DEVELOPMENT OF PCR-BASED TRANSPOSABLE ELEMENT ASSAYS FOR VERIFICATION OF PEARL MILLET-NAPIERGRASS HYBRIDS (*Pennisetum glaucum* [L.] R. BR X *Pennisetum purpureum* SCHUMACH.)

A Thesis

by

CHRISTIAN C. CENTENO

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

MASTER OF SCIENCE

Chair of Committee,	Stephan Hatch
Co – Chair of Committee,	David Stelly
Committee Member,	Russell Jessup

Intercollegiate Faculty Chair, Dirk Hays

August 2017

Major Subject: Molecular and Environmental Plant Sciences

Copyright 2017 Christian C. Centeno

ABSTRACT

Interspecific hybridization can serve a number of purposes in plant breeding. When significant investments are required to produce and/or use hybrids, it is important to accurately differentiate between hybrid and non-hybrid seed or progeny. However, recognition of hybrids versus non-hybrids is problematic for some parental species combinations, e.g., hybrids between pearl millet (*Pennisetum glaucum*, 2n=2*x*=14: AA) and napiergrass (*P. purpureum*, 2n=4x=28: A'A'BB). This cross can be made reciprocally to produce pearl millet-napiergrass ('PMN') hybrid taxa (2n = 3*x* = 21 chromosomes; AA'B genome) from *P. glaucum* x *P. purpureum*, or kinggrass (KG) hybrid taxa (2n = 3*x* =21; AA'B genome) from *P. purpureum* x *P. glaucum*. Identification of these hybrids is reportedly complicated by homeology among parental genomes, similarity of parental C-values, genetic similarity between parental species, morphological similarities and insufficient molecular methods. In this research, we explored hybrid identification through several approaches -- morphological, cytogenetic, flow cytometric and molecular genetics.

Based on ANOVA and *t*-tests after replicated sampling from a genotypic panel that included one *P. glaucum*, two *P. purpureum*, four PMN hybrid taxa and one KG hybrid taxa, the morphological traits -- spikelet primary bristle length, average length of bristles, number of bristles, and length of spikelets -- were found to be insufficient as individual indicators of hybridity and did not allow for the construction of a taxonomic key. In contrast, chromosome number determinations from spreads of root-tip mitotic

cells sufficed to distinguish PMN (2n=21), pearl millet (2n=14), napiergrass (2n=28) types, but the overall procedures were time- and resource-consuming. C-values were determined by flow cytometry but the differences between triploid hybrids and the parental species were non-significant.

This research explored a molecular approach based on the *Tuareg* MITE in *Pennisetum*, which is widely distributed and highly polymorphic in *Pennisetum* genomes. Quantitative differences in PCR-based amplification should be diagnostic of genotype, at least for some *Tuareg* sequence-specific primers. Possible *Tuareg* sequence targets were determined using a sequence-based approach. Candidate sequences were assessed by electrophoretic analysis of PCR amplicons for presence/absence, band number and size distributions. Two markers, *PgTb1* and *Tr54*, were individually assessed in detail. Among the sampled materials, the *PgTb1* provided the most time and cost-efficient method of accurately identifying the interspecific *Pennisetum* hybrid taxa. *PgTb1* was effective at separating the PMN and KG hybrids from both parents in a reliable manner. Tr54 was able to separate the hybrids from the napiergrass mean, and was able to separate the *average* of a group of such hybrids from pearl millet, but due to variation among individual hybrids, Tr54 assays were unable to reliably separate *individual* hybrids from the pearl millet PEGL 09TX04. Thus, *PgTb1* is deemed the superior test marker for hybrid identification.

DEDICATION

This thesis is dedicated to my mom, Pat Lozano-Centeno, without whom I would not have even thought about or gone to college and made it this far. I would also like to thank my fiancé Paloma, thank you for your daily encouragement and sacrifices. Dad and Diana, thank you for answering my phone calls and trying to understand my frustrations. Grandma Centeno, Grandpa and Grandma Lozano, thank you for constantly praying for me and providing me support to eat and live. Grandpa Centeno, I know you are up there watching over me, and this thesis is me paying my dues like you said to do, so that my hard work done now will let me enjoy the fruits of my labor later.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Hatch, co-chair Dr. Stelly and committee member Dr. Jessup for their guidance and support throughout the course of this research. I would also like to thank the late Dr. B Greg Cobb, who taught me everything I know about Endnote™ and formatting my thesis.

Thank you to all my friends and colleagues at Texas A&M University, you all have provided me the support and guidance needed to complete this research.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This research was supervised by Professor and Chair Dr. Stephan Hatch of the Department of Ecosystem Science and Management, Co-Chair and Professor Dr. David Stelly, and Committee Member and Associate Professor Dr. Russell Jessup of the Department of Soil and Crop Sciences for providing access to their labs, reagents, and guidance. Thank you for navigating me through this graduate program and connecting me with colleagues that helped further this research.

I would like to recognize Dr. Nithya Subramanian for helping me learn how to extract DNA using the kit method, undergraduate student lab technician Andre Valenzuela for helping standardize DNA content, Dr. George Hodnett for teaching me how to prepare spreads of root-tip chromosomes, and Dr. Terje Raudsepp for allowing me to use her lab's fluorescent microscope and software for digital imaging. All other work conducted for the thesis was completed independently by Christian C. Centeno.

Funding Sources

The following research was supported by Texas A&M University by the Graduate Diversity Fellowship, and the National Science Foundation, which granted the Bridge to Doctorate Graduate Student Fellowship Award No.: HRD-1406755 Title: "Texas A&M University System Louis Stokes Alliance for Minority Participation (TAMUS LSAMP) Bridge to the Doctorate (BTD) Cohort X (2014-2016) Program; these also provided funding for tuition, fees, and a generous stipend. Thank you Dr. Hatch for nominating me for these fellowships that I was awarded.

NOMENCLATURE

qPCR	Quantitative Polymerase Chain Reaction
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
PMN	Pearl Millet – Napiergrass
PAGE	Polyacrylamide Gel Electrophoresis
MITEs	Miniature-Inverted Repeat Transposable Elements
TEs	Transposable Elements
GISH	Genomic *in situ* Hybridization
PIF	P Instability Factor (PIF)
TIRs	Terminal Inverted Repeats
FISH	Fluorescent *in situ* Hybridization
Sb	*Sorghum bicolor*

TABLE OF CONTENTS

Page

ABSTRACT ii

DEDICATION iv

ACKNOWLEDGEMENTS v

CONTRIBUTORS AND FUNDING SOURCES....vi

NOMENCLATURE.... viii

TABLE OF CONTENTS ix

LIST OF FIGURES....xi

LIST OF TABLES xii

INTRODUCTION....1

LITERATURE REVIEW....3

- Multiple Uses of Crops 3
  - Pennisetum Species 3
  - Hybridization to Improve Crop Usage 4
  - Taxonomy Difficulties of Hybridized Crops....6
- Understanding Transposable Elements 7
  - Discovery of Transposable Elements 7
  - Tb1 Evolutionary Branching 8
  - Tuareg....9
- Developing Molecular Tools for PMN Hybrids 10
  - qPCR 10
  - Root-Tip Smears and Flow Cytometry 11

OBJECTIVES 13

MATERIALS AND METHODS 14

- Morphological Survey....14
- Chromosome Number 15
- Flow Cytometry....17

DNA Extraction and Testing 18
Molecular Survey with Tuareg MITEs 20

RESULTS AND DISCUSSION 25

Morphological Survey 25
Individual Morphology Statistics 25
Group Means Morphology Statistics 30
Chromosome Number and Flow Cytometry 34
Molecular Survey 39

CONCLUSION 48

REFERENCES 49

APPENDIX A 56

APPENDIX B 69

LIST OF FIGURES

Page

Figure 1. Diagrammatic representation of the interspecific crossing scheme used to produce a pearl millet-napiergrass (PMN) hybrid 5

Figure 2. Common evolutionary origins of the *Teosinte-branched1* (*Tb1*) homologs in modern maize, teosinte, pearl millet and wild *Pennisetum glaucum*.............. 9

Figure 3. Mitotic metaphase chromosome spreads from root-tips of pearl millet, napiergrass 'Merkeron', and an interspecific PMN hybrid. 35

Figure 4. Graphic display of qPCR values at cycles 30-60 using the *PbTb1* Taqman assay in pearl millet, napiergrass and a kinggrass interspecific hybrid and several PMN interspecific hybrids. 41

Figure 5. Graphic display of group means for PgTb1 Taqman assay qPCR values for pearl millet (1), napiergrass (2) and their interspecific hybrids (5). 42

Figure 6. Graphic display of qPCR values at cycles 30-60 using the Tr54 Taqman assay in pearl millet, napiergrass and a kinggrass interspecific hybrid and several PMN interspecific hybrids. 43

Figure 7. Graphic display of group means for Tr54 Taqman assay qPCR values for pearl millet (1), napiergrass (2) and their interspecific hybrids (5). 44

LIST OF TABLES

Page

Table 1. Test panel of eight *Pennisetum* genotypes - origin, identification, parentage, ploidy, chromosome number and reproductive status. 15

Table 2. *Tuareg* clones from Genbank used in PCR marker survey, forward and reverse sequences and base pair lengths are annotated in the table. 21

Table 3. Taqman® assay probe and primer sequences for *Tuareg* markers *PgTb1* and Tr54. 23

Table 4. Pairwise comparison *t* values for differences in mean Primary Bristle Length between individual genotypes and corresponding probability values. 27

Table 5. Pairwise comparison *t* values for differences in mean Average Length of Bristles between individual genotypes and corresponding probability values. . 28

Table 6. Pairwise comparison *t* values for differences in mean Number of Bristles between individual genotypes and corresponding probability values. 29

Table 7. Pairwise comparison *t* values for differences in mean Length of Spikelet between individual genotypes and corresponding probability values. 30

Table 8. Pairwise comparison *t* values for differences in mean group Primary Bristle Length group means between individual genotypes and corresponding probability values. 32

Table 9. Pairwise comparison *t* values for differences in mean average Bristle Length group means between individual genotypes and corresponding probability values. 32

Table 10. Pairwise comparison *t* values for differences in mean Number of Bristles group means between individual genotypes and corresponding probability values. 33

Table 11. Pairwise comparison *t* values for differences in mean Length of Spikelet group means between individual genotypes and corresponding probability values. 33

Table 12. Flow cytometric nuclear DNA content ratios (2C) between *Sorghum* and individual *Pennisetum* genotypes. 37

Table 13. Estimated DNA content values (2C) of *Sorghum* and the eight individual *Pennisetum* genotypes. 38

Table 14. ANOVA for DNA content differences among eight *Pennisetum* genotypes. 38

Table 15. Calculation of qPCR mean differences of PMN genotypes from single pearl millet (PEGL09TX04) accession. 45

Table 16. Statistical analysis of qPCR PMN differences from single pearl millet accession. 45

Table 17. Calculation of qPCR mean differences of PMN genotypes from the two napiergrass accessions. 46

Table 18. Statistical analysis of qPCR PMN differences from the two napiergrass accessions. 47

INTRODUCTION

This research addresses the need to have low-cost and time-effective means that differentiate between interspecific hybrid taxa and parental species, as well as among interspecific hybrids. Many herbaria abstain from collecting cultivated crops and interspecific hybrids for accession because herbaria strive to preserve "native and naturalized plants" (BRIT, 2010). The resulting lack of interspecific hybrids in herbaria can make it more difficult to identify hybrids in the field and when keying. In addition, the use of keys based on taxonomic characteristics is generally very time-consuming and tedious, so while "keying" may be a satisfactory secondary method of verification for generating uniform hybrids from the parents, simpler and faster methods would generally be desirable.

Molecular tools such as sequence-based DNA markers typically allow for the differentiation between interspecific hybrids and parental species, and can be especially helpful when parental species contain one or more genomes that are closely homologous. The *objective of this research* is to develop molecular assays that utilize variation of transposable elements content and distribution in pearl millet, napiergrass, and PMN hybrids, and to compare results from the markers to results from more traditional methods, including, morphological traits, chromosome counts and DNA content levels. Hybrid inflorescences will be examined for morphological differences. Root tip chromosome spreads from actively growing samples will be analyzed to determine chromosome number (Staginnus, Huettel et al., 2001; Wipff & Hatch, 1994). DNA

content levels will be determined by flow cytometry. Analysis of transposable elements (TEs) presence and copy number within the genomes of the pearl millet-napiergrass (PMN) hybrids (this includes kinggrass from here on) and the parental species, could allow for rapid demonstration of the differences within and among the genotypic groups.

The *central hypothesis* is a rapid marker assay can be developed and successfully used to show inheritance of TEs from the parents to the hybrids with an inheritance pattern that is easily recognizable by the number of repetitive elements that are quantified by the use of qPCR. This hypothesis and research effort build on results of previous research (Dowling, Burson et al., 2013; Dowling, Burson et al., 2014; Jessup, 2013; Jessup & Dowling, 2015; Remigereau, Robin et al., 2006). Successful quantification of TEs might allow for rapid decision-making for PMN selection in early generation populations. Some miniature inverted repeat transposable elements (MITEs) may be especially useful targets if they occur hybrids but not in all parents or ancestors. Development of an assay for hybridity would enable \breeders and producers to select PMN and their parents for crop uniformity and ecological stability enhancement.

LITERATURE REVIEW

Rapid technological advances have sparked agriculturalists to change the way they implement recommended management practices (Follett, 2001; Lal, 2008; Lemus & Lal, 2005). The need for affordable and efficient identification methods for hybrid crops makes the proposed molecular and vegetative studies important for discerning hybrids from their parents.

Multiple Uses of Crops

Increasing water scarcity, potential risk for climate change, and degradation of agricultural lands further stress the urgency for development of crops with improved resource use efficiency under suboptimal conditions. (Jessup & Dowling, 2015). The potential benefits of novel and under-utilized crops with such resilience can include tremendous effects on sustainability of ecosystems, drought and winter stress tolerance, bioenergy sources, feedstock quality, and improved food security (Jessup & Dowling, 2015; Moose & Mumm, 2008).

Pennisetum Species

Pearl millet is primarily grown in Africa and Southeast Asia, often in environments that have dry and poor soil health, drought and cold temperatures, low photoperiods and rainfall, where it can be used as a grain crop for human consumption, fodder for livestock, and its residual biomass can then be used for fuels (Bogdan, 1977; Moser, Burson et al., 2004). In the United States, South America and Australia, pearl millet is predominantly grown for high quality feed and grazing for livestock (Bogdan, 1977).

