CLASSIFICATION OF ORNAMENTAL ACCESSIONS OF MISCANTHUS WITH MOLECULAR TECHNIQUES

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Plant Biology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2012

Urbana, Illinois

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Abstract

The perennial C4 grass genus *Miscanthus* has a long history of use as valued landscaping and garden ornamentals. More recently, interest in domestic energy security and sustainable fuel sources have brought it to the forefront as a sustainable and productive bioenergy feedstock. In particular, the sterile inter-specific hybrid *Miscanthus* x *giganteus,* has proven to be particularly productive over a wide range of habitats and sustainable. But it lacks genetic variation, a serious limitation to wide scale production. New forms of *M. x giganteus* could be achieved by crosses of the parent species. Not only are the parents of the hybrid uncertain, but the degree of diversity in accessions of the parent species in the USA is unknown, as is the relationship to other putative *Miscanthus* spp. Identification at the species level depends predominantly on floral characteristics, which is a major limitation for plants which may not flower in some locations and which are supplied as vegetative propagules. DNA based methods should overcome these limitations

This study tested the efficacy of three emergent DNA based methods for inter- and intra-specific separation. Simultaneously it also tested the proper categorization of accessions within species and the degree of diversity of commercially available *Miscanthus* accessions within the United States.

1) High Resolution Melting analysis was used to determine if accessions of *Miscanthus* could be distinguished at the species level. HRM was able to distinguish species and furthermore, it was found that material provided as *M.* x *giganteus* 'Kurt Bluemel' and *M. sinensis* 'Hercules' in fact corresponded to *M. sacchariflorus*. The method is low cost at scale and rapid. It could be particularly valuable for establishing the veracity of material supplied as a named accession.

2) Simple Sequence Repeats were used to look at both interspecific and intraspecific relationships of over 80 accessions of *Miscanthus* by using primers designed from sugarcane. That study showed that there was a clear separation of accessions at the species level and there was a low level of similarity between accessions even within the same species. This also showed that genetic variation in the ornamental accessions of *M. sinensis* commercially available in the USA, was high compared to material recently collected from known sites that were geographically widely separated.

3) A high throughput method of single nucleotide detection was applied to over 300 *Miscanthus* accessions that identified 803 SNP markers that allowed for individual fingerprints of each plant to be obtained. Overall, this method proved the most effective. It separated all accessions and provided clear evidence that at least three had been misclassified at the species level. It also confirmed the SSR study finding that there was wide diversity in the extant ornamental collections of *M. sinensis.*

Overall, this study established that the emergent DNA based screening methods are highly effective in both inter-specific and intra-specific differentiation of *Miscanthus* an important pre-requisite to an effective breeding program.

This dissertation is dedicated to:

My siblings, Bunmi, Funmi and Patience for always being supportive

ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor, Steve Long, whose expertise, understanding, and patience, added considerably to my graduate experience. I would like to thank the other members of my committee, Dr. Steve Moose, Dr. Tom Voigt and Dr. Ray Ming for the assistance they provided at all levels of my graduate studies.

A very special thanks goes out to Dr. Kankshita Swaminathan, without whose motivation and encouragement I would not have been able to keep my sanity during my time as a graduate student. She provided me with direction, technical support and became both a mentor and friend. I doubt that I will ever be able to convey my appreciation fully, but I owe her my eternal gratitude.

I would also like to thank Dr. Roger Kroes, Dr. Joesph Moskal and Dr. Nigel Otto for providing me with my first experience working in a laboratory and conducting independent research. If it weren't for their openness to allow a young high school student into their midst I would not have seen my passion for science flourish. In addition I would like to acknowledge Dr. Shawna Naidu for being open enough to allow a student who knew nothing about plants to work with her as a McNair scholar in undergrad. Without her giving me the opportunity to work under her, I would not have met Dr. Long.

The research in this dissertation would not have been possible without funding from the Energy Biosciences Institute, the National GEM Consortium Fellowship, and the American Society of Plant Biologist Travel Award.

I am especially grateful to past and current members of the Long Lab, Moose Lab, Voigt Lab and Agronomy Team that provided continual support and feedback on my research, field work and writing. Special thanks to Emily Doherty, Andy Wycislo and Becky Arundale for answering questions in regards to field related issues. I would also like to thank Won Byoung Chae for the intellectual conversations about *Miscanthus* taxonomy which showed me that my work had a place in the scientific community. Thank you to Rachel Shekar and Lindsay Clark for reading and commenting on chapters of this dissertation. I would like to thank Ashley Spence for being a friend through this whole process. She was always willing to coax me off the ledge when times became challenging in both research and life.

