded from GenBank (National Center for Biotechnological Information (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Simple sequence repeats were identified and primer sequences designed using the SSRLocator software package located online at http://www.ufpel.edu.br/faem/fitotecnia/fitomelhoramento/faleconosco.html. These PCAR ('Pennisetum-Ciliare-Apomictic-Repeat') marker sequences were used to survey the parental pearl millet and napiergrass genotypes. All the maternal parents' DNA was placed in a bulk each with an equal volume of 50µL and concentration of 50 ng μL^{-1}, and the bulk female was surveyed in association with each of the four paternal parents. Selected SSRs contained at least ten dinucleotide or five tri-, tetra-, or pentanucleotide repeats (Jessup, et al. 2002). Primer design was based on the standards of 50% guanine-cytosine content, minimum melting temperature of 50 °C, absence of secondary structure, length of 20–27 nucleotides, and amplified PCR product range of 100–400 base pairs (bp) in length. The desired length of markers to be surveyed was between 100-200 bp long. A total of 58 SSR markers were surveyed from the compilation of sequences from buffelgrass that are between 100-400 bp. Polymerase chain reactions were performed in a total volume of 20 µL using 11.8 µL of PCR H_2O, 1 µL of 50 ng

μL^{-1} DNA, 2 µL of 1X Promega $MgCl_2$-free PCR buffer, 2 µL 2.5 mM $MgCl_2$, 1 µL of 4mM deoxynucleoside triphosphates (dNTPs), 1 µL of 2 mM of each forward and reverse primer, and 0.2 µL of 5 U μL^{-1} *Taq* polymerase. The reactions were conducted in 96-well plates and temperature cycling was carried out using a PTC-220 Dyad Thermal Cycler (MJ Research Inc., Waltham, MA). The PCR began with an initial denaturation at 95 °C for 3 minutes; followed by 40 touchdown decrement cycles at 95 °C for 25 seconds, 55 °C for 25 seconds, and 70 °C for 45 seconds; and concluded with an elongation stage of 72 °C for 10 minutes. The final hold was at 4 °C indefinitely. Amplification of the PCR products was completed using polyacrylamide gel electrophoresis (PAGE) on a large MEGA-GEL (C.B.S. Scientific, Del Mar, CA) high-throughput unit and nondenaturing gels with final concentrations of 40mL acrylamide, 10mL 10X TBE (tris-borate-EDTA) Buffer, 1.4mL ammonium persulfate, and 80µL TEMED (Tetramethylethylenediamine) as described by Wang et al. (2003). The polyacrylamide gels were stained with ethidium bromide for 40 minutes prior to loading the DNA into the wells. Prior to loading the wells, 2µL of gel loading buffer (containing 35 mL of 50% glycerol, 2.5 mL of 10X TBE, 2 mL of 0.5M EDTA, 0.5 mL of 20% SDS, 10 mL de-ionized H_2O, and 0.05 g of bromophenol blue) was added to the 96 well plate and centrifuged briefly to a maximum 340 rpm. After the PCR product and 1.5µL of 50 bp ladder was loaded onto the gel rig, electrophoresis was carried out on a two hour run at a maximum amperage and wattage of 350 mA and 400 W, respectively. Since the desired bp length of the surveyed SSRs were 100-200, a run time of two hours was ideal. Identifying alleles that are 200 bp or larger required runs of three hours or

more. Once the gels completed the electrophoresis process, they were then photographed using UV light to illuminate the allele bands. The brightness, contrast and white levels of the photographs were manipulated using Adobe Photoshop®. The pictures where then scored for the presence or absence of allele bands according to the procedure set forth by Rodriguez et al. (2001). Loci that appeared to be homozygous dominant, hemizygous, and some heterozygotes were identified. The markers were then compared to other gel results to determine the best suitable for hybrid analysis using the same PAGE protocol.