Napiergrass has been used historically as a feedstock in Africa, India, Puerto Rico, and small countries in South America for cattle, it has also been used in the Philippines to choke out the invasive species *Imperata cylindrica* (L.) P. Beauv.; a secondary use is grazing, shortly after three months of being planted (Bogdan, 1977). Napiergrass was chosen for biofuels because of its production of high biomass; C_4 nature and ability to withstand temperate conditions; nitrogen fixing ability - a key attribute for maintaining soil health and preventing erosion, and increased water use efficiency; ability to grow in short photoperiods and withstand cold temperatures (Bogdan, 1977; Jessup & Dowling, 2015; Samson, Mani et al., 2005).

Hybridization to Improve Crop Usage

The first of successful hybridization of PMN was recorded by Burton (1944), leading to a triploid 2n = 3x =21. Pearl millet-napiergrass (PMN) (*Pennisetum glaucum* [L.] R. Br (AA genome; 2n=2x=14, common name is pearl millet) x *Pennisetum purpureum* Schumach. (A'A'BB genome; 2n=4x=28, common names are napiergrass and cultivar 'Merkeron'), is a triploid hybrid (2n = 3x = 21; AA'B genome). The reciprocal hybridization is also feasible, with *P. purpureum* x *P. glaucum* referred to as kinggrass (abbreviated as KG; 2n = 3x =21). Sterility of the F1 PMN hybrid was observed after pollinations with napiergrass pollen resulted in no seed (Burton 1944). PMN produces more biomass than the parents but less than KG (Dowling et al., 2014).

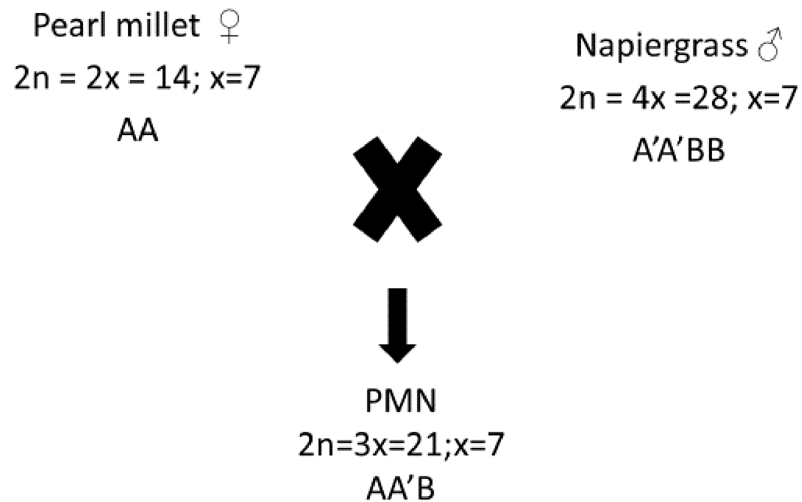


Figure 1. Diagrammatic representation of the interspecific crossing scheme used to produce a pearl millet-napiergrass (PMN) hybrid. Diploid pearl millet is used as the female parent and pollinated with pollen from napiergrass, a tetraploid, to produce the F1 PMN hybrid, a sterile triploid with AA'B genomic constitution. The reciprocal cross results in Kinggrass, which also is a sterile AA'B triploid.

PMN hybrids serve many multidisciplinary purposes. They are used widely as a forage commodity in Africa and India, as biofuel energy and feedstock, and a tool for ecological sustainability for increasing water use efficiency and preventing soil desiccation (Dowling et al., 2013; Dowling et al., 2014; Jessup, 2013; Jessup, Burson et al., 2003; Jessup & Dowling, 2015). The perennial nature of PMN allows for soil erosion control and mitigation, wildlife habitat restoration, carbon sequestration and conservation agricultural practices (Adler, Grosso et al., 2007; Follett, 2001; Lal, 2008; Lemus & Lal, 2005). One reason PMN has so many functions is that it is sterile, which prevents the crop from become excessively invasive. PMN hybrids are grown from large

seed, which are easy to plant, tend to have high biomass (important for its use as a biofuel), and produces a crop at low cost. Plus, they are drought and winter tolerant.

Taxonomy Difficulties of Hybridized Crops

It has been suggested that using classical taxonomic methods for identifying hybrid taxa from their parents is confusing when there is no scientific name or accession for the hybrids (Burke, Tang et al., 2002; Staton, Bakken et al., 2012; Wipff & Hatch, 1994). With advances in molecular plant breeding, hybrids and their counterparts can be difficult to discern, especially when hybrids resemble the parents, and there is a lack of uniform descriptions, formal designation of accessions, and scientific names for hybrid lines (Chapman, 1990). Common names are insufficient resulting in confusion of what is what, and varies by location (Wipff & Hatch, 1994).

Identification of the PMN requires an understanding of plant taxonomy because the parent species are morphologically similar, and they contain similar A or A-like (A’) genomes. Discernible quantitative phenotypic traits can be lacking when comparing hybrids to similar parents within the same genus. A taxonomic method for distinguishing hybrid taxa from their parents has yet to be documented.

Understanding Transposable Elements

Discovery of Transposable Elements

TEs play major roles in plant development, genetic structure, and evolution (Lönnig & Saedler, 2002; Staginnus et al., 2001). Maize cytogeneticist Barbara McClintock, a Nobel laureate, discovered “controlling elements”, now known as “transposable elements”, that have or can have the ability to move or induce the movement of certain other elements (MacRae, Learn et al., 1990; McClintock, 1947). She was able to deduce that their movement was associated with and caused discrete phenotypic changes in maize and has since been extended in observation and theory to hybrids and the evolution of species (Lönnig & Saedler, 2002; McClintock, 1993). Transposable elements which are repetitive elements or sequences were subsequently found to constitute a type of repetitive sequence (McClintock, 1947). The first TE to be described was in maize by McClintock; it was an activator (*Ac*) sequence that is autonomous (MacRae et al., 1990). *Ac* falls into the Class II transposons because it relies on a copy-paste mechanism DNA; its sequences include terminal inverted repeats (TIRs) that enable autonomous behavior (Staginnus et al., 2001). *Ac* sequences have been found in pearl millet (MacRae et al., 1990). Miniature-inverted repeat transposable elements (MITEs) are derived from Class II transposons and also contain TIRs, but MITES are smaller (< 600 bp) than conventional TEs; thousands of copies of MITES exist in a typical genome, and are thought to play major roles in the evolution genome structure and genetic regulation (Staginnus et al., 2001). MITEs are separated into two categories based on movement within the genome. One of these, the *Tourist*-like MITES

(PIF/Harbinger/Tourist) use PONG and PIF transposases (Remigereau et al., 2006; Staginnus et al., 2001) and is relevant to this *Pennisetum* project.

Tb1 Evolutionary Branching

The *Teosinte-branched1* (*Tb1*) gene was first recognized as a spontaneous recessive mutant in maize, and a mutation at this locus was later found to be among the five or so key domestication-related traits differing between maize and its wild relative teosinte (Doebley, Stec et al., 1995). Subsequent analysis related enhanced expression of *Tb1* was tied to evolutionary insertion of a transposable element in the promoter region (Studer, Zhao et al., 2011). One of many descendent studies on maize *Tb1* and its homologs involved pearl millet, and led to the discovery of the *Tuareg* MITE family in pearl millet (Dussert, Remigereau et al., 2013; Remigereau et al., 2006).

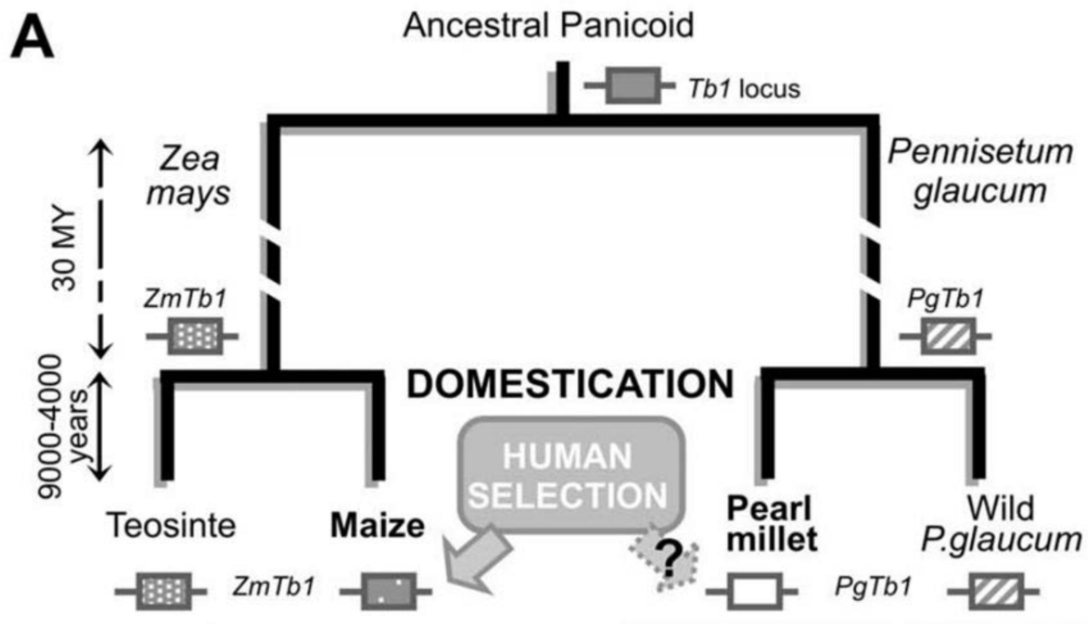


Figure 2. Common evolutionary origins of the *Teosinte-branched1* (*Tb1*) homologs in modern maize, teosinte, pearl millet and wild *Pennisetum glaucum.* The diagram depicts evolutionary branching of an ancestral *Tb1* of an Panicoid ancestor into *Zea* (maize) and *Pennisetum* (pearl millet) lineages (Remigereau, Lakis et al., 2011).

Tuareg

Tuareg is a MITE is related to the PIF family in maize and occurs in pearl millet, (Remigereau et al., 2006). TEs were reported in *Pennisetum ciliare* (L.) Link, when studies on allelic interactions led researchers to focus on understanding the nature of TEs in *Pennisetum* (Akiyama, Conner et al., 2004; Ozias-Akins, Akiyama et al., 2003). *Tuareg* MITEs were chosen for this research because of their close relationship to pearl millet.

Developing Molecular Tools for PMN Hybrids

The ability to customize probes and expedite analysis of large amounts of data using computers has increased diversity and numbers of variables (genes, transcripts, etc.) that can be detected and potentially used for discerning parent species from interspecific hybrids. Analysis by qPCR is normally used to reveal quantitative differences in DNA copy number, and might be used at a tool to differentiate more effectively among genotypes and aid in their identification, in place of or in addition to traditional vegetative keying and other pre-molecular methods. A number of molecular methods, including general PCR, GISH and FISH have been used to identify TEs in *Pennisetum* (Dos Reis, Mesquita et al., 2014; Dussert et al., 2013; MacRae et al., 1990; Ozias-Akins et al., 2003). Previous work using genomic *in situ* hybridization (GISH) was able to discern the A, A' and B genomes and compare the size of chromosomes to each of the genomes studied, it was also concluded that the A and A' genomes are ancestral in origin (Dos Reis et al., 2014). Molecular methods such as PCR, qPCR, FISH and GISH might be used to identify transposable elements that have sufficiently marked differences in distributions among the PMN/KG parental species and interspecific hybrids to be employed as a diagnostic classification tool (Dos Reis et al., 2014; Dowling et al., 2013; Huttley, MacRae et al., 1995; Staginnus et al., 2001)

qPCR

Transposable elements have been little used for marker-assisted selection in plant breeding programs. Specific TEs might be used to differentiate between interspecific hybrids and parental species that differ in presence/absence or copy number. In such

cases, methods such as qPCR could be used to develop TE-based surveys that delineate hybrids and parents. Taqman®, molecular beacons and other probes can be constructed based on genomic DNA sequences (Bonnet, Tyagi et al., 1999; Chandra-Shekara, Pegadaraju et al., 2010; Hwang, Seo et al., 2004; Terzi, Infascelli et al., 2004; Tyagi & Kramer, 1996). Using *PgTb1* and *Tr54* to distinguish these PMN hybrid taxa from their parents by analyzing their abundance and distributions has yet to be done,.

Root-Tip Smears and Flow Cytometry

Cytological methods such as chromosome counting, karyotyping, meiotic pairing analysis can be used to understand chromosome and genome biology/behavior (Burke et al., 2002; Staginnus et al., 2001; Wipff & Hatch, 1994). Although root-tip chromosome spreads preparation can be tedious, it is cost-effective and in some situations, reveals taxonomic and genetically important information.

The aim of this study is to compare morphological, cytogenetic, and one or more molecular methods for wide hybrid verification within *Pennisetum* and determine which one is most effective. Specifically, pearl millet-napiergrass hybrids derived from parental species pearl millet and napiergrass will be evaluated, along with the parental species pearl millet and napiergrass.

There is thus a significant need to distinguish PMN hybrids from their parental species but no cytogenetic method reported to date is both effective and practical to use on a large scale. Morphological traits of parents and hybrids overlap significantly, and near equivalency of parental nuclear genome sizes essentially prevents discrimination by means of flow cytometry (Martel, De Nay et al., 1997). Cytological counting can be

used to distinguish these hybrids from parents but is time consuming (Burton, 1944). Flow cytometry has failed to be a useful tool in identifying PMN from the parents due to the fact that in spite of two-fold differences in ploidy level and chromosome number, these species have similar “C values”, i.e., DNA content per unreplicated haploid nucleus (Dos Reis et al., 2014; Dowling et al., 2013; Martel et al., 1997; Techio, Davide et al., 2010).

OBJECTIVES

The following approaches will be evaluated for their ability to discern between the interspecific hybrid taxa and the parents:

Objective 1: Compare and contrast vegetative taxonomic differences and develop a key for based on morphological traits, especially length of bristles, length of primary bristle, lengths of florets per spikelet.

Objective 2: Use of root-tip chromosome spreads from actively growing specimens to look at chromosome number at mitotic metaphase-I.

Objective 3: Utilization of flow cytometry on genotypes collected to evaluate DNA content.

Objective 4: Development and use of a qPCR marker survey to test presence and copy number of TEs.