My research would not have gone as smoothly without the assistance from numerous undergrads that helped with field measurement, sample collection and preparation. I would like to acknowledge Ross Polk for his help with maintaining the field and setting up the *Miscanthus* database. I

would also like to thank Ornella Ngamboma for her work on scoring, rescoring and then scoring again the SSR data. In addition I would like thank Mike Ferrari, Derek Athy, Neil Thomas, Lecretia Williams and Chris Deamont for their help in collecting field data.

I would like to thank my friends and family for always being understanding through this process. My parents Wale and Remi Oladeinde have always been strong advocates for education and their constant encouragement allowed me to pursue my dream of obtaining a PhD. I would also like to thank my sister Patience for her constant distractions that kept me joined to the real world when I was too busy with school work. My sister Bunmi, has always been supportive and even though she moved to Nigeria 3 years ago she managed to keep me grounded in life and reinforced the priority that family is important.

Last but not least I would like to thank my two best friends. I have been truly blessed to have Tiara Wright and Jide Akinrinmade in my life. Even though I often got too consumed with school and my life in Champaign to visit, call or even text these two people always made sure to reach out to me. I can truly say that I love each one of them unconditionally for all the support they have given me.

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Chapter 1: General Introduction

Miscanthus Andersson. is a genus of perennial C4 grasses (Poacea) that is native to East Asia and Southeast Africa (Greef et al. 1997). *Miscanthus* is a member of the tribe Andropogoneae within the grass subfamily *Panicoideae*. Other members of the tribe include a number of highly productive grasses and the major crops: *Zea mays L.* (maize), *Sorghum bicolor* (L.) Moench (sorghum) and *Saccharum officinarium L.* (sugarcane) (Hodkinson et. al., 2002; Amalraj and Balasundaram , 2005). All species of this tribe use the NADP-malic enzyme (ME) version of C4 photosynthesis (Anderson et al. 2011). C4 photosynthesis also confers high water and high nitrogen use efficiencies as well as high light energy conversion efficiencies that increase their potential for generating large quantities of biomass with low to no input (Lewandowski et al. 2000). Among variants of C4 photosynthesis, the NADP-malic enzyme form appears the most efficient form of C4 photosynthesis in terms of both energy transduction efficiency and nitrogen use (Ehleringer and Pearcy 1983; Taub and Lerdau 2000; Ghannoum et al. 2005). All species of the genus *Miscanthus* are perennial and have cane-like stems. However, a consensus on the taxonomical method of classifying the species has yet to be achieved. *Miscanthus sensu lato* (in a broad sense) contains approximately 14–20 species (Hodkinson, Chase, Lledó, et al. 2002; Clifton-Brown et al. 2008; Sun et al. 2010) while *Miscanthus sensu stricto* (in a strict sense) contains 11-12 species (Hodkinson and Chase 2002; Clifton-Brown et al. 2008; Vermerris 2008). Even though taxonomical studies on *Miscanthus* have been ongoing since 1856 (Amalraj and Balasundaram 2005; Sun et al. 2010) there is still no consensus on the definition of the two groups, the number of species, subspecies, varieties and cultivars or the taxonomic system of identification to be used.

The interest in biomass feedstocks for bioenergy came to the forefront in the US when in 2005 the Billion Ton Study indicated that, by using perennial grasses, alongside crop residues and some forestry the US had sufficient land and potential biomass resources to achieve over 1Bt of biomass (Perlack et al.

2005). Converted to cellulosic ethanol, this would be sufficient to displace more than 30% of U.S. petroleum by 2030 use (Perlack et al. 2005). This has been confirmed by the recent update of the report which benefitted from more detailed experience with the potential feedstocks (Perlack and Stokes 2011). The report played a key part in the development of the Energy Independence and Security Act of 2007 which was passed into law by the 110th Congress of the USA. This mandated 140 billion liters of biofuels by 2022, of which 79 billion liters must be derived from non-cornstarch products (e.g. sugar or lingo-cellulose). Assuming a conversion efficiency of biomass to ethanol of 380 l t^{-1} (Heaton et al. 2008) this would require 20 Pg (20 billion metric tons) of lingo-cellulosic biomass annually by 2022. To achieve this with minimum impact on agricultural land, other land and ecosystem services, requires productive and sustainable perennials that require few or no inputs. Fall harvested perennial C4 grasses appear to fit this requirement well, of which *Miscanthus* species appear particularly promising (Heaton et al. 2008).