CHAPTER IV

RESULTS AND DISCUSSION

Pearl Millet x Napiergrass Hybridization

Both *cms* and fertile pearl millet genotypes produced full heads of seed when pollinated by napiergrass. The size, shape, and color of the potential hybrid seed were different from the self-pollinated seed of the fertile pearl millet lines. These differences could be noticed without magnification. All parental lines used in these crosses are listed in Table 1. In Table 2 are the number of inflorescences pollinated for all hybrid genotypes and the approximate total number of seeds which resulted from the crosses. The number of seed recovered from each genotype with large numbers of seed was calculated based on an average weight of 100 seed. A large range of seed sizes and weights were observed, and the size classes were fourfold in each hybrid genotype (Figure 1). The seed sizes from the two different genotypes were similar with the largest class (1/13 Round) having slightly more seed than the rest. The weight differences from the largest to the smallest seed class varied by more than 30%. Seed in all of the seed classes had high percentages of germination (Table 3.) Seed size did not influence the germination. Most seed in the smallest seed class (1/17 Round) had wrinkled seed coats and appeared to have abnormal development, but nearly 100% germination was observed for this class and across all seed size classes in both PMN genotypes studied (Table 3).

Mature inflorescences were collected from individual PMN F_1 hybrids in each of the six genotypes. When the panicles were threshed, no seeds were recovered in all of the PMN genotypes indicating that all the F_1 hybrids were sterile (Table 4). These finding supports previous reports of sterility in PMN hybrids by Burton (1942), Burton and Powell (1968), Gonzalez and Hanna (1984), Juahar and Hanna (1998), and Barbosa et al. (2003). Even though members of the A genome from pearl millet and the A' genome from napiergrass pair with one another during metaphase I of meiosis, the seven chromosomes of the B genome of napiergrass do not have anything to pair with, and they are present as seven univalent. These univalent lag behind the A and A' chromosomes during meiosis I and II and often are not incorporated into the resulting gametes with result in sterility (Jauhar, 1981). Jauhar (1968) did observe intragenomic as well as intergenomic pairing of chromosomes where chromosomes from different gene pools paired during meiosis. Despite the latter finding of Jauhar (1968), the former highly predictable behavior of comparable subgenome pairing results in triploid (2n=3x=21) genome formula of AA'B or some combination thereof.

Flow Cytometry

Histograms showing the 2C and 4C peaks for diploid Tift D2A1 pearl millet, Merkeron napiergrass, Tift D2A1 and Merkeron napiergrass, and diploid Tift D2A1 pearl millet and a putative pearl millet x napiergrass hybrid are shown in Figures 2A, 2B, 2C, and 2D, respectively. The 2C and 4C peaks for the pearl millet line (Figure 2A) and Merkeron napiergrass (Figure 2B) are quite evident, and the location of the 4C peaks in relation to the 2C peaks on the X axis are as they should be even though the 4C peaks for

napiergrass are quite broad. When pearl millet and napiergrass were analyzed together there was a single 2C peak. The 4C peak is probably further to the right and is not shown on this histogram. This demonstrates that the two species have similar DNA contents and this agrees with earlier findings (Martel et al. 1997). When the putative PMN hybrids were analyzed with pearl millet as the internal standard, the 2C peaks of both plants were located very closely to one another and they produced a single broad peak (Figure 2D). This indicated that pearl millet and the F_1 PMN hybrids have similar DNA contents, and because of the proximity of 2C peaks, it was not possible to separate them from one another. Consequently, the putative hybrids could not be identified as true hybrids using flow cytometry.

Pearl Millet x Napiergrass Hybrid Verification

From the 58 EST-SSRs surveyed in the bulked segregant analysis, several were heterozygous dominant, and many others were homozygous dominant and hemizygous at the particular loci. Three of the paternal napiergrass parents that are of novel origin contained four markers that were identified to be hemizygous at their particular loci that span across all three of these napiergrass genotypes. Two particular markers occurred in all four genotypes even Merkeron. Five EST-SSRs were found to be novel for PEPU09FL01, PEPU09FL02, and PEPU09FL03. Specifically, of all 58 markers surveyed, seven total hemizygous EST-SSRs were identified for Merkeron, seven for PEPU09FL01, eight for PEPU09FL02, and six for PEPU09FL03. These markers took precedent over any that were heterozygous to remove a portion of error due to segregation in the electrophoresis analysis. Four of the best were chosen for marker-