MATERIALS AND METHODS

Morphological Survey

Morphological keying was done with the use of a stereo microscope, needle-nose forceps, dissecting needle and metric ruler (Walters & Keil, 1996). Data was collected from the spikelets, including primary bristle length, average length of bristles, number of bristles, and length of spikelet. Five spikelets randomly selected from each genotype were chosen for measurement, all of which were taken in millimeters. For "primary bristle length", the primary bristle of each spikelet was measured five times per genotype. For "average length of bristles", lengths were measured for four randomly chosen bristles, then averaged to produce one average value per spikelet. For "number of bristles" (per spikelet), all of its bristles of a given spikelet were counted, excluding the primary bristle, and this was done five to six times per sample depending on number of florets per spikelet. For "length of spikelet", the length of each spikelet was measured from the pedicle to the apex of the lemma and palea. In the cases where two florets existed per spikelet and the second the respective feature(s), the measurements for the first floret were used. Measurements were recorded in Microsoft Excel then imported into JASP to determine significance between genotypes and genotypic groups. To judge statistical significance, a 95% confidence interval was used in ANOVA and t-tests. Pair-wise tables of t-tests were constructed to facilitate statistical overview and determine if there might be sufficient basis to support developing a "key" based off the morphological traits between genotypes.

Plant materials utilized in this study included one pearl millet, two napiergrass and four PMN hybrids, one KG hybrid, i.e., five interspecific hybrids, overall (Table 1).

Table 1. Test panel of eight *Pennisetum* genotypes - origin, identification, parentage, ploidy, chromosome number and reproductive status.

Origin	Genotype identification	Maternal parent	Paternal parent	Ploidy and chromosome number (2n)	Reproduction
P. glaucum x *P. purpureum*	PMNV13TX01	PEGL 09TX04	PEPU ('MERKERON')	3x = 21	Sterile
P. glaucum x *P. purpureum*	PMNV13TX06	PEGL 09TX04	PEPU ('MERKERON')	3x = 21	Sterile
P. glaucum x *P. purpureum*	PMNV14TX03	PEGL 09TX04	PEPU ('MERKERON')	3x = 21	Sterile
P. purpureum x P. glaucum	PMNV14TX14	PI 508273	PEPU ('MERKERON')	3x = 21	Sterile
P. purpureum x P. glaucum	KGV13TX01	PEPU 09TX01	PEGL 09TX04	3x = 21	Sterile
P. glaucum	PEGL 09TX04	N/A	N/A	2x = 14	Sexual
P. purpureum	PEPU 09TX01	N/A	N/A	4x = 28	Sexual
P. purpureum	MERKERON	N/A	N/A	4x = 28	Sexual

Chromosome Number

Fresh actively growing roots were collected and prepped and viewed at mitotic metaphase-I to visualize the chromosomes (Staginnus et al., 2001; Wipff & Hatch, 1994). The protocol for root-tip chromosome spreads was by Hodnett (2016). Making root-tip chromosome spreads was a two-day process, this was done for all genotypes tested. On day 1 root-tips were harvested between 9 am and 11 am, then, submerged in alpha bromo naphthalene for 45 minutes, then the alpha bromo naphthalene was removed and replaced with 3:1 (ethanol:acetic acid) fixative overnight (about 12 hours).

On day 2 water bath to 37°C the fixative was removed and replaced with ddH_2O, then replaced with ddH_2O every 15 minutes, 3 times. Using a scapula and stereomicroscope, the opaque portion of the root tip was excised, as it is the region of highest mitotic index. Root tips were then placed in different microfuge tubes according to relative root size, then 0.2 M HCl was added and incubated for 30 minutes in a hot-water bath (37°C). Using a glass mini-pipette, the HCl carefully was removed and the root tips were gently washed with distilled water multiple times for a total duration of 15 minutes. Using the glass mini-pipette the water was removed and an enzyme mixture (30% cellulose, 15% pectinase) was added to each microtubes, which was then incubated in a warm water bath (37°C) for 30-40 minutes. The duration of incubation depended on size of the root tips: thicker root tips were incubated for 40 minutes and thinner root tips for 30 minutes. After the enzymatic hydrolysis and incubation, the samples of root tips were carefully washed with distilled water 3 to 4 times to remove excess enzyme.

For chromosome preparations, a root tip was individually placed on a sterile glass slide, and then 1 drop of freshly mixed 3:1 ethanol:acetic acid was added to the root- tip, which was , immediately macerated using needle nose forceps; the slide was air-dried. Using a phase contrast microscope, slides were scanned for chromosome spreads, and if present, those slides were stained. Slides of chromosome spreads were stained according to methods developed by the Stelly Lab (Crane, Price et al., 1993; Halfmann, Stelly et al., 2007) using a coplin staining jar containing Azure B (0.2% Azure B in 0.1 M ph7 phosphate buffer), each slides was immersed in the staining solution for 30 seconds, then washed by them briefly under a running water faucet to

remove the remaining stain, and the air dried using the exhaust outlet from a laboratory air compressor.

Digital images of the root-tip chromosome spreads were visualized on a Zeiss Axioplan2 fluorescence microscope at 1000x magnification while using oil immersion optics, and images were captured using Ikaros V 5.2.23 by MetaSystems GmbH, then exported as .TIF files.

Flow Cytometry

Nuclear DNA content values were determined for all entries from leaf materials. Leaf samples were macerated in Galbraith's buffer, and incubated with propidium iodide, and visualization through a Partec CyFlow flow cytometer to analyze 2 C peaks (normal DNA content) between the parents as previously described (Dowling et al., 2013). All eight genotypes were tested individually, with *Sorghum bicolor* serving as the reference standard across samples because its genome was significantly different in genome size from the research materials and its genome has been successfully annotated (Paterson, Bowers et al., 2009). Diploid *Sorghum bicolor* (2C 1.67 pg, 818 Mb 1C) accession (BTX623) was tested individually and used as the standard. Histogram peaks were gated at (*i*) 2C, (*ii*) 4C and (*iii*) 8C, representing (*i*) diploid unreplicated nuclei, (*ii*) diploid replicated nuclei, plus endoreduplicated unreplicated nuclei, and (*iii*) endoreduplicated replicated nulcei, respectively. Each entry was evaluated separately and in combination with the *Sorghum* standard (Price, Dillon et al., 2005).

DNA Extraction and Testing

DNA was extracted using Macherey-Nagel NucleoSpin® Plant II kits (Ref 7407770.50 Lot 1601/002) and following the Macherey-Nagle Genomic DNA from Plant User Manual. Wet leaf tissue was collected from all eight genotypes, then cell lysis was performed using the Buffer PL2 option. Deviations from the manufacture's protocol included the following. For initial extractions, (1) 100g of fresh plant leaf material was collected; (2) the mid rib was discarded and not included in the extraction process; (3) tissue was homogenized using steel beads shaken at 4.0 m/s for 20 seconds on a general lab shaker. In subsequent DNA extractions, (1) 100g of wet weight plant leaf material without the mid rib was collected, (2) tissue was frozen with liquid nitrogen, (3) tissue was homogenized using a mortar and pestle. For all extractions, (4) samples were centrifuged (Step 3) for 15 minutes at 11,000 rpm.

DNA concentrations were quantified in two separate labs with two different spectrophotometers - a DeNovix DS-11 Spectrophotometer and an Eppendorf Biophotometer Spectrophotometer UV/VIS. Readings were taken from DeNovix DS-11 Spectrophotometer as ng/uL, then samples were diluted using ddH_2O water to 50 ng/uL to make DNA stock solutions. Readings were taken from the Eppendorf Biophotometer Spectrophotometer UV/VIS according to the protocol listed in Burson (2014), where stock DNA was not diluted, and a blank cuvette with 50 uL of ddH_2O was used to "zero out" the spectrophotometer. The cuvette was then flushed with ddH_2O, then an empty spray bottle was used to dry the cuvette, after which 50 uL of DNA solution was added to the cuvette. After the reading the DNA concentration, DNA was pipetted back into its

respective centrifuge tube, this was repeated for each genotype tested. DNA concentrations measured on the Eppendorf Biophotometer were read as ug/mL, and were diluted to 50 ng/uL with deionized water to make DNA stock solutions.

The quality of each DNA extract was also assessed by PCR. The DNA extract from each genotypes was used a template for PCR using primers that amplify two previously reported EST-SSR markers, PCAR 19 and PCAR 33 (Burton, 1944; Dowling et al., 2013). In these tests, i.e. observing the expected ampliconic bands in the electrophoretic gels would indicate if the extracts sufficed for PCR-based amplification and subsequent use for Taqman® assays.

The PCR marker (SSR) survey was followed the protocol of Dowling et al. (2013). Changes made to the protocol included the following: DNA of each sample was added to the individual wells instead of directly to the master mix. Two replicates of ten reactions were run for each EST-SSR marker, where each set of 10 included eight genotypes, a reference genotype 'Merkeron' and a non-template control. Thus, a total of 20 reactions were run per marker.

The master mix for 20 reactions was as follows: ddH_2O 236 uL, $MgCl_2$ 40 uL, Buffer manufactured by Promega M190G 19933532 Thermophillic DNA 10X Buffer 40 uL, dNTPs 20 uL, Taq GenScript 5U/uL 1000 U Cat. No. E00007 4 uL, left primer for PCAR marker 20 uL, right primer for PCAR marker 20 uL. The volume of DNA extract in each assay differed among sample types: 1 uL of DNA from the hybrids; 2.5 uL of DNA from species samples - PEGL 09TX04, PEPU 09TX01 and 'Merkeron'. The PCR marker survey with SSR markers PCAR 19 and 33 EST-SSRs was used to confirm

hybridization between the parents (Dowling et al., 2013) by visualizing in polyacrylamide gels the electrophoretically separated fluorescent bands that corresponded to the ampliconic SSRs.

Molecular Survey with Tuareg MITEs

Tuareg MITEs clone DNA oligonucleotide primers were made using Genbank® accessions and be produced by Eurofins Genomics (Table 2). The oligios manufactured by Eurofins Genomics were standardized as per the product order form so that the experiment had balanced reagents. The PCAR 19 and 30 EST-SSRs developed by Dowling was used to confirm homeology between the parents (Dowling et al., 2013). Testing the *Tuareg* MITEs clones was deemed financially valuable due to the cost of ordering Taqman® probes. Gels gave a visual image of clones that shared homeology by fluorescing at a single location, the clones that shared that homeology had a high probability for being compatible and present in this selection of genotypes.

Table 2. *Tuareg* clones from Genbank used in PCR marker survey, forward and reverse sequences and base pair lengths are annotated in the table.

Tuareg Clone	Primer	Product bp	GenBank® Accession ID
PgTb1-F	GGTGCTCATCAACCCTCAAC	140	DQ190504.1
PgTb1-R	GCGATGACCAAACCAAGTTCA		
ReB2N28-F	GCTTCTCCACTGGCTTTTACA	150	DQ190505.1
ReB2N28-R	GTCACATGTGGGGAAGAAGC		
Tr34-F	CCTGCCAAACCCCTAACAAA	153	DQ190506.1
Tr34-R	GCAAAATGGCTGTGGTTGTG		
Tr7-F	GCTTCTCCACTGGCTTTTACA	172	DQ190508.1
Tr7-R	GCAAAATGGCTGTGGTTGTG		
Re296-F	GGTGGCAGAGCTTTTCCAAA	191	DQ190513.1
Re296-R	GCTGTGGTTGTGGCTTCTAC		
Tr54-F	GCCCTGCCAAAACCCTAAAA	220	DQ190516.1
Tr54-R	CAGCTGTGACTGAGGCTACT		
Tr9-F	GCTTCTTCACTGGCTTCTACA	150	DQ190523.1
Tr9-R	GTCACATGTGGGGAAGAAGC		
Tr30-F	CCCTGCCAAACACCTAACAA	226	DQ190530.1
Tr30-R	GGTTGTGGCTGCAATGGTAA		
Tr39-F	TCTCGAGGTTTCTTCCCCAC	153	DQ190534.1
Tr39-R	CTGTGGTTGTGGCTGAAACA		
ZmTr33-F	TGATCCTCCCCTCAATCCCT	155	DQ191787.1
ZmTr33-R	GGCTTTAGTTCTGGCTCACG		

Tuareg sequences were identified using the accessions provided by GenBank® (Remigereau et al., 2006). *Tuareg* sequences from pearl millet were downloaded from GenBank® (National Center for Biotechnological Information; http://www.ncbi.nlm.nih.gov/Genbank/index.html), and a subset of nine were arbitrarily selected. Primers for *PgTb1* were as described in Remigereau et al., 2006. Primer design the nine additional *Tuareg* selections was performed utilizing Primer3web

version 4.0.0 (http://bioinfo.ut.ee/primer3/). Conditions were 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 polymerase chain reaction (PCR) product range of 100 - 400 base pairs (bp) in length; then purchased by Eurofins Genomics.

PCR was used to test suitability of primer pairs for individual *Tuareg* MITE-associated sequences, and to test the quality of stock DNA from each of the eight genotypes. The *Tuareg* MITEs survey protocol was identical as the PCR marker survey except tested primers were used as a size reference listed in Table 2. A 50 bp DNA ladder was used as a reference and placed before *PgTb1*, ReB2N2, Tr7, Tr54, and Tr9. *Tuareg* MITEs tested were *PgTb1*, ReB2N2, Tr34, Tr7, Re296, Tr54, Tr9, Tr30, Tr39, and Zm39.

Taqman® probes were designed for PCR-selected *Tuareg* sequences by loading their GenBank sequences into Beacon Designer 8.0, opening a Taqman® probe search window and adjusting the length (bp) of each sequence until the quality of the search indicated 'Best-Standard' or 'Good-Standard' (Table 3). The length for the *Tuareg* sequences: *PgTb1* 18-25 bp and Tr54 16-25 bp long.

Table 3. Taqman® assay probe and primer sequences for *Tuareg* markers *PgTb1* and Tr54.