Three perennial grass species, *M.* ×*giganteus*, *M. sacchariflorus*, and *M. sinensis*, have been identified as having high potential for biomass production (Jones and Walsh 2001).

One perennial grass that has special potential as a biomass feedstock is *Miscanthus* x *giganteus* Greef & Deuter ex Hodkinson & Renvoize (Beale and Long 1995; Lewandowski et al. 2000; Hodkinson and Renvoize 2001; Heaton et al. 2004, 2008; Dohleman et al. 2009). It has been known as *Miscanthus sinensis* '*Giganteus*', *Miscanthus ogiformis* (Honda) Adati as well as *Miscanthus sacchariflorus* var. brevibarbis (Clifton-Brown et al. 2008). This sterile hybrid was collected in southern Japan in 1932 and transferred to Denmark (Stewart et al. 2009) and was cultivated and distributed for landscaping since then. However, in the 1980s and onwards its tall stature attracted interest as a potential biomass feedstock for temperate environments (Jones and Walsh 2001). Indeed, replicated trials in S. England at 52° N, showed a peak biomass of 30 Mg ha^{-1} and a fall harvested dry biomass of 20 Mg ha^{-1} without any

addition of fertilizer; this remains the highest yield recorded for any crop in the UK (Beale and Long 1995) Promising yields have been recorded across much of W. Europe (Lewandowski et. al., 2000). *M. x giganteus* has proved successful in trials in Europe and more recently in Illinois (Heaton et al. 2008; Dohleman et al. 2009). A mechanistically based growth and production model, parameterized on this clone predicts that it could yield well (>25 Mg ha^{-1}) through much of the eastern half of the 48 states (Miguez et al. 2011). However, as noted above *M.* x *giganteus* is a sterile clone, this has the advantage that it minimizes the risk of any invasive spread. This however, also has two major disadvantages if the crop is to be grown at scale. First, because all plants will be genetically identical, save somatic mutations and any epigenetic differences, it will be potentially vulnerable to an epidemic of any pest or disease of the crop which (Agindotan et al. 2010; Ahonsi et al. 2011; Mekete et al. 2011) emerges. Secondly, one genotype cannot be edaphically and climatically suited to all conditions. A successful crop will therefore require a breeding program in which more forms of *M.* x *giganteus* are produced to introduce diversity in both biotic and abiotic tolerance. Achieving this requires knowledge of both the origins of *M.* x *giganteus* and variability within current collections of the assumed parent species, and identification of species sufficiently closely related that they might be used in breeding programs.

M. x *giganteus* is an allotriploid plant with 2n =3x = 57 chromosomes which makes it sterile and requires vegetative propagation from its rhizomes. There have been two prominent hypotheses as to the allotripolod origin of *M.* x *giganteus*. The first being that an allotetraploid (*M. sinensis* x *M. sacchariflorus*) and a diploid of either *M. sinensis* or *M. sacchariflorus* hybridized in Japan in a region where both plant species overlap in growth and flowering time. The other hypothesis is that *M.* x *giganteus* originated from a diploid *M. sinensis* and *M. sacchariflorus* and one of the parents produced an unreduced gamete (Hodkinson, Chase, Takahashi, et al. 2002). Internal transcribed spacer (ITS) rDNA sequencing has demonstrated that *M.* x *giganteus* is almost certainly a hybrid produced from *M. sinensis*

and *M. sacchariflorus*. From this study it was also determined that the maternal genome donor was *M. sacchariflorus* based on plastid DNA markers (Hodkinson, Chase, Takahashi, et al. 2002).

The most obvious feature that distinguishes *M. sinensis* from *M. sacchariflorus* and *M.* x *giganteus* is that the former is tufted, also known as clump forming, while the latter two produce vigorous lateral offshoots, making it more rhizomatous or spreading. Other features that separate these species are that *M. sinensis* has awned spikelets, no presence of adventitious roots and lack of branching on the stems while *M. sacchariflorus* and *M.* x *giganteus* is opposite in these phenotypic features (Sun et al., 2010). Both *M. sinensis* and *M. sacchariflorus* can be propagated vegetatively or through seed, although seed viability varies. All three species were originally introduced into to the United States for landscape planting, which has made it the prominent garden and landscaping ornamental that it is today. However, this also make it difficult to determine the origins for many accessions of *Miscanthus* currently distributed because of their long history as horticultural crops that have been passed from nursery to nursery and garden to garden (Hodkinson et al. 2002b).