assisted hybrid verification. An example of one specific marker for hybrid verification is shown in Figure 3. Table 5 shows the overall summary of markers tested across parents and progeny including the ratio of hybrids confirmed. The secondary gene pool in napiergrass was said to form seven univalents during meiosis. The incompatibility of the primary and secondary genomes of *Pennisetum* in these hybrids make analysis straightforward, and analysis of hybrids was done with the assumption that the hemizygous markers identified came from the B genome of napiergrass. This method of hybrid analysis will return a high success rate. Likewise, while scoring these gels many loci were hemizygous or heterozygous dominant in pearl millet. Utilizing those markers could possibly assist breeding efforts for other potential wide hybrids with pearl millet or in the reciprocal hybrid of napiergrass x pearl millet, also known as kinggrass.

CHAPTER V

CONCLUSIONS

Hybridization between pearl millet and napiergrass in a greenhouse setting is possible at College Station, TX. Flowering in napiergrass occurs almost sequentially as the day-length becomes shorter, and pollinations can be made the entire winter when the day-length is less than the required 10 ½ or 11 hrs of day light. The size and weight differences of PMN hybrid seeds play an important part in the goal to expedite a seeded PMN product sometime in the future. Larger seeds make planting and handling easier for farmers, and larger seed would be more likely to break the soil crust as well as persist longer in the field under unfavorable conditions than smaller seed. Additional research is needed to investigate the phenotypic and morphological differences in plants from these different seed classes to determine if there is any consistency or correlation between the two or if it is solely an environmental factor. As has been reported in the literature, these hybrids are completely sterile. This is a favorable characteristic to contain, and along with selection of very late flowering or non-flowering phenotypes a highly desirable 'seeded-yet-sterile' dedicated energy crop is constructed. Flow cytometry is not a valid method to distinguish napiergrass and pearl millet from their interspecific hybrids. The total DNA content of pearl millet and napiergrass were essentially the same even though distinct 2C and 4C peaks were seen from the diploid pearl millet, and it was not possible to make any distinctions between the two much less their hybrids. The use of EST-SSRs is a valid method to confirm hybrids in PMN, and the identification of new microsatellites that are unique to napiergrass in regards to these

particular pearl millet genotypes. The best case scenario would be where these results were ubiquitous across all genotypes of pearl millet as it is almost certain that cultivars and wild accessions of napiergrass contain the same major EST-SSR at the given loci. This method of hybrid verification can be utilized in the reciprocal cross of napiergrass x pearl millet or other wide hybrids with pearl millet as the male parent. The use of these EST-SSRs can be a starting point for DNA sequencing of napiergrass and their use in a marker-assisted breeding program.

REFERENCES

Aljanabi, S. M. and I. Martinez. 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucl. Acids. Res. 25 (22):4692-4693.

Barbosa, S., L.C. Davide, and A.V. Pereira. 2003. Cytogenetics of *Pennisetum purpureum* Schumack x *Pennisetum glaucum* L. hybrids and their parents. Ciênc. Agrotec. 27:26-35.

Bhandari, A.P., Sukanya, D.H., and Ramesh, C.R. 2006. Application of isozyme data in fingerprinting napiergrass (*Pennisetum purpureum* Schum.) for germplasm management. Genet. Resour. Crop Evol. 53: 253–264.

Boddorff, D., and W.R. Ocumpaugh. 1986. Forage quality of pearl millet x napiergrass and dwarf napiergrass. Soil Crop Sci. Soc. Fla. Proc. 45:170-173.

Burton, G.W. 1942. A cytological study of some species in the Tribe *Paniceae*. Am. J. Bot. 29:355-361.

Burton, G.W. 1944. Hybrids between napier grass and cattail millet. J. Hered. 35:227-232.

Burton, G.W. 1989. Registration of 'Merkeron' napiergrass. Crop Sci. 29:1327.