	Forward	Reverse	Probe
PgTb1	GGCTTTTCCAAGTGCTTC	GTGGCTAAAACGGCTG	TTTACAGAAGCCCTGCCAAACC
Tr54	CTACGGAGGTGAATCC	GTGACTGAGGCTACTC	TAGTGGCTTCCTGCGGCTTC

The MxPro 3005P qPCR instrument by Agilent Genomics was used to perform qPCR, and the output data were utilized by the MxPro software. A 100-fold DNA dilution series (1 ng, 100 pg, 50 pg, to 10 pg) was assessed to optimize the amount of template DNA needed for each Taqman® assay. The qPCR reagents included the Brilliant III Ultra Fast QPCR Master Mix by Agilent Technologies CAT#600880 and the Taqman® Gene Expression Assays by Applied Biosystems, purchased via Thermo Fisher Scientific. Master mix and thermal profile for qPCR mostly followed the protocol given by Agilent Technologies (2015). Changes made to the protocol included the following: using 15 uL of 2X QPCR Master Mix; 1 uL of DNA from 1:50 dilution of stock DNA when testing *PgTb1*, or 1 uL of DNA from 1:100 dilution of stock DNA when testing Tr54; 4 uL each of the forward and reverse primers; and 1 uL of Taqman® probe. The master mix for one probe and 18 reaction included 72 uL forward primer, 72 uL reverse primer, 270 uL 2X Master Mix, 18 uL Taqman® probe, 90 uL ddH_2O, 5.4 uL passive reference dye (already diluted, 1 uL reference dye : 499 uL ddH_2O), ROX and FAM were selected in the software. DNA was thawed for 1 hour and reagents were thawed for 15 mins before using them for the master mix. Once DNA samples were completely thawed, each microtube was gently flicked before 1 uL aliquots were loaded into plate wells. Primers and Taqman® probes were thawed out on the styrofoam edge

of an ice box while waiting to be added to the master mix. After the 1 uL aliquots of sample DNA were loaded, 29.3 uL of the master mix was added to each well. The plate was sealed with a clear adhesive, and then centrifuges on a Beckman Coulter Allegra™ 6R Centrifuge at ~340 rpm. Centrifuged PCR plates were moved to a cold block until inserted into the qPCR machine. Setting up the PCR plate in the qPCR machine followed the protocol by Agilent Technologies (2009, 2015), with the following changes: under the Amplification Segment, Fast 2 Step was changed to Normal 2 Step; Segment 2 was changed to 60 cycles instead of 40 cycles, and instead of only pre-selecting to capture just "End points" at the end of each cycle at Segment 2, it was changed to that "All points" were collected at the end of each cycle in Segment 2. Data were exported to Microsoft Excel 2016, and then imported into JASP 0.8.0.1 for statistical analysis.

RESULTS AND DISCUSSION

Morphological Survey

Data collected from the spikelets included primary bristle length, average length of bristles, number of bristles, and length of spikelets. Measurements were recorded in Microsoft Excel then imported into JASP to determine significance between genotypes. Statistical analysis of morphological traits between the genotypes did not have consistent results to build a taxonomic key that would be accurate with future samples.

Data collection from the spikelets is to be very tedious. Statistical analysis included the means of the hybrid genotypes combined, the napiergrass genotypes combined, and the individual pearl millet sample, the following is annotated in the results as GROUP MEANS. Data from each genotype sampled were annotated in Microsoft Excel under the morphological characteristic that was surveyed. Statistical tests for morphological tests performed in JASP, including ANOVA and paired t-tests.

Individual Morphology Statistics

Individual morphology statistics suggest that it is difficult to find a significant trait that varies across all samples. For primary bristle length (Table 4), ANOVA test and t-test did not prove significant. For average length of bristles (Table 5), ANOVA did not show significance, and the t-test only showed significance (<.001), only between PEGL 09TX04 and PEPU 09TX01. For number of bristles (Table 6), the ANOVA and t-test did not prove significant. For length of spikelet (Table7), the ANOVA and t-test did were non-significant. Table 5 allows for quick pair-wise comparisons of means between individual genotypes. Differences between the parents (PEGL 09TX04, PEPU 09TX01,

MERKERON) and the hybrids were low and insufficient to develop a key. Table 6 allows for quick pair-wise comparisons of individual genotypes for differences in mean Number of Bristles. Differences between the parents (PEGL 09TX04, PEPU 09TX01, MERKERON) and the hybrids were mostly insignificant and collectively insufficient to develop a key. Table 7. there was not enough significance between the parents (PEGL 09TX04, PEPU 09TX01, MERKERON) and the hybrids; for there to be enough significance to develop a key, the parental columns would have asterisks denoting significance in each of the hybrid(s) row.

Table 4. Pairwise comparison *t* values for differences in mean Primary Bristle Length between individual genotypes and corresponding probability values.

t/p	PEGL 09TX04	PEPU 09TX01	MERKERON	PMNV 13TX01	PMNV 13TX06	PMNV 14TX03	PMNV 14TX14
PEGL 09TX04							
PEPU 09TX01	-2.734[&]						
	0.052*						
MERKERON	-3.833	0					
	0.019**	1					
PMNV 13TX01	-0.757	0.764	1.021				
	0.491	0.487	0.365				
PMNV 13TX06	-4.546	-0.586	-4.811	-1.626			
	0.01**	0.589	0.009**	0.179			
PMNV 14TX03	-3.023	0.723	1.375	-0.564	3.323		
	0.039	0.51	0.241	0.603	0.029**		
PMNV 14TX14	1	2.804	3.985	1.076	4.859	4.162	
	0.374	0.049*	0.016**	0.342	0.008**	0.014**	
KGV 13TX01	-4.512	-1.486	-3.942	-3.53	-3.47	-5.685	-5.241
	0.011*	0.211	0.017**	0.024**	0.026*	0.005**	0.006**

[&] Data in the table were consolidated from pair-wise comparisons between means and t-tests.
* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Table 5. Pairwise comparison *t* values for differences in mean Average Length of Bristles between individual genotypes and corresponding probability values

t/p	PEGL 09TX04	PEPU 09TX01	MERKERON	PMNV 13TX01	PMNV 13TX06	PMNV 14TX03	PMNV 14TX14
PEGL 09TX04							
PEPU 09TX01	-9.122[&]						
	<0.001***						
MERKERON	-5.789	-0.707					
	0.004**	0.519					
PMNV 13TX01	-4.225	-2.058	-1.688				
	0.013**	0.109	0.167				
PMNV 13TX06	-5.658	1	1.136	2.211			
	0.005**	0.347	0.319	0.092*			
PMNV 14TX03	-7.303	0.946	0.958	2.598	0.452		
	0.002**	0.398	0.392	0.06*	0.675		
PMNV 14TX14	-31.027	-1.089	-0.294	1.96	-1.341	-1.933	
	<0.001***	0.338	0.783	0.121	0.251	0.125	
KGV 13TX01	-0.284	2.818	3.256	2.692	3.011	2.605	2.96
	0.79	0.048	0.031**	0.055*	0.04	0.06*	0.042

[&] Data in the table were consolidated from pair-wise comparisons between means and t-tests.
* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Table 6. Pairwise comparison *t* values for differences in mean Number of Bristles between individual genotypes and corresponding probability values.

t/p	PEGL 09TX04	PEPU 09TX01	MERKERON	PMNV 13TX01	PMNV 13TX06	PMNV 14TX03	PMNV 14TX14
PEGL 09TX04							
PEPU 09TX01	5.895[&]						
	0.002**						
MERKERON	5.193	-0.394					
	0.007**	0.735					
PMNV 13TX01	-0.679	-4.351	-3.086				
	0.534	0.012**	0.037*				
PMNV 13TX06	0.827	-2.481	0.314	1.661			
	0.455	0.068	0.769	0.172			
PMNV 14TX03	7.443	-0.335	0.314	8.358	3.231		
	0.002**	0.754	0.769	0.001***	0.032*		
PMNV 14TX14	1.718	-3.942	-1.307	1.719	0.483	-1.705	
	0.161	0.017**	0.261	0.161	0.654	0.163	
KGV 13TX01	-2.453	-5.929	-6.402	-2.331	-11.978	-6.714	-3.154
	0.07	0.004**	0.003**	0.08	<0.001***	0.003**	0.034*

[&] Data in the table were consolidated from pair-wise comparisons between means and t-tests.
* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Table 7. Pairwise comparison *t* values for differences in mean Length of Spikelet between individual genotypes and corresponding probability values The chart allows for quick comparisons of individual genotypes against each other. The t value is listed above the p-value.

t/p	PEGL 09TX04	PEPU 09TX01	MERKERON	PMNV 13TX01	PMNV 13TX06	PMNV 14TX03	PMNV 14TX14
PEGL 09TX04							
PEPU 09TX01	-2.092[&]						
	0.105						
MERKERON	-3.9	-1.425					
	0.018*	0.227					
PMNV 13TX01	-0.356	0.461	1.769				
	0.74	0.669	0.152				
PMNV 13TX06	-2.86	-0.967	1.633	-1.58			
	0.046*	0.388	0.178	0.189			
PMNV 14TX03	-3.055	-0.953	2.449	-1.491	0.535		
	0.038*	0.394	0.07	0.21	0.621		
PMNV 14TX14	0.688	2.138	3.814	0.704	2.794	3.47	
	0.529	0.099	0.019*	0.521	0.049*	0.026*	
KGV 13TX01	-2.228	-0.514	3.162	-1.281	2.449	1.633	-2.449
	0.09	0.634	0.034*	0.269	0.07	0.178	0.07

[&] Data in the table were consolidated from pair-wise comparisons between means and t-tests.
* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Group Means Morphology Statistics

The group means morphology statistics and statistical tests in Tables 8-11 indicate that none of the sampled traits differs significantly between the parent species versus the hybrids and is relatively uniform among the hybrids. Primary bristle length ANOVA and t-test were nonsignificant. Average length of bristles ANOVA and t-test were nonsignificant. ANOVA indicated that the Number of Bristles variation was

nonsignificant, but the t-test between napiergrass and PEGL 09TX04 showed very high significance (<.001). Length of spikelet ANOVA and t-test did not prove significant. Table 8 there was not enough significance between the parents (PEGL 09TX04, Napiergrass) and the hybrids; for there to be enough significance to develop a key, the parental rows would have asterisks denoting significance in the hybrid column. Table 9 there was not enough significance between the parents (PEGL 09TX04, napiergrass) and the hybrids; for there to be enough significance to develop a key a, the parental rows would have asterisks denoting significance in the hybrid column. There was not enough significance (Table 10) between the parents (PEGL 09TX04, napiergrass) and the hybrids; for there to be enough significance to develop a key, the parental rows would have asterisks denoting significance in the hybrid column. There was not enough significance (Table 11) between the parents (PEGL 09TX04, napiergrass) and the hybrids; for there to be enough significance to develop a key, the parental rows would have asterisks denoting significance in the hybrid column.

Table 8. Pairwise comparison *t* values for differences in mean group Primary Bristle Length group means between individual genotypes and corresponding probability values. The chart allows for quick comparisons of grouped genotypes against each other. The t value is listed above the p-value.

t/p	**Hybrids**	**Napiergrass**
Hybrids		
Napiergrass	0.383	
	0.449	
PEGL 09TX04	-2.494	-4.02
	0.067	0.016**

[&] Data in the table were consolidated from pair-wise comparisons between means and t-tests.
* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Table 9. Pairwise comparison *t* values for differences in mean average Bristle Length group means between individual genotypes and corresponding probability values. The chart allows for quick comparisons of grouped genotypes against each other. The t value is listed above the p-value.

t/p	**Hybrids**	**Napiergrass**
Hybrids		
Napiergrass	-0.041[&]	
	0.969	
PEGL 09TX04	-8.204	-3.487
	0.001***	0.018*

[&] Data in the table were consolidated from pair-wise comparisons between means and t-tests.
* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Table 10. Pairwise comparison *t* values for differences in mean Number of Bristles group means between individual genotypes and corresponding probability values. The chart allows for quick comparisons of grouped genotypes against each other. The t value is listed above the p-value.

t/p	Hybrids	Napiergrass
Hybrids		
Napiergrass	-9.265[&] <0.001***	
PEGL 09TX04	0.682 0.533	-9.261 <0.001***

[&] Data in the table were consolidated from pair-wise comparisons between means and t-tests.
* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

Table 11. Pairwise comparison *t* values for differences in mean Length of Spikelet group means between individual genotypes and corresponding probability values. The chart allows for quick comparisons of grouped genotypes against each other. The t value is listed above the p-value. There was not enough significance (Table 11) between the parents (PEGL 09TX04, napiergrass) and the hybrids; for there to be enough significance to develop a key, the parental rows would have asterisks denoting significance in the hybrid column.

t/p	Hybrids	Napiergrass
Hybrids		
Napiergrass	1.323[&] 0.256	
PEGL 09TX04	-1.512 0.205	-3.942 0.017*

[&] Data in the table were consolidated from pair-wise comparisons between means and t-tests.
* Significant at the 0.05 probability level.
** Significant at the 0.01 probability level.
*** Significant at the 0.001 probability level.

The individual (Tables 4 -7) and group (Tables 8 -11) means morphology statistics strongly suggest that it is difficult to discern hybrid from their parent genotypes using classical morphological taxonomic techniques for these traits.

Chromosome Number and Flow Cytometry

The thus the ability to prepare mitotic chromosome spreads was very dependent on rapidly growing roots and the mitotic index. The success of chromosome preparations was unpredictable and problematic. The process after collecting the root-tips was simple but tedious and not time-efficient. Acquiring acceptable spreads had a low success. However, once obtained, they indeed enabled a chromosome number determination, and these were made for each entry. Representative images are illustrated (Figure 3).

Figure 3. Mitotic metaphase chromosome spreads from root-tips of pearl millet, napiergrass “Merkeron”, and an interspecific PMN hybrid. (A) PEGL 09TX04 pearl millet spread showing 14 chromosomes. (B) PMNV 13TX06 interspecific hybrid spread showing 21 chromosomes. (C) Napiergrass cultivar ‘Merkeron’ spread showing 28 chromosomes. All spreads stained with Azure B and shown at 1000X magnification.