Miscanthus is closely related to *Saccharum* to the extent that inter-generic and fertile hybrids may be obtained. This has been exploited for the improvement of sugarcane (*Saccharum officinarum*) by hybridization with *M. sinensis*, and then several rounds of backcrossing with *S. officinarum* to introduce, for example, disease resistance traits (Loureiro et al. 2011). The most prominent phenotypic difference between *Saccharum* and *Miscanthus* is the position of the spikelets on the flowering panicle and the fragility of the rachis (Hodkinson et al., 2002b). Because *Miscanthus* and *Saccharum* are so similar, solely morphologically based characterization of these genera may have led to errors in classification between the two genus. Further, since both genera may flower rarely or never in some environments, and both are propagated vegetatively, a reliable means of classification other than flower morphology would be highly advantageous and should now be possible through emergent DNA based methods. A

secondary need, if breeding is commercialized, will be for the developers of advantaged hybrids to be able to protect their intellectual property. This may only be possible if cheap DNA based methods can be developed to identify unique germplasm in the vegetative state.

There is now some information at the molecular level to help evaluate past morphologically based taxonomies of *Miscanthus* and related genera. Key studies have been done to help in classifying familiarity but have been unable differentiate between closely related varieties within a species. The end result of the studies done on *Miscanthus* showed that there was a clear separation between *M. sinensis* and *M. sacchariflorus* while the other species that were included in the study fell into clusters closer to *M. sinensis* but with unclear separation (Jones and Walsh, 2001). The ability to identify the inter-relationships among species have been conducted, but these studies have unable to detect differences between varieties (Hodkinson et. al., 2002a; Hodkinson, M W Chase, Lledo, et al., 2002) . For example *M. sinensis* var. condensatus was not distinguishable from other M. *sinensis* accessions samples.

Molecular studies of *Miscanthus* have been conducted using isozyme analysis to assess genetic diversity (Zub 2011) as well as amplified fragment length polymorphism (AFLP) (Greef et al. 1997) both at the inter and intra specific levels. These studies were limited in terms of the number of cultivars sampled and that only 6 primers sets were used in the AFLP study and 13 primers in the isozyme, this however reflected the fact that these methods of 15 years ago were very time consuming compared to the methods available today. Hodkinson et al in 1997 used DNA sequences from the internal transcribed spacer of nuclear ribosomoal DNA (nrITS) to distinguish inter specific relationships within *Miscanthus* but were unable to differentiate between cultivars or varieties. Later work by this group used AFLPs and inter-simple sequence repeats (ISSR) for DNA fingerprinting (Hodkinson et al., 2002a). These studies were limited in the number of actual primer pairs that could be used to assess diversity

within *Miscanthus* and the reproducibility in the testing platforms. A total of three AFLP primers and 2 ISSR primers which produced 26 markers were used to fingerprint 75 *Miscanthus* accessions (Hodkinson et al., 2002a). It showed that *M. floridulus* and *M. sinensis* ssp. condensatus could not be distinguished from *M. sinensis*. However, *M. x giganteus* and *M. sacchariflorus* groups were identifiable.

More recent studies have used chloroplast microsatellites (cpSSRs) developed from the complete chloroplast genome of sugarcane to help in differentiating species within the *Miscanthus* genus (Cesare et al. 2010). The six cpSSR markers developed for this study were highly polymorphic and were tested on 164 *Miscanthus* genotypes. Although sample size was robust the number of markers used still did not allow for clear intra-specific differentiation. With the recent release of SSR markers from the model grass species *Brachypodium distachyon* these have also been tested on *M. sinensis* accessions (Zhao et al. 2011). Out of the 57 SSR markers selected for testing on *Miscanthus* 86% of them were effective. The phylogenetic tree grouped the 21 *Miscanthus sinensis* accessions into 3 clusters that correlated with the geographical distribution and ecotype classification (Zhao et al. 2011). SSR markers have been used increasingly to assess *Miscanthus* diversity, but as yet they have not been used to assess the level of diversity already present in the USA, in the form of ornamental accessions (Hung et al. 2008; Zhao et al. 2011; Ho et al. 2011).