Burton, G.W., and J.B. Powell. 1968. Pearl millet breeding and cytogenetics. Adv. Agron. 20:49-69.

Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P. and van den Belt,

M. 1997. The value of the world's ecosystem services and natural capital. Nature. 387:253-260.

Cox, T.S., L. R. House, and K. J. Frey. 1984. Potential of wild germplasm for increasing yield of grain sorghum. Euphytica. 33(3):673-684.

Dujardin, M. and W.W. Hanna. 1983. Apomictic and sexual pearl millet × *Pennisetum squamulatum* hybrids. J. Hered. 74:277-279.

Dujardin, M. and W.W. Hanna. 1985. Cytology and reproductive behavior of pearl millet-napiergrass hexaploids x *Pennisetum squamulatum* trispecific hybrids. J.Hered. 76:382-384.

Germplasm Resources Information Network (GRIN). National Plant Germplasm System (NPGS). Available at http://www.ars-grin.gov/cgi-bin/npgs/swish/accboth?query=pearl+millet&submit=Search%21&sort=swishrank&si=0&si=1&.cgifields=si&.cgifields=reverse.html. (verified 24 Aug. 2011).

Gonzalez, B. and W.W. Hanna. (1984). Morphological and fertility responses in isogenic triploid and hexaploid pearl millet x napiergrass hybrids. J. Hered. 75:317-318.

Guretzky, J. 2007. Switchgrass establishment requires patience [Online]. Ag News Views. The Samuel Roberts Noble Foundation. Ardmore, OK. Available at http://www.noble.org/Ag/Research/Articles/Switchgrass/index.htl. (verified 30 Aug 2011)

Hanna, W.W. 1982. Apomictic interspecific hybrids between pearl millet and *Pennisetum orientale* L. C. Rich. Crop Sci. 22:857-859.

Harland, J.R. and J.M.T. de Wet. 1971. Toward a rational classification of cultivated plants. Taxon. 20:509-517.

Harris, K., Anderson, W., and R. Malik. 2009. Genetic relationships among napiergrass (*Pennisetum purpureum* Schum.) nursery accessions using AFLP markers. Plant Genet. Resour. 8:63-70.

Holm, C. 2010. ABC Science [Online]. Available at http://www.abc.net.au/science/articles/2010/10/29/3050800.htm?site=science&topic=enviro.com. (verified 31 Aug 2011).

ICRISAT. Crop description of pearl millet [Online]. Available from http://www.icrisat.org/what-we-do/crops/PearlMillet.html. (verified 31 Aug 2011).

Jauhar, P.P. 1981. Cytogenetics and breeding of pearl millet and related species. A.R. Liss, New York.

Jauhar, P.P., and W.W. Hanna. 1998. Cytogenetics and genetics of pearl millet. Adv. Agron. 64:2-21.

Jessup, R.W. Burson, B. L. Burow, G. B. Wang, Y. W. Chang, C. Li, Z. Paterson, A. H. and M.A. Hussey. 2002. Disomic inheritance, suppressed recombination, and allelic interactions govern apospory in buffelgrass as revealed by genome mapping. Crop Sci. 42:1688-1694.

Karlsson, S.B., and I.K. Vasil. 1986. Morphology and ultrastructure of embryogenic cell suspension cultures of *Panicum maximum* (guinea grass) and *Pennisetum purpureum* (napier grass). Am. J. Bot. 73:894-901.

Khanna, M., H. Önal, B. Dhungana, and M. Wander. 2010. Economics of herbaceous energy crops for electricity generation: Implications for greenhouse gas mitigation. Biom. Bioen. 35:1474-1484.

Kort, J., M. Collins, and D. Ditsch. 1998. A review of soil erosion potential associated with biomass crops. Biom. Bioen. 14:351-359.

Lewandowski, I., J.C. Clifton-Brown, J.M.O. Scurlock, and W. Huisman. 2000. Miscanthus: European experience with a novel energy crop. Biom. Bioen. 19:209-227

Lewandowski, I., J. M. O. Scurlock, E. Lindvall, and M. Christou. 2003. The development and current status of perennial rhizomatous grasses as energy crops in the US and Europe. Biom. Bioen. 25:335-361.