Flow cytometry will be utilized on the genotypes collected. We used a single sample of *Sorghum bicolor* inbred BTX623 as a common reference *Sorghum* genome (1C) contains about 780 Mbp (Price et al., 2005). Thus values for the reference were well separated for these *Pennisetum* samples. C-values estimates for the *Pennisetum* samples were based on the genome size of *Sorghum* and the ratio of flow cytometric peaks (2C) for the *Pennisetum* sample versus BTX623 (Table 12). Gates were placed three times. The gates are labeled M1, M2, and M3. M1 is the *Sorghum* 2C DNA content, M2 is the target sample 2C DNA content, and M3 is the *Sorghum* 4C content. A 2C (*Sorghum*) : 2C (*Pennisetum*) ratio was developed from testing the *Sorghum* accession with the 8 genotypes (Table 12). The ratios were calculated by dividing the 2C PMN genotype second gate value and by the 2C *Sorghum* value from the first gate (Table 12). The 2C DNA (pg) for the genotypes tested was calculated by taking the respective number produced in Table 12 and multiplied by *S. bicolor* 2C DNA 1.67 pg. DNA per Haploid (1C) Genome was calculated by the 2C DNA (pg) divided by 2. DNA per Haploid (1C) Genome (Mbp) was calculated by the DNA per Haploid (1C) Genome (pg) divided by 980 Mbp (Cavalier-Smith, 1985; Price et al., 2005). The 2C and 4C peaks of the hybrids and the parents were identifiable using flow cytometry (Table 12 & 13). ANOVA was performed by using the 2C DNA (pg) means (Table 13), results indicated that there is no significant distinction between pearl millet, PMN, and napiergrass. The mean 2C values of the eight genotypes tested using ANOVA produced standard deviation of 0.07 and standard error of 0.02478 (Table14). The mean 2C DNA 4.6 pg (0.07 st.dev.) suggest that there is no clear distinction of the PMN hybrids from

Pennisetum purpureum which is less than 1 standard deviation. These flow cytometry results indicate that this method was not a suitable in this instance. Screenshots of the graphs and associated tabular data are in Appendix B.

Table 12. Flow cytometric nuclear DNA content ratios (2C) between *Sorghum* and individual *Pennisetum* genotypes .

2C (*Sorghum*): 2C (*Pennisetum*)	**Ratios**
Sb x PEGL 09TX04	2.69
Sb x PEPU 09TX 01	2.80
Sb x Merkeron	2.81
Sb x PMNV 13TX01	2.75
Sb x PMNV 13TX06	2.77
Sb x PMNV 14TX03	2.74
Sb x PMNV 14TX14	2.78
Sb x Kinggrass	2.71

Table 13. Estimated DNA content values (2C) of *Sorghum* and the eight individual *Pennisetum* genotypes.

Species	Genotype Identification	2C DNA (pg)	DNA per Haploid (1C) Genome (pg)	DNA per Haploid (1C) Genome (Mbp)
Sorghum bicolor	BTX 623	1.67	0.84	818
Pennisetum glaucum	PEGL 09TX04	4.49	2.25	2201
Pennisetum purpureum	MERKERON	4.69	2.35	2299
Pennisetum purpureum	PEPU 09TX01	4.68	2.34	2291
P. glaucum X P. purpureum	PMNV 13TX01	4.59	2.30	2250
P. glaucum X P. purpureum	PMNV 14TX03	4.58	2.29	2242
P. glaucum X P. purpureum	PMNV 13TX06	4.63	2.31	2267
P. glaucum X P. purpureum	PMNV 14TX14	4.64	2.32	2275
P. purpureum x P. glaucum	KGV 13TX01	4.53	2.26	2218

Table 14. ANOVA for DNA content differences among eight *Pennisetum* genotypes.

	Samples	2C DNA
Valid	8	8
Mean		4.604
Std. Error of Mean		0.02478
Std. Deviation		0.07009

Molecular Survey

The EST-SSRs PCAR 13 and PCAR 33 when tested with these samples on the PAGE showed fluorescence, this allowed for the determination that the samples had a localization of gene concentration. The localization of gene concentration was seen by the isolated fluorescence producing a band at one single location of the gel.

Use of the PCR marker survey to test clones of *Tuareg* MITEs with the selected hybrids and parents to select *Tuareg* markers to be then made into Taqman® probes was successful in selecting two of *Tuareg* MITEs. Selected clones *PgTb1* and Tr 54 produced fluorescence at a single location, this determined their compatibility with the samples tested.

Beacon Designer 8.0 was used to design Taqman® probes from the selected clones. *PgTb1* and Tr54 sequences from GeneBank were loaded into Beacon Designer and were then sent to Applied Biosystems by Thermo Fisher Scientific to produce the Taqman® Gene Expression Assays.

Dilution experiments indicated that the Taqman® assays were most effective when sample DNA content ranged from 50-100 pg/uL, which indicates roughly 10-20 G1-phase nuclear equivalents (2C level). Diluted samples were susceptible to degradation and therefore handled carefully and expeditiously. Taqman® results from individual diluted samples were somewhat varied so multiple dilutions were used to insure consistent results. If plates were prepared ahead of time, they were stored at -20°C and used within 12 hours. PCR plates used on the qPCR machine are only good to use

once, prepping plates ahead of time is reasonable if stored in -20°C freezer and used within 12 hours.

Taqman® assays of diluted samples were examined for OMN and the parental species representatives, MxPro exported data were analyzed using Microsoft Excel, including graphs and charts. Data were collected for individual diluted samples, then collectively (averaged; MxPro software uses the term collective when selecting export style of data) between the replications, each PCR plate had two replications for each sample tested and had a no template control (NTC). The NTC showed that it is important to change the pipette tips in between aliquoting the master mix between samples; when this was done, DNA and Taqman® signal levels for the NTC were zero.

Initial statistical analysis was done using data points from cycle 1 to 60 but was changed to only include cycles 46 to 60 when exponential growth occurred. Points before cycle 46 were either above or below 0 and caused lack of degrees of freedom, which would give errors in the analysis process, therefore any cycle that was below 0 was replaced with 0 when calculating the means. Cycles 46 – 60 suggest to be the most statistically most powerful.

PgTb1 was the most informative marker when replicated twice or four times, distinct differences between the hybrids and parents. Although statistical testing relied on the data from cycles 46-60 and 4 replications per marker, the figures display data from cycles 30-60. Mean *PgTb1* qPCR fluorescence from four replications at each cycle and for each genotype is depicted in Figure 4. The PgTb1 Group means were produced by taking the means of the individual hybrids and averaging them at each individual

cycle to produce a mean value at each cycle. This was also done for the napiergrass mean. Pearl millet (PEGL 09TX04) data was produced using the same data from Figure 4.

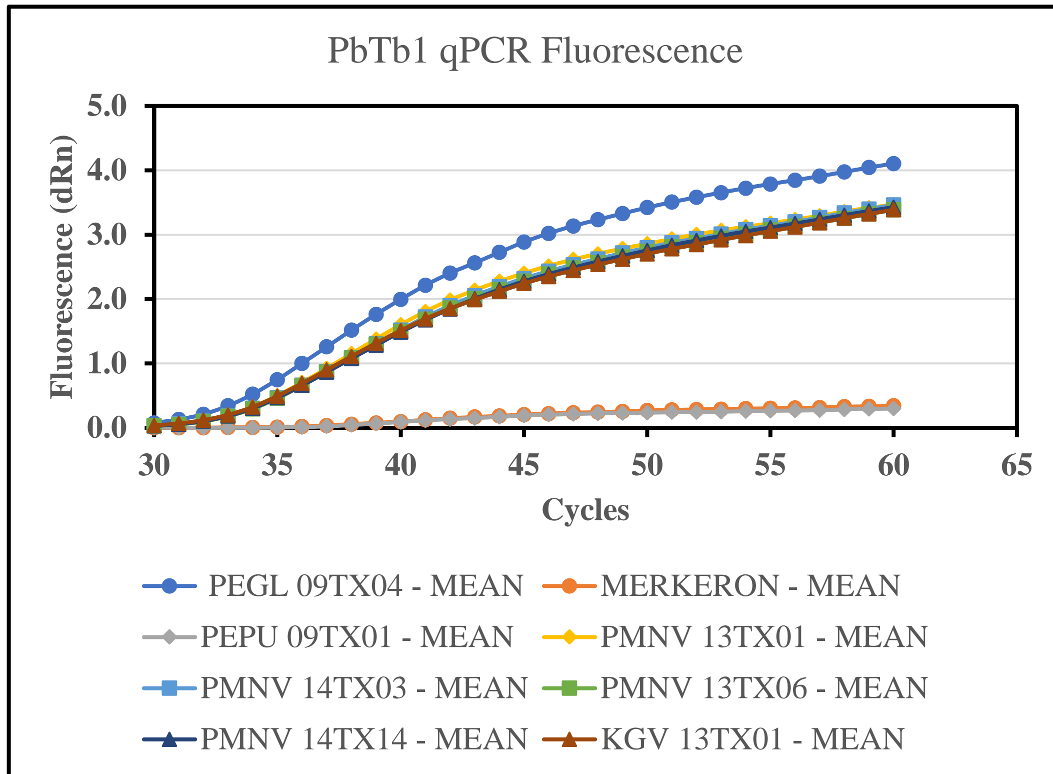


Figure 4. Graphic display of qPCR values at cycles 30-60 using the *PbTb1* Taqman assay in pearl millet, napiergrass and a Kinggrass interspecific hybrid and several PMN interspecific hybrids. See Table 1 for list of genotypes and identity codes. N = 4, number of replications.

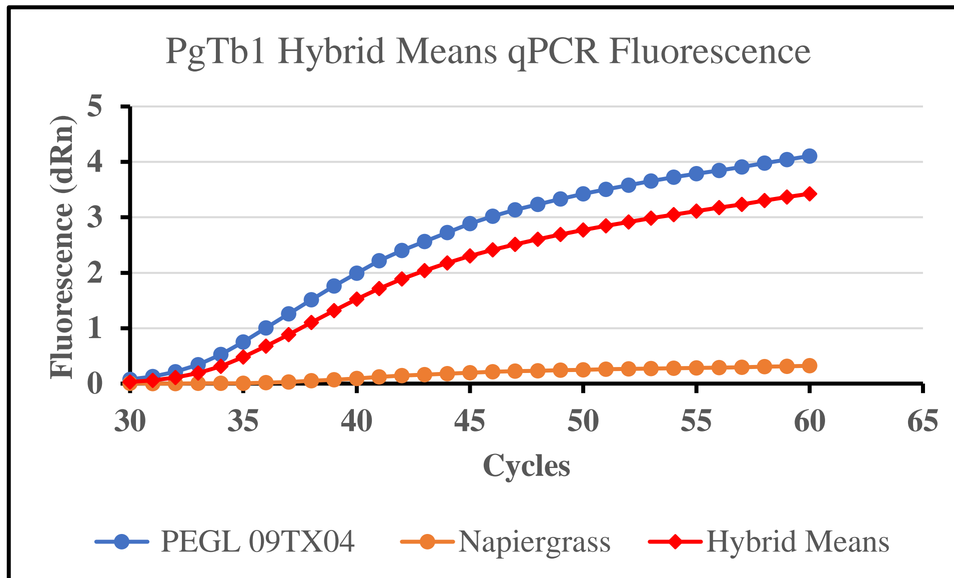


Figure 5. Graphic display of group means for *PgTb1* Taqman assay qPCR values for pearl millet (1), napiergrass (2) and their interspecific hybrids (5). Individual samples are identified in Figure 7. N = 4, number of replications.

Analogous analysis of Tr54 by qPCR demonstrated that it too enabled discrimination of the hybrids from the parents, degree of differentiation was weaker than for *PgTb1*.

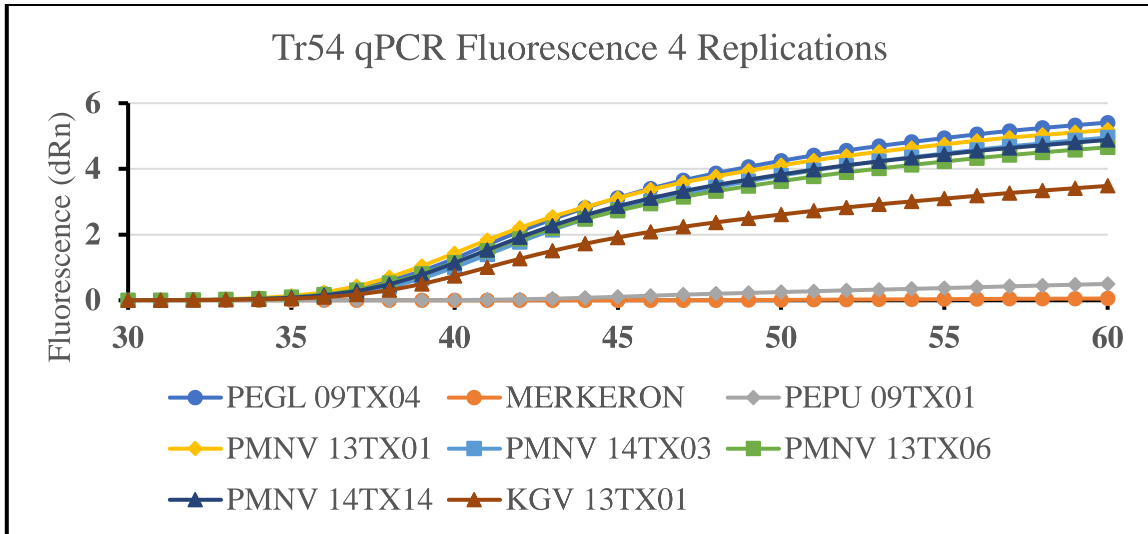


Figure 6. Graphic display of qPCR values at cycles 30-60 using the Tr54 Taqman assay in pearl millet, napiergrass and a kinggrass interspecific hybrid and several PMN interspecific hybrids. N = 4, number of replications.

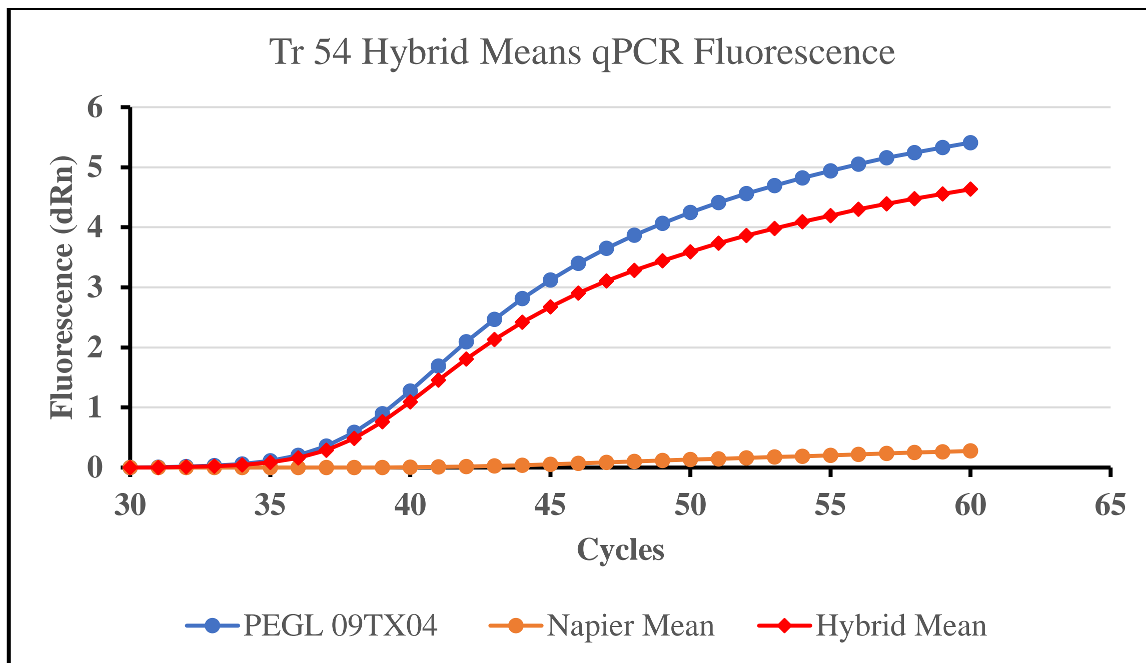


Figure 7. Graphic display of group means for Tr54 Taqman assay qPCR values for pearl millet (1), napiergrass (2) and their interspecific hybrids (5). N = 4, number of replications.