Lowe, A.J., W. Thorpe, A. Teake, and J. Hanson. 2003 Characterization of germplasm accession of napiergrass (*Pennisetum purpureum* and *P. purpureum* x *P. glaucum* hybrids) and comparison with farm clones using RAPD. Genet.Resour. Crop Evol. 50: 121–132

Lu, H., J. Zhang, K.B. Liu, N. Wu, Y. Li, K. Zhou, M. Ye, T. Zhang, H. Zhang, X. Yang, L. Shen, D. Xu, and Q. Li. 2009. Earliest domestication of common millet (*Panicum miliaceum*) in East Asia extended to 10,000 years ago. Proc. Natl. Acad. Sci. USA. 106:7367-7372.

Martel, E., D. De Nay, S. Siljak-Yakovlev, S. Brown, and A. Sarr. 1997. Genome size variation and basic chromosome number in pearl millet and fourteen related *Pennisetum* species. J. Hered. 88:139-143.

McLaughlin, S. B. and M. E. Walsh. 1998. Evaluating environmental consequences of producing herbaceous crop for bioenergy. Biom. Bioen.. 14:317-324.

Osgood, R.V., W.W. Hanna, and T.L. Tew. 1997. Hybrid seed production of pearl millet x napiergrass triploid hybrids. Crop Sci. 37:998-999.

Pantulu, J.V. and N.K. Rao. 1982. Cytogenetics of pearl millet. Theor. Appl. Genet. 61:1-17.

Perlack, R.D., L.L. Wright, A.F. Turhollow, R.L. Graham, B.J. Stokes, and D.C. Erbach. 2006. Biomass as a feedstock for a bioenergy and bioproducts industry: The technical feasibility of a billion-ton annual supply. DOE. ORNL. Oak Ridge, TN.

Powell, J.B. and G.W. Burton. 1966. A suggested commercial method of producing an inter-specific hybrid forage in *Pennisetum.* Crop Sci. 6:378-379.

Rao, N.K., L.J. Reddy, and P.J. Bramel. 2003. Potential of wild species for genetic enhancement of some semi-arid food crops. Genet. Resour. Crop Evol. 50:707 – 721.

Renard, G., J. Proud, G. Hanson, M. Mulaa, B. Pallangyo, and B. Kabirizi. 2011. ASARECA [Online]. Available at http://www.asareca.org/index.php?page&as=228 (verified 31 Aug. 2011).

Rodriguez, S., G. Visedo, and C. Zapata. 2001. Detection of errors in dinucleotide repeats typing by nondenaturing electrophoresis. Electrophoresis 22:2656–2664.

Sissine, F. Congressional Research Service. 2007. Energy Independence Security Act 2007: A Summary Major Provisions. Available at: http://energy.senate.gov/public/_files/HR6EnergyBillSummary.pdf.

Smart, A.J., and L.E. Moser, 1999. Switchgrass seedling development as affected by seed size. Agron. J. 91:335-338.

Sobrinho, FD. 2005. Agronomic evaluation of interspecific hybrids of elephant grass and pearl millet. Pesq. AgroPec. Bras. 40:873-880.

Sotomayor-Rios, A., S. Torres-Cardona, W.W. Hanna, and A. Quiles-Belen. 1989. Response of a *Pennisetum* interspecific hybrid and local cultivar 'Millo Blanco', *Sorghum bicolor* L. Moench., to cutting intervals and locations. p. 585-586. *In* Proc. XVI Int. Grassland Congr. 4-11 Oct. 1989. Nice, France.

Stapf, O., and C.E. Hubbard, 1934. *Pennisetum.* p. 954-1070. *In* D. Prain (ed.) Flora of Tropical Africa. Bibliotheca Botanica. London, England.

Stebbins, G.L. 1958. Longevity, habitat, and release of genetic variability in the higher plants. Cold Spring Harbor Symp. Quant. Biol. 23:365-378. Academic Press. New York.

Thomas Jefferson Agricultural Institute. [Online]. Available at http://www.jeffersoninstitute.org/pearl_millet.php. (verified 2 Sep. 2011).

Thompson, J.B. 1919. Napier and merker grasses, two new forage crops for Florida. Bull. Univ. Fla. Agric. Exp. Stn. 317-153. Gainsville.

United States Dep. Agric. (USDA). National Agric. Statistics Service. 2007 Census Agric. Conservation and Agric. Practices [Online]. Available at http://www.agcensus.usda.gov/Publications/2007/Online_Highlights/Fact_Sheets/conservation.pdd.

United States Dep. of Agric. (USDA). USDA and DOE fund 10 research projects to accelerate bioenergy crop production and spur economic impact. Press Release. 11 Aug. 2011, Release No. 0352.11. http://www.nifa.usda.gov/newsroom/newsroom.html.

United Nations (UN). Dep. Econ. Social Affairs. Population Division. Population Estimates Projections Section. World Populations Prospects, the 2010 Revision. 11 Oct. 2010. [Online]. Available at http://esa.un.org/unpd/wpp/index.com.

Wang, D., J. Shi, S. R. Carlson, P. B. Cregan, R. W. Ward, and B. W. Diers. 2003. A low-cost, high-throughput polyacrylamide gel electrophoresis system for genotyping with microsatellite DNA markers. Crop Sci. 43:1828-1832.

Wisconsin Grasslands Bioenergy Network (WGBN). Agric. Eco. Res. Group [Online]. Available at http://www.wgbn.wisc.edu/key-concepts/critical-issues#marginal-lands.com. Dep. of Agron., UW-Madison. (verified 2 Sep. 2011).

Woodard, K.R. and G.M. Prine. 1993. Dry matter accumulation of elephantgrass, energycane, and elephantmillet in a subtropical environment. Crop Sci. 33:818-824.

APPENDIX

Figure 1. Four Seed Size Classes in Two Pearl Millet x Napiergrass Hybrids.

A) TIFT D2 A1 x 'Merkeron' (Classes arranged from 1/13 Round-1/17 Round, and the seed weight per 100 seed were 7.36mg, 3.88mg, 3.02mg, and 2.14mg, respectively.

B) ICMA 89111 x 'Merkeron' (Classes arranged from 1/13 Round-1/17 Round, and the seed weight per 100 seed were 7.78mg, 4.35mg, 3.65mg, and 2.36mg, respectively.