The mean differences of the PMN hybrids from pearl millet PEGL 09TX04 for markers PgTb1 and Tr54 were calculated and compared to the standard error of the difference between means to assess their statistical significance (Table 15-16). The test ratios relative to the standard error of the difference indicated that differences between the mean of the hybrids and the pearl millet accession PEGL 09TX04 were significant for both markers, and especially high for PgTb1. Values of PgTb1 were relatively consistent across individual hybrids and statistically separated from the pearl millet accession PEGL 09TX04. However, values for Tr54 were more variable among individual genotypes, and not consistently separable statistically from the pearl millet

accession PEGL 09TX04. The data thus suggest PbTb1 is far more preferable than Tr54 as a marker to distinguish individual hybrids from PEGL 09TX04.

Table 15. Calculation of qPCR mean differences of PMN genotypes from single pearl millet (PEGL09TX04) accession.

	Mean Differences of Hybrid Genotypes from PEGL 09TX04				
	PMNV 13TX01	PMNV 14TX03	PMNV 13TX06	PMNV 14TX14	KGV 13TX01
PgTb1	-0.581	-0.630	-0.695	-0.666	-0.717
Tr54	-0.158	-0.451	-0.656	-0.449	-1.719

Table 16. Statistical analysis of qPCR PMN differences from single pearl millet accession.

qPCR PMN differences from PEGL 09TX04		
	PgTb1	Tr54
Average (n=5), absolute value	0.658	0.687
Standard Deviation	0.054*	0.604
Standard Error (SE)	0.024*	0.270
Ratio of Average difference to SE	27.187	2.543
Ratio expected at p=0.0005	0.208	2.325
Ratio expected at 0.005	0.111	1.243

Analogously, the mean differences of the PMN hybrids from the napiergrass mean for each of the markers PgTb1 and Tr54 were calculated and compared to the standard error of the difference between means to assess their statistical significance (Table 17-18). The test ratios relative to the standard error of the difference indicated

that differences between the means of the hybrids and the napiergrass mean were significant for both markers, and especially high for PgTb1. Values of PgTb1 were relatively consistent across individual hybrids and statistically separated from the napiergrass mean. In spite of the variability of values for Tr54 across individual hybrid genotypes, they were consistently separable statistically from the napiergrass mean. The data thus suggest PbTb1 is far more preferable than Tr54 as a marker to distinguish individual hybrids from the napiergrass mean, but that Tr54 also suffices for that purpose.

Taking the PgTb1 and Tr54 results for all genotypes together (Tables 15-18), PbTb1 is deemed to be effective at separating the PMN and KG hybrids from both parents in a seemingly reliable manner. Tr54, on the other hand, seems capable of reliably separating the hybrids from the napiergrass mean, but cannot reliably separate individual hybrids from the pearl millet PEGL 09TX04, in spite of the fact that it was able to separate the *average* of a group of such hybrids from pearl millet.

Table 17. Calculation of qPCR mean differences of PMN genotypes from the two napiergrass accessions.

	Mean Differences of Hybrid Genotypes from Napiergrass				
	PMNV 13TX01	PMNV 14TX03	PMNV 13TX06	PMNV 14TX14	KGV 13TX01
PgTb1	2.768	2.718	2.653	2.683	2.631
Tr54	4.257	3.965	3.760	3.966	2.696

Table 18. Statistical analysis of qPCR PMN differences from the two napiergrass accessions.

qPCR PMN differences from Napiergrass

	PgTb1	Tr54
Average (n=5), absolute value	2.691	3.729
Standard Deviation	0.054*	0.604
Standard Error (SE)	0.024*	0.270
Ratio of Average difference to SE	111.194	13.808
Ratio expected at p=0.0005	0.208	2.325
Ratio expected at 0.005	0.111	1.243

CONCLUSION

This research effort established a TE-based molecular assay that can be used to reliably distinguish PMN hybrids from their parents. The best-performing assay was based on qPCR of *Tuareg* MITE *PgTb1*, that exhibited a simple amplicon electrophoretic band pattern. The usefulness of the molecular assay is underscored by the fact that reliable classification of PMN hybrids versus their parents was not feasible by morphological keying, nor by flow cytometric analysis of nuclear DNA content levels. Root-tip mitotic chromosome spreads reliably distinguished PMN from their parents, but the requisite preparations, protocols and analysis procedures were laborious, time-consuming and thus expensive. Improved or alternative solutions might include additional or alternative probes, target loci and/or different methods of analysis that are cheaper and/or faster to implement, and/or are able to discriminate more accurately among hybrid individuals with relatively smaller morphological differences. Use of a larger PMN population from the selected parents would likely facilitate efforts to refine the assay's discriminating power. Statistical tests would have been more robust if the pearl millet and napiergrass values used for calculating the differences were based on data from several genotypes and supported by a statistical description of the respective distributions for the two markers.

REFERENCES

Adler, P. R., Grosso, S. J. D., & Parton, W. J. (2007). Life-cycle assessment of net greenhouse-gas flux for bioenergy cropping systems. *Ecological Applications, 17*(3), 675-691.

Akiyama, Y., Conner, J. A., Goel, S., Morishige, D. T., Mullet, J. E., Hanna, W. W., & Ozias-Akins, P. (2004). High-resolution physical mapping in *Pennisetum squamulatum* reveals extensive chromosomal heteromorphism of the genomic region associated with apomixis. *Plant Physiology, 134*(4), 1733-1741.

Bogdan, A. (1977). *Tropical pasture and fodder plants*. London Longman.

Bonnet, G., Tyagi, S., Libchaber, A., & Kramer, F. R. (1999). Thermodynamic basis of the enhanced specificity of structured DNA probes. *Proceedings of the National Academy of Sciences, 96*(11), 6171-6176.

BRIT, B. R. I. o. T. (2010). Plant Collection & Preservation. 3/25/2010. Retrieved from http://www.brit.org/sites/default/files/public/Herbarium/Plant_Collection_and_Preservation_01.pdf

Burke, J. M., Tang, S., Knapp, S. J., & Rieseberg, L. H. (2002). Genetic analysis of sunflower domestication. *Genetics, 161*(3), 1257-1267.

Burton, G. W. (1944). Hybrids between napier grass and cattail millet. *Journal of Heredity, 35*(8), 227-232.

Cavalier-Smith, T. (1985). Cell volume and the evolution of eukaryotic genome size. *The evolution of genome size,* pp. 105-184.

Chandra-Shekara, A. C., Pegadaraju, V., Thompson, M., Vellekson, D., & Schultz, Q. (2010). A novel DNA-based diagnostic test for the detection of annual and intermediate ryegrass contamination in perennial ryegrass. *Molecular Breeding, 28*(2), 217-225. doi:10.1007/s11032-010-9475-4

Chapman, G. P. (1990). *Reproductive versatility in the grasses*: Cambridge University Press.

Crane, C. F., Price, H. J., Stelly, D. M., Czeschin Jr, D. G., & McKnight, T. D. (1993). Identification of a homeologous chromosome pair by in situ DNA hybridization to ribosomal RNA loci in meiotic chromosomes of cotton (Gossypium hirsutum). *Genome, 36*(6), 1015-1022.

Doebley, J., Stec, A., & Gustus, C. (1995). teosinte branched1 and the origin of maize: evidence for epistasis and the evolution of dominance. *Genetics, 141*(1), 333-346.

Dos Reis, G. B., Mesquita, A. T., Torres, G. A., Andrade-Vieira, L. F., Pereira, A. V., & Davide, L. C. (2014). Genomic homeology between *Pennisetum purpureum* and *Pennisetum glaucum* (Poaceae). *Comp Cytogenet, 8*(3), 199-209. doi:10.3897/CompCytogen.v8i3.7732

Dowling, C., Burson, B., Foster, J., Tarpley, L., & Jessup, R. (2013). Confirmation of Pearl Millet-Napiergrass Hybrids Using EST-Derived Simple Sequence Repeat (SSR) Markers. *American Journal of Plant Sciences, 04*(05), 1004-1012. doi:10.4236/ajps.2013.45124

Dowling, C., Burson, B., & Jessup, R. (2014). Marker-assisted verification of Kinggrass (*Pennisetum purpureum* Schumach. x *Pennisetum glaucum* [L.] R. Br.). *Plant Omics, 7*(2), 72 - 79.

Dussert, Y., Remigereau, M. S., Fontaine, M. C., Snirc, A., Lakis, G., Stoeckel, S., . . . Robert, T. (2013). Polymorphism pattern at a miniature inverted-repeat transposable element locus downstream of the domestication gene *Teosinte-branched1* in wild and domesticated pearl millet. *Mol Ecol, 22*(2), 327-340. doi:10.1111/mec.12139

Follett, R. (2001). Soil management concepts and carbon sequestration in cropland soils. *Soil and Tillage Research, 61*(1), 77-92.

Halfmann, R., Stelly, D., & Young, D. (2007). Towards improved cell cycle synchronization and chromosome preparation methods in cotton. *Journal of cotton science*.

Hodnett, G. (2016, September 27 2016). [Chromosome Spreads].

Huttley, G. A., MacRae, A., & Clegg, M. T. (1995). Molecular evolution of the *Ac/Ds* transposable-element family in pearl millet and other grasses. *Genetics, 139*(3), 1411-1419.

Hwang, G. T., Seo, Y. J., & Kim, B. H. (2004). A highly discriminating quencher-free molecular beacon for probing DNA. *Journal of the American Chemical Society, 126*(21), 6528-6529.

Jessup, R. (2013). 'Seeded-Yet-Sterile Perennial Biofuel Feedstocks. *Advances in Crop Science and Technology., 1*(2). doi:10.4172/acst.1000e102

Jessup, R., Burson, B., Burow, G., Wang, Y.-W., Chang, C., Li, Z., . . . Hussey, M. (2003). Segmental allotetraploidy and allelic interactions in buffelgrass (Pennisetum ciliare (L.) Link syn. Cenchrus ciliaris L.) as revealed by genome mapping. *Genome, 46*(2), 304-313.

Jessup, R., & Dowling, C. (2015). “Seeded-yet-Sterile” Perennial Grasses: Towards Sustainable and Non-invasive Biofuel Feedstocks. *Bioenergy and Biological Invasions: Ecological, Agronomic and Policy Perspectives on Minimizing Risk, 5*, 97.

Lal, R. (2008). Crop residues as soil amendments and feedstock for bioethanol production. *Waste Management, 28*(4), 747-758.

Lemus, R., & Lal, R. (2005). Bioenergy crops and carbon sequestration. *Critical Reviews in Plant Sciences, 24*(1), 1-21.

Lönnig, W.-E., & Saedler, H. (2002). Chromosome rearrangements and transposable elements. *Annual review of genetics, 36*(1), 389-410.

MacRae, A. F., Learn, G. H., Karjala, M., & Clegg, M. T. (1990). Presence of an Activator (Ac)-like sequence in Pennisetum glaucum (pearl millet). *Plant molecular biology, 15*(1), 177-179.

Martel, E., De Nay, D., Siljak-Yakoviev, S., Brown, S., & Sarr, A. (1997). Genome size variation and basic chromosome number in pearl millet and fourteen related Pennisetum species. *Journal of Heredity, 88*(2), 139-143.

McClintock, B. (1947). Cytogenetic Studies of Maize and Neurospora. *Carnegie Institution of Washington Yearbook 46, 46*, 7.

McClintock, B. (1993). *The significance of responses of the genome to challenge*: Singapore: World Scientific Pub. Co.

Moose, S. P., & Mumm, R. H. (2008). Molecular plant breeding as the foundation for 21st century crop improvement. *Plant Physiol, 147*(3), 969-977. doi:10.1104/pp.108.118232

Moser, L. E., Burson, B. L., & Sollenberger, L. E. (2004). *Warm-season (C4) grasses*: American Society of Agronomy.

Ozias-Akins, P., Akiyama, Y., & Hanna, W. W. (2003). Molecular characterization of the genomic region linked with apomixis in Pennisetum/Cenchrus. *Functional & integrative genomics, 3*(3), 94-104.

Paterson, A. H., Bowers, J. E., Bruggmann, R., Dubchak, I., Grimwood, J., Gundlach, H., . . . Poliakov, A. (2009). The Sorghum bicolor genome and the diversification of grasses. *Nature, 457*(7229), 551-556.

Price, H. J., Dillon, S. L., Hodnett, G., Rooney, W. L., Ross, L., & Johnston, J. S. (2005). Genome evolution in the genus Sorghum (Poaceae). *Annals of Botany, 95*(1), 219-227.

Remigereau, M.-S., Lakis, G., Rekima, S., Leveugle, M., Fontaine, M. C., Langin, T., . . . Robert, T. (2011). Cereal domestication and evolution of branching: evidence for soft selection in the Tb1 orthologue of pearl millet (Pennisetum glaucum [L.] R. Br.). *PLoS One, 6*(7), e22404.

Remigereau, M. S., Robin, O., Siljak-Yakovlev, S., Sarr, A., Robert, T., & Langin, T. (2006). Tuareg, a novel miniature-inverted repeat family of pearl millet (Pennisetum glaucum) related to the PIF superfamily of maize. *Genetica, 128*(1-3), 205-216. doi:10.1007/s10709-005-5703-y

Samson, R., Mani, S., Boddey, R., Sokhansanj, S., Quesada, D., Urquiaga, S., . . . Ho Lem, C. (2005). The potential of C4 perennial grasses for developing a global BIOHEAT industry. *BPTS, 24*(5-6), 461-495.