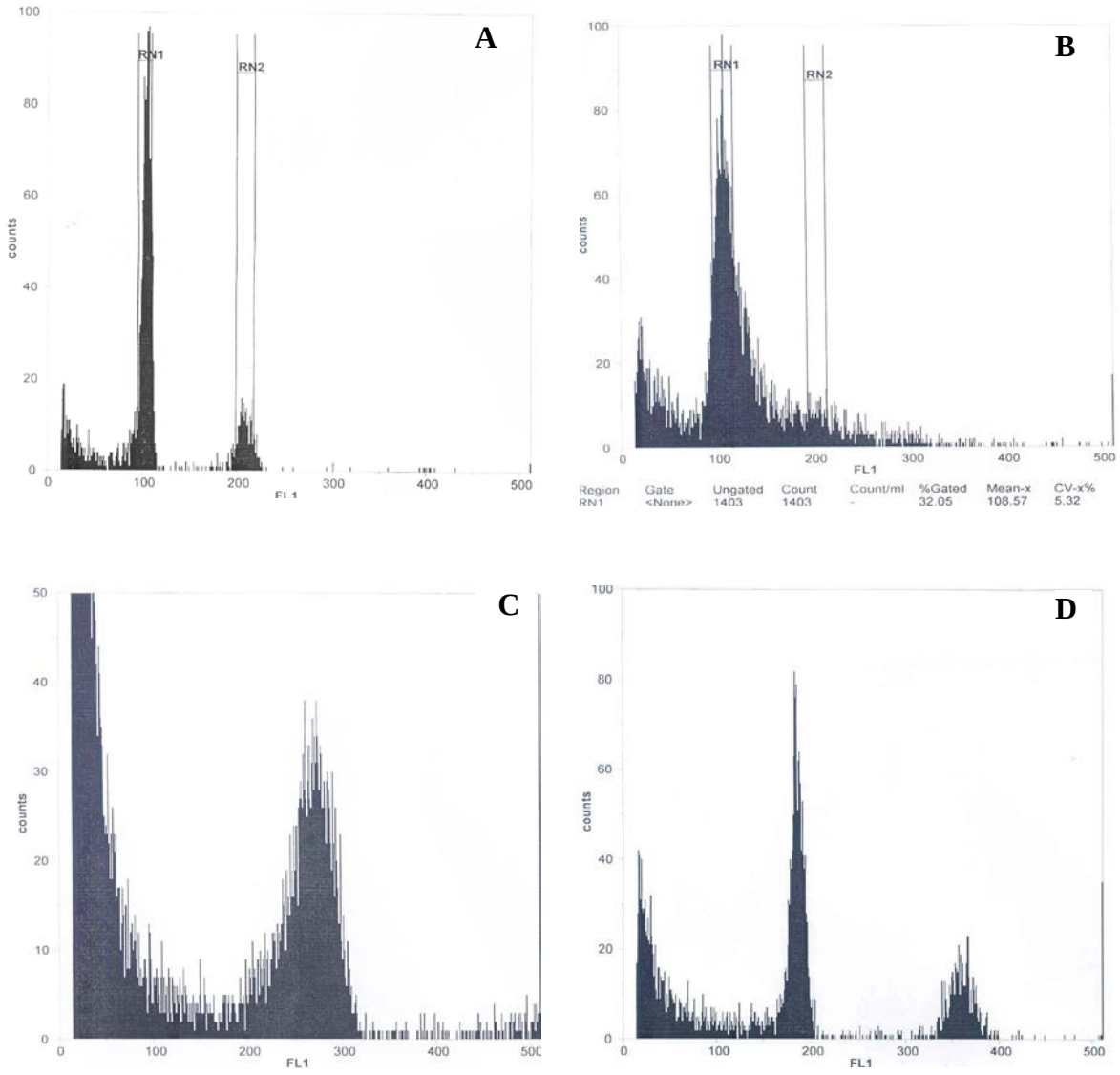

Figure 2. Flow Cytometry Hybrid Verification Histograms.
X axis is the photon intensity associated with DNA content. Y axis is the particle or nuclei count.
A) PEGL 508273
B) Merkeron
C) PEGL 508273 w/ Merkeron
D) PEGL 508273 w/ PMN (508273 x Merkeron)

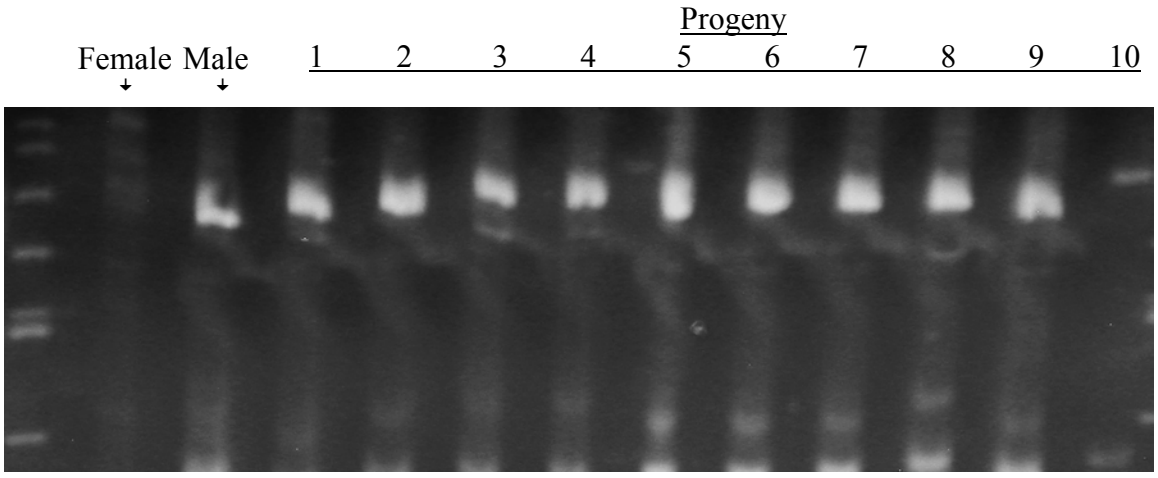

Figure 3. PCR Products of PMN Hybrid PEGL09TX04 x PEPU09FL01 showing 10/10 Hybrids in These Progeny.

Figure 4. Germination Tray of the Four Seed Size Classes for Pearl Millet x Napiergrass Genotype TIFT D2A1 x Merkeron

Table 1. Parental Genotypes.

Species	***ID***	***Source: GPS coordinates/GRIN PI No.***
P. purpureum	PEPU09FL01	(30.66050 N 86.21496 W)
	PEPU09FL02	(29.34651 N 82.21921 W)
	PEPU09FL03	(29.34651 N 82.21921 W)
	'Merkeron'	*(30.54863 N 96.44158 W)*
	TIFT 85D2A1	PI 508273
P. glaucum	ICMA 89111	PI 599192
	ICMA 88006	PI 596507
	TIFT 8677	PI 564585
	No. 467	PI 288787
	No. 44	PI 295167
	PEGL09TX04	(30.54863 N 96.44158 W)

Table 2. Summary of PMN Crosses Made during Winter 2010

Cross	Inflorescences Pollinated	Seed Produced
	No.	
TIFT D2A1 x Merkeron	45	~27,000
TIFT D2A1 x PEPU09FL01	12	200
TIFT D2A1 x PEPU09FL02	5	85
TIFT D2A1 x PEPU09FL03	7	100
ICMA 89111 x Merkeron	35	~14,000
ICMA 89111 x PEPU09FL01	9	175
ICMA 89111 x PEPU09FL02	5	100
ICMA88004 x Merkeron	4	350
PEGL288787 x Merkeron	13	~7,500
TIFT 8677 x Merkeron	8	350
TIFT 8677 x PEPU09FL01	2	100
PEGL295167 x Merkeron	14	~5,700
PEGL09TX04 x Merkeron	6	100
PEGL09TX04 x PEPU09FL01	6	100

Table 3. Percent Germination of Seed Size Classes from Two Pearl Millet x Napiergrass Hybrids

	Seed Size Class			
Genotypes	**1/13**	**1/14**	**1/15**	**1/17**
TIFT D2 A1 x Merkeron	100%	100%	90%	90%
ICMA 89111 x Merkeron	100%	90%	100%	100%

Table 4. Seed Production of Open-Pollinated Pearl Millet x Napiergrass Hybrids

Cross	**Inflorescences**	**Florets**	**Seed**
		No.	
PEGL09TX04 x Merkeron	22	5,531	**0**
ICMA89111 x PEPU09FL01	37	10,475	**0**
PEGL09TX04 x PEPU09FL01	2	863	**0**
TIFT D2 A1 x Merkeron	7	1,951	**0**
ICMA89111 x Merkeron	5	1,435	**0**
TIFT 8677 x PEPU09FL01	15	4,560	**0**

Figure 5. Survey of Napiergrass Specific Expressed Sequence Tags-Simple Sequence Repeats across Pearl Millet x Napiergrass Hybrids

Cross	Hemizygous Paternal-Specific Markers	Hybrids Confirmed/Progeny Tested
	No.	
ICMA89111 x Merkeron	4	10/10
ICMA89111 x PEPU09FL01	4	10/10
PEGL09TX04 x Merkeron	4	9/10
PEGL09TX04 x PEPU09FL01	4	10/10
TIFT D2 A1 x Merkeron	4	10/10
TIFT 8677 x PEPU09FL01	4	10/10

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