Staginnus, C., Huettel, B., Desel, C., Schmidt, T., & Kahl, G. (2001). A PCR-based assay to detect En/Spm-like transposon sequences in plants. *Chromosome Research, 9*(7).

Staton, S. E., Bakken, B. H., Blackman, B. K., Chapman, M. A., Kane, N. C., Tang, S., . . . Burke, J. M. (2012). The sunflower (Helianthus annuus L.) genome reflects a recent history of biased accumulation of transposable elements. *Plant J, 72*(1), 142-153. doi:10.1111/j.1365-313X.2012.05072.x

Studer, A., Zhao, Q., Ross-Ibarra, J., & Doebley, J. (2011). Identification of a functional transposon insertion in the maize domestication gene tb1. *Nature genetics, 43*(11), 1160-1163.

Techio, V. H., Davide, L. C., Cagliari, A., Barbosa, S., & Pereira, A. V. (2010). Karyotipic asymmetry of both wild and cultivated species of Pennisetum. *Bragantia, 69*(2), 273-279.

Terzi, V., Infascelli, F., Tudisco, R., Russo, G., Stanca, A. M., & Faccioli, P. (2004). Quantitative detection of Secale cereale by real-time PCR amplification. *LWT - Food Science and Technology, 37*(2), 239-246. doi:10.1016/j.lwt.2003.08.005

Tyagi, S., & Kramer, F. R. (1996). Molecular beacons: probes that fluoresce upon hybridization. *Nature biotechnology, 14*(3), 303-308.

Walters, D. R., & Keil, D. J. (1996). *Vascular plant taxonomy*: Kendall Hunt.

Wipff, J. K., & Hatch, S. L. (1994). A systematic study of Digitaria sect. Pennatae (Poaceae: Paniceae) in the New World. *Systematic Botany*, 613-627.

APPENDIX A

A-1 Analysis of Variance (ANOVA) of four morphological traits among the eight genotypes in the test panel.

	Cases	Sum of Squares	df	Mean Square	F	p
Primary Bristle Length	Genotypes	824.7	7	117.81	5.291	< .001
	Residual	734.8	33	22.27		
Average Length of Bristles	Genotypes	193.5	7	27.641	8.572	< .001
	Residual	109.6	34	3.225		
Number of Bristles	Genotypes	3340	7	477.16	13.36	< .001
	Residual	1214	34	35.71		
Length of Spikelet	Genotypes	76.66	7	10.951	3.354	0.008
	Residual	107.73	33	3.265		

A-2 Univariate statistics (mean, standard deviation and standard error of the mean) for four morphological traits among the eight genotypes in the test panel Morphology ANOVA Descriptives– Individual Genotypes; consolidated ANOVA descriptives table for each of the morphological characteristics tested using individual genotype data. Statistical analysis provided mean, standard error or mean, standard deviation, and minimum and maximum. There was no significance between individual genotypes when testing a single morphological trait.

	Primary Bristle Length		Average Length of Bristles		Number of Bristles		Length of Spikelet	
	Genotypes	**Measurement**	**Genotypes**	**Measurement**	**Genotypes**	**Measurement**	**Genotypes**	**Measurement**
Mean	4.439	10.63	4.357	7.595	32.43	4.357	4.439	4.122
Std. Error of Mean	0.363	0.9752	0.3636	0.4196	1.626	0.3636	0.363	0.3353
Std. Deviation	2.324	6.244	2.356	2.719	10.54	2.356	2.324	2.147

A-3 Hybrid Mean Morphology ANOVA; consolidated ANOVA table comprising of the morphological traits and the grouped means of the hybrids, napiergrass, and pearl millet.

	Cases	Sum of Squares	df	Mean Square	F	p
Primary Bristle Length	Genotypes	208.8	2	104.4	7.659	0.006
	Residual	177.2	13	13.63		
Average Length of Bristle	Genotypes	39.65	2	19.824	13.63	< .001
	Residual	20.36	14	1.454		
Number of Bristles	Genotypes	695.1	2	347.57	22.78	< .001
	Residual	213.6	14	15.26		
Length of Spikelet	Genotypes	18.67	2	9.336	4.551	0.032
	Residual	26.67	13	2.051		

A-4 Hybrid Mean Morphology ANOVA Descriptives; consolidated ANOVA table descriptives comprising of the morphological traits and the grouped means of the hybrids, napiergrass, and pearl millet.

	Primary Bristle Length		**Average Number of Bristles**		**Number of Bristles**		**Length of Spikelet**	
	Genotype	**Measurement**	**Genotype**	**Measurement**	**Genotype**	**Measurement**	**Genotype**	**Measurements**
Mean	2	9.769	2.059	6.618	2.059	31.32	2	3.9
Std. Error of Mean	0.2041	1.268	0.2006	0.4697	0.2006	1.828	0.2041	0.4346
Std. Deviation	0.8165	5.073	0.8269	1.937	0.8269	7.536	0.8165	1.739

A-5 Individual Genotypes Morphology Paired t-test; chart used individual genotypes to test against each other to produce paired sample t-test; statistical analysis produced p-values for each paired sample together.

Paired Samples T-Test			Primary Bristle Length			Average Length of Bristle			Number of Bristles			Length of Spikelet		
			t	df	p	t	df	p	t	df	p	t	df	p
PEGL 09TX04	-	PEPU 09TX01	-2.734	4	0.052	-9.122	5	< .001	5.895	5	0.002	-2.092	4	0.105
PEGL 09TX04	-	MERKERON	-3.833	4	0.019	-5.789	4	0.004	5.193	4	0.007	-3.9	4	0.018
PEGL 09TX04	-	PMNV 13TX01	-0.757	4	0.491	-4.225	4	0.013	-0.679	4	0.534	-0.356	4	0.74
PEGL 09TX04	-	PMNV 13TX06	-4.546	4	0.01	-5.658	4	0.005	0.827	4	0.455	-2.86	4	0.046
PEGL 09TX04	-	PMNV14TX03	-3.023	4	0.039	-7.303	4	0.002	7.443	4	0.002	-3.055	4	0.038
PEGL 09TX04	-	PMNV 14TX14	1	4	0.374	-31.027	4	< .001	1.718	4	0.161	0.688	4	0.529
PEGL 09TX04	-	KGV 13TX01	-4.512	4	0.011	-0.284	4	0.79	-2.453	4	0.07	-2.228	4	0.09
PEPU 09TX01	-	MERKERON	0	4	1	-0.707	4	0.519	-0.364	4	0.735	-1.425	4	0.227
PEPU 09TX01	-	PMNV 13TX01	0.764	4	0.487	-2.058	4	0.109	-4.351	4	0.012	0.461	4	0.669
PEPU 09TX01	-	PMNV 13TX06	-0.586	4	0.589	1	4	0.374	-2.481	4	0.068	-0.967	4	0.388
PEPU 09TX01	-	PMNV14TX03	0.723	4	0.51	0.946	4	0.398	-0.335	4	0.754	-0.953	4	0.394
PEPU 09TX01	-	PMNV 14TX14	2.804	4	0.049	-1.089	4	0.338	-3.942	4	0.017	2.138	4	0.099
PEPU 09TX01	-	KGV 13TX01	-1.486	4	0.211	2.818	4	0.048	-5.929	4	0.004	-0.514	4	0.634
MERKERON	-	PMNV 13TX01	1.021	4	0.365	-1.688	4	0.167	-5.713	4	0.005	1.769	4	0.152
MERKERON	-	PMNV 13TX06	-4.811	4	0.009	1.136	4	0.319	-3.086	4	0.037	1.633	4	0.178
MERKERON	-	PMNV14TX03	1.375	4	0.241	0.958	4	0.392	0.314	4	0.769	2.449	4	0.07
MERKERON	-	PMNV 14TX14	3.985	4	0.016	-0.294	4	0.783	-1.307	4	0.261	3.814	4	0.019
MERKERON	-	KGV 13TX01	-3.942	4	0.017	3.256	4	0.031	-6.402	4	0.003	3.162	4	0.034
PMNV 13TX01	-	PMNV 13TX06	-1.626	4	0.179	2.211	4	0.092	1.661	4	0.172	-1.58	4	0.189

PMNV 13TX01	-	PMNV14TX03	-0.564	4	0.603	2.598	4	0.06	8.358	4	0.001	-1.491	4	0.21
PMNV 13TX01	-	PMNV 14TX14	1.076	4	0.342	1.96	4	0.121	1.719	4	0.161	0.704	4	0.521
PMNV 13TX01	-	KGV 13TX01	-3.53	4	0.024	2.692	4	0.055	-2.331	4	0.08	-1.281	4	0.269
PMNV 13TX06	-	PMNV14TX03	3.323	4	0.029	0.452	4	0.675	3.231	4	0.032	0.535	4	0.621
PMNV 13TX06	-	PMNV 14TX14	4.859	4	0.008	-1.341	4	0.251	0.483	4	0.654	2.794	4	0.049
PMNV 13TX06	-	KGV 13TX01	-3.47	4	0.026	3.011	4	0.04	-11.978	4	< .001	2.449	4	0.07
PMNV14TX03	-	PMNV 14TX14	4.162	4	0.014	-1.933	4	0.125	-1.705	4	0.163	3.47	4	0.026
PMNV14TX03	-	KGV 13TX01	-5.685	4	0.005	2.605	4	0.06	-6.714	4	0.003	1.633	4	0.178
PMNV 14TX14	-	KGV 13TX01	-5.241	4	0.006	2.96	4	0.042	-3.154	4	0.034	-2.449	4	0.07

A-6 Individual Genotypes Morphology Paired t-test Descriptives; chart produced mean, standard deviation (SD), standard error (SE) for the individual genotypes that were tested

	Primary Bristle Length				Average Length of Bristles				Number of Bristles				Length of Spikelet			
	N	**Mean**	**SD**	**SE**	**N**	**Mean**	**SD**	**SE**	**N**	**Mean**	**SD**	**SE**	**N**	**Mean**	**SD**	**SE**
PEGL 09TX04	5	4.6	4.336	1.939	6	4.583	0.683	0.279	6	36.33	5.82	2.376	5	2.4	2.191	0.98
PEPU 09TX01	6	13.167	6.646	2.713	6	8	0.274	0.112	6	22	4.336	1.77	6	4.333	2.16	0.882
MERKERON	5	12.4	0.548	0.245	5	8.2	0.837	0.374	5	24	6.205	2.775	5	5.8	0.447	0.2
PMNV 13TX01	5	8.6	8.355	3.736	5	12	4.305	1.925	5	40.2	6.419	2.871	5	3.2	3.033	1.356
PMNV 13TX06	5	14.2	0.837	0.374	5	7.7	0.671	0.3	5	34	5.958	2.665	5	5.4	0.548	0.245
PMNV 14TX03	5	10.5	2.915	1.304	5	7.55	0.855	0.382	5	23.4	3.912	1.749	5	5.2	0.447	0.2
PMNV 14TX14	5	3.4	4.669	2.088	5	8.35	0.548	0.245	5	31	8.216	3.674	5	1.8	2.49	1.114
KGV 13TX01	5	17.6	2.881	1.288	5	4.9	2.453	1.097	5	49.8	6.301	2.818	5	4.8	0.447	0.2

A-7 Hybrid Mean Morphology Paired t-test; chart consolidated each morphological characteristic tested with the group means of the hybrids, napiergrass, and pearl millet; paired sample t-test provided t value, degrees of freedom, and p-value.

Paired Samples t-Test			Primary Bristle Length			Average Brisle Length			Number of Bristles			Length of Spikelet		
			t	**df**	**p**	**t**	**df**	**p**	**t**	**df**	**p**	**t**	**df**	**p**
PEGL 09TX04	-	Napier	-4.02	4	0.016	-3.487	5	0.018	9.261	5	< .001	-3.942	4	0.017
PEGL 09TX04	-	Hybrids	-2.494	4	0.067	-8.204	4	<0.001	0.682	4	0.533	-1.512	4	0.205
Napier	-	Hybrids	0.838	4	0.449	-0.041	4	0.969	-9.265	4	< .001	1.323	4	0.256

A-9 Hybrid Mean Morphology Paired t-test Descriptives; chart consolidated each morphological characteristic tested with the group means of the hybrids, napiergrass, and pearl millet; paired sample t-test descriptives provided mean, standard error of mean, standard error deviation, minimum and maximum.

	Primary Bristle Length			**Average Bristle Length**			**Number of Bristles**			**Length of Spikelet**		
	PEGL 09TX0	**Napier**	**Hybrids**	**PEGL 09TX0**	**Napier**	**Hybrids**	**PEGL 09TX0**	**Napier**	**Hybrids**	**PEGL 09TX0**	**Napier**	**Hybrids**
Mean	4.6	13.17	10.86	4.583	7.417	8.1	36.33	22.67	35.68	2.4	5	4.08
Std. Error of	1.939	1.553	1.218	0.2789	0.6813	0.4525	2.376	1.054	0.7392	0.9798	0.4282	0.3137
Std. Deviatio	4.336	3.804	2.724	0.6831	1.669	1.012	5.82	2.582	1.653	2.191	1.049	0.7014

A-10 qPCR ANOVA; Taqman® probes ANOVA consolidated in one chart to easily compare p-values.

	Cases	Sum of Squares	df	Mean Square	F	p
PgTb1	Samples	182.07	7	26.01	337.1	< .001
	Residual	8.641	112	0.077		
Tr54	Samples	360.77	7	51.538	214.8	< .001
	Residual	26.88	112	0.24		

A-11 qPCR ANOVA Descriptives; Taqman® probes ANOVA descriptives produced mean, standard error of mean, standard deviation, minimum and maximum to easily compare the two probes together.

	PgTb1 Measurement	Tr54 Measurement
Mean	2.37	3.057
Std. Error of Mean	0.1156	0.1648
Std. Deviation	1.266	1.805
Minimum	0.2017	0.00007818
Maximum	4.106	5.411

A-12. qPCR Group means ANOVA, statistical analysis of the group means of the hybrids, napiergrass, and pearl millet; chart allowed easy comparison of the two Taqman® probes p-value.

	Cases	Sum of Squares	df	Mean Square	F	p
PgTb1	Samples	91.37	2	45.683	43.48	< .001
	Residual	94.57	90	1.051		
Tr54	Samples	169.355	2	84.678	361.1	< .001
	Residual	9.848	42	0.234		

A-13 qPCR Group means ANOVA descriptives; the group means of the hybrids, napiergrass and pearl millet chart provides mean, standard error or mean, standard deviation, minimum and maximum.

	PgTb1 Measurement	Tr54 Measurement
Mean	1.537	2.889
Std. Error of Mean	0.1474	0.3008
Std. Deviation	1.422	2.018
Minimum	0.000558	0.0697
Maximum	4.106	5.411

A-14 qPCR Individual Paired T-test; individual genotypes tested with each Taqman® probe to produce the p-value for each genotyped tested against another.

			PgTb1			Tr54		
Paired Samples T-test			**t**	**df**	**p**	**t**	**df**	**p**
PEGL 09TX04	-	MERKERON	42.895	14	< .001	28.744	14	< .001
PEGL 09TX04	-	PEPU 09TX01	42.136	14	< .001	31.638	14	< .001
PEGL 09TX04	-	PMNV 13TX01	54.468	14	< .001	10.549	14	< .001
PEGL 09TX04	-	PMNV 14TX03	132.187	14	< .001	71.015	14	< .001
PEGL 09TX04	-	PMNV 13TX06	93.299	14	< .001	27.041	14	< .001
PEGL 09TX04	-	PMNV 14TX14	201.839	14	< .001	22.919	14	< .001
PEGL 09TX04	-	KGV 13TX01	149.826	14	< .001	34.117	14	< .001
MERKERON	-	PEPU 09TX01	14.598	14	< .001	-12.295	14	< .001
MERKERON	-	PMNV 13TX01	-41.001	14	< .001	-30.605	14	< .001
MERKERON	-	PMNV 14TX03	-36.788	14	< .001	-26.826	14	< .001
MERKERON	-	PMNV 13TX06	-37.332	14	< .001	-28.979	14	< .001
MERKERON	-	PMNV 14TX14	-35.659	14	< .001	-29.538	14	< .001
MERKERON	-	KGV 13TX01	-35.116	14	< .001	-26.129	14	< .001
PEPU 09TX01	-	PMNV 13TX01	-40.193	14	< .001	-34.241	14	< .001
PEPU 09TX01	-	PMNV 14TX03	-36.174	14	< .001	-29.504	14	< .001
PEPU 09TX01	-	PMNV 13TX06	-36.677	14	< .001	-32.576	14	< .001
PEPU 09TX01	-	PMNV 14TX14	-35.086	14	< .001	-33.099	14	< .001
PEPU 09TX01	-	KGV 13TX01	-34.563	14	< .001	-30.045	14	< .001
PMNV 13TX01	-	PMNV 14TX03	7.647	14	< .001	30.789	14	< .001
PMNV 13TX01	-	PMNV 13TX06	28.493	14	< .001	53.535	14	< .001
PMNV 13TX01	-	PMNV 14TX14	10.944	14	< .001	60.618	14	< .001
PMNV 13TX01	-	KGV 13TX01	17.532	14	< .001	44.052	14	< .001
PMNV 14TX03	-	PMNV 13TX06	22.007	14	< .001	11.081	14	< .001
PMNV 14TX03	-	PMNV 14TX14	21.097	14	< .001	-0.121	14	0.905
PMNV 14TX03	-	KGV 13TX01	48.701	14	< .001	28.405	14	< .001
PMNV 13TX06	-	PMNV 14TX03	-22.007	14	< .001	-11.081	14	< .001
PMNV 13TX06	-	PMNV 14TX14	-6.804	14	< .001	-42.64	14	< .001
PMNV 13TX06	-	KGV 13TX01	5.432	14	< .001	40.616	14	< .001
PMNV 14TX14	-	KGV 13TX01	24.672	14	< .001	41.221	14	< .001

A-15 qPCR Individual Paired T-test Descriptives; chart provides mean, standard error of mean, standard deviation, minimum and maximum for each Taqman ®probe and individual genotype in the paired sample t-test.

	PEGL 09TX04		MERKERON		PEPU 09TX01		PMNV 13TX01		PMNV 14TX03		PMNV 13TX06		PMNV 14TX14		KGV 13TX01	
	PgTb1	**Tr54**	**PgTb1**	**Tr54**	**PgTb1**	**Tr54**	**PgTb1**	**Tr54**	**PgTb1**	**Tr54**	**PgTb1**	**Tr54**	**PgTb1**	**Tr54**	**PgTb1**	**Tr54**
Mean	3.618	4.59	0.2866	0.02524	0.2518	0.3235	3.037	4.431	2.988	4.139	2.923	3.934	4.141	2.953	2.87	2.901
Std. Error of Mean	0.08726	0.1636	0.009626	0.004897	0.007431	0.02912	0.07668	0.1488	0.08302	0.1582	0.08021	0.1397	0.1441	0.08436	0.1137	0.08403
Std. Deviation	0.338	0.6336	0.03728	0.01897	0.02878	0.1128	0.297	0.5762	0.3215	0.6125	0.3107	0.541	0.5582	0.3267	0.4404	0.3254
Minimum	3.021	3.4	0.2204	0.0007818	0.2017	0.1393	2.515	3.367	2.433	3.012	2.39	2.945	3.105	2.387	2.085	2.35
Maximum	4.106	5.411	0.3453	0.05373	0.2984	0.498	3.465	5.188	3.462	4.957	3.388	4.658	4.881	3.429	3.483	3.386

A-16 Consolidated flow cytometry gates from output graphs found in Appendix B. Gates were taken three times, M1 is the *Sorghum bicolor* 2C DNA content value, M2 is the target genotype sample 2C DNA content value, and M3 is the 4C *Sorghum bicolor* DNA content value.

Samples	Gates	Means
Sb x PEGL 09TX04	M1	94667
	M2	254423
	M3	190900
Sb x PEPU 09TX01	M1	100434
	M2	281631
	M3	204805
Sb x Merkeron	M1	103427
	M2	290782
	M3	207922
Sb x PMNV 13TX01	M1	100744
	M2	276689
	M3	201921
Sb x PMNV 13TX06	M1	111530
	M2	308432
	M3	225305
Sb x PMNV 14TX03	M1	99101
	M2	272009
	M3	200195
Sb x PMNV 14TX14	M1	103474
	M2	286678
	M3	213458
Sb x KGV 13TX01	M1	109622
	M2	296567
	M3	219037

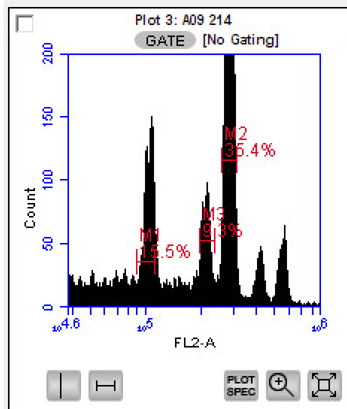
A-17 qPCR Group means t-test; the paired samples t-test chart comprises both Taqman® probes used in this research while comparing the grouped means of the hybrids, napiergrasses, and pearl millet. Each comparison output had a significant p-value.

			PgTb1			Tr54		
			t	**df**	**p**	**t**	**df**	**p**
PEGL 09TX04	-	Napiergrass	42.51	14	< .001	30.08	14	< .001
PEGL 09TX04	-	Hybrids	109.95	14	< .001	29.84	14	< .001
Napiergrass	-	Hybrids	-36.78	14	< .001	-30.06	14	< .001

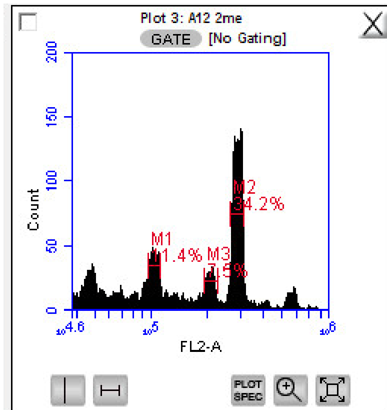
A-18 qPCR Group means T-test Descriptives; the paired samples t-test descriptives comprises both Taqman® probes used in this research while producing mean, standard error of mean, standard deviation, minimum and maximum statistical analysis for each of grouped means ie., hybrids, napiergrasses, and pearl millet.

	PEGL 09TX04		**Napiergrass**		**Hybrids**	
	PgTb1	**Tr54**	**PgTb1**	**Tr54**	**PgTb1**	**Tr54**
Mean	3.618	4.59	0.2692	0.1743	2.96	3.903
Std. Error of Mean	0.08726	0.1636	0.008516	0.01699	0.08165	0.1409
Std. Deviation	0.338	0.6336	0.03298	0.06582	0.3162	0.5456
Minimum	3.021	3.4	0.211	0.0697	2.415	2.903
Maximum	4.106	5.411	0.3219	0.2758	3.426	4.633

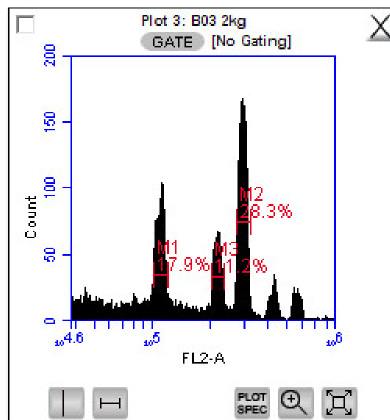
APPENDIX B



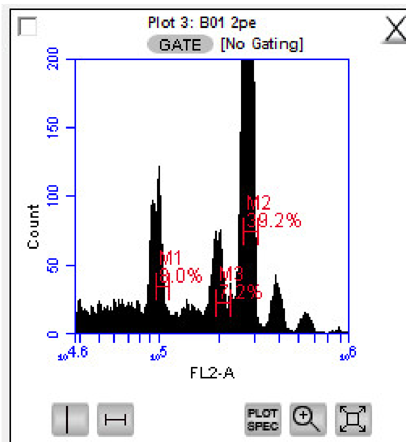
B-1 Sb x PMNV 14TX14; *Sorghum bicolor* and PMN samples mixed together in flow cytometer. Gated mean values were taken three times, M1 is the *Sorghum bicolor* 2C DNA content value, M2 is the PMN 2C DNA content value, and M3 is the 4C *Sorghum bicolor* DNA content value.



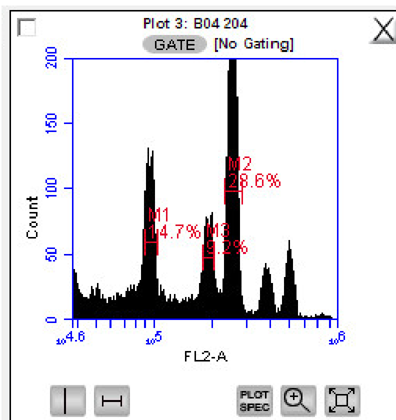
B-2 Sb x Merkeron; *Sorghum bicolor* and Merkeron samples mixed together in flow cytometer. Gated mean values were taken three times, M1 is the *Sorghum bicolor* 2C DNA content value, M2 is the Merkeron 2C DNA content value, and M3 is the 4C *Sorghum bicolor* DNA content value.



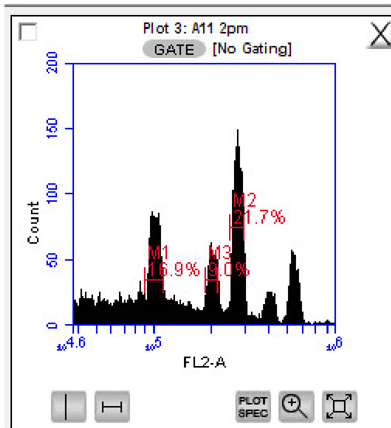
B-3 Sb x KGV 13TX01; *Sorghum bicolor* and PMN samples mixed together in flow cytometer. Gated mean values were taken three times, M1 is the *Sorghum bicolor* 2C DNA content value, M2 is the PMN 2C DNA content value, and M3 is the 4C *Sorghum bicolor* DNA content value.



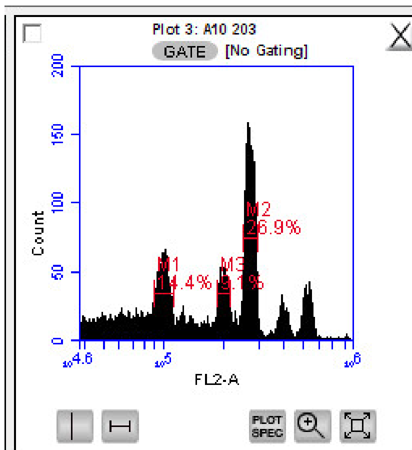
B-4 Sb x PEPU 09TX01; *Sorghum bicolor* and napiergrass samples mixed together in flow cytometer. Gated mean values were taken three times, M1 is the *Sorghum bicolor* 2C DNA content value, M2 is the napiergrass 2C DNA content value, and M3 is the 4C *Sorghum bicolor* DNA content value.



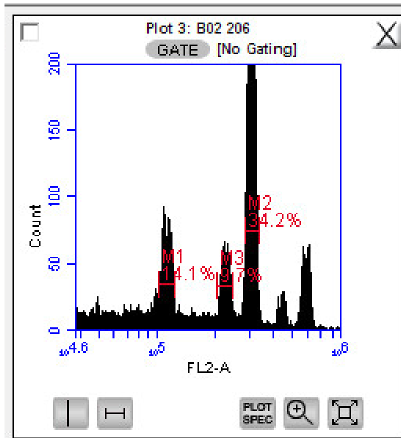
B-5 Sb x PEGL 09TX04; *Sorghum bicolor* and pearl millet samples mixed together in flow cytometer. Gated mean values were taken three times, M1 is the *Sorghum bicolor* 2C DNA content value, M2 is the pearl millet 2C DNA content value, and M3 is the 4C *Sorghum bicolor* DNA content value.



B-6 Sb x PMNV 13TX01; *Sorghum bicolor* and PMN samples mixed together in flow cytometer. Gated mean values were taken three times, M1 is the *Sorghum bicolor* 2C DNA content value, M2 is the PMN 2C DNA content value, and M3 is the 4C *Sorghum bicolor* DNA content value.



B-7 Sb x PMNV 14TX03; *Sorghum bicolor* and PMN samples mixed together in flow cytometer. Gated mean values were taken three times, M1 is the *Sorghum bicolor* 2C DNA content value, M2 is the PMN 2C DNA content value, and M3 is the 4C *Sorghum bicolor* DNA content value.



B-8 Sb x PMNV 13TX06; *Sorghum bicolor* and PMN samples mixed together in flow cytometer. Gated mean values were taken three times, M1 is the *Sorghum bicolor* 2C DNA content value, M2 is the PMN 2C DNA content value, and M3 is the 4C *Sorghum bicolor* DNA content value.