Historically, *Miscanthus* species and varieties were distributed by horticultural suppliers, or from gardener to gardener. Classification has been based on morphological information, and sometimes depends on floral features. Currently, limited information is available about the genetic diversity within the genus and extant collections in the USA (Hodkinson, Chase, Takahashi, et al. 2002; Stewart et al. 2009). The complete DNA sequence of an organism's genome would be the ultimate tool for characterization of a genotype and provide the means to determine exact similarity between individuals. Only a few plants have been completely sequenced and preference has been for small diploid genomes

(Armstead et al. 2009) compared to higher ploidy leveled plants such as *M.* x *giganteus* that has a genome size 7.5 Gbp (Swaminathan et al. 2010). Since complete sequencing has been undertaken for only a few plant species, sequencing of genes, parts of genes or non-coding single sequence repeats remains a more practical means. Techniques for these approaches have developed rapidly opening new potential opportunities, although the relative merits of different approaches in typing plant collections remain uncertain. The aim of this research is to explore these new methods in analyzing inter- and intra-specific genetic diversity and relationships within *Miscanthus*. Further to identify unique germplasm, for example in protecting breeder's rights or a utility patent, a quick but unambiguous DNA based genotyping method needs to be identified. These aims are addressed here by testing three emerging DNA typing methods on 330 *Miscanthus* accessions held at the University of Illinois.

1) High Resolution Melt analysis (HRM) is a technique used to detect mutations and polymorphism differences in double stranded DNA (dsDNA). The technique uses a fluorescently labeled dye that binds specifically to dsDNA. As temperature increases the dsDNA becomes single stranded and the fluorescent dye falls off. The sequence of the DNA base pairs affects the melting temperature and the differences in the melting profile are used to distinguish samples from one another. This technique is cost effective if the user already has access to the machinery and requires a short amount of time from set up to analysis. HRM analysis is used in Chapter 2 to assess if species level differences of *Miscanthus* can be distinguished.

2) Simple Sequence Repeats (SSRs) are two to six base pairs of repeating DNA sequences. Primers can be designed to amplify SSRs and then they are subsequently used as molecular markers. In Chapter 3 sugarcane derived SSR primers were used to assess the genetic diversity between and within *Miscanthus* species. SSRs can be run on a gel based system or using a capillary DNA analyzer which makes it a technique that anyone can use.

3) Single Nucleotide Polymorphisms (SNPs) are abundant in both human and plant genomes. A custom 1,536 SNP arrays was used in Chapter 3 to genotype 393 *Miscanthus* accessions and produce a unique fingerprint profile for each sample. The SNP array uses allele specific primers with fluorescent dyes to detect differences at each locus. The high-throughput nature of this technique allows for multiple 96 samples to be genotyped at 1,536 loci at once.

The 330 accessions includes both a broad collection of ornamental accessions both from local gardens and suppliers and from two major national suppliers (Kurt Bluemel Inc., Baldwin, MD and Emerald Coast Growers, Pensacola, FL), 240 *Miscanthus sinensis* accessions that were planted from seed (Jelitto Perennial Seeds, Louisville, KY), and some material recently collected from known locations along the length of the Japanese Islands.

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Chapter 2: High Resolution Melt Analysis can be used as a rapid screening method to identify and separate *Miscanthus* accessions.

Abstract

Miscanthus is a genus of perennial C4 grasses that are native to East Asia and Southeast Africa. *Miscanthus* accessions have been widely distributed across the United States for horticultural use as ornamental plants. More recently, *M. sinensis, M. sacchariflorus* and their inter-specific hybrid, *M. x giganteus* have attracted interest as sustainable bioenergy feedstocks. While some lines have proved sterile and productive, others may be less productive or invasive. As large scale plantings are considered, it has become critical that growers can definitively identify the species, variety and accession that they are planting. Taxonomical identification of accessions has relied, until now, on phenotypic characteristics. Collecting phenotypic information is time consuming and depends on flowering that may or may not appear at a given location within a given year. High Resolution Melting (HRM) of double-stranded DNA is a potentially rapid and cost-effective means of detecting unique differences in DNA between individuals without the need of sequencing. To test its efficiency in practice one plant from each of eleven accessions of *Miscanthus* was assessed for genotypic variations by using 96 primer sets by HRM. The primers were designed from transcriptome information obtained from two *M. sinensis* accessions, 'Gross Fontaine' and 'Undine'. HRM proved effective in separating all accessions and identifying their likely relationships. Morphology and flowering time correlated with HRM findings. Four out of the five plants labeled as *M. x giganteus* species grouped together. However, one plant labeled as *M. sinensis* and another labeled as *M. x giganteus* were actually found to be *M.*

sacchariflorus. This result underscores the problem of past mislabeling or misidentification that needs to be resolved prior to large-scale planting of these bioenergy crops. Overall, the HRM platform can be used as a rapid means of genotyping *Miscanthus* accessions.

Introduction

Miscanthus is a genus of perennial C4 rhizomatous grasses that are native to East Asia and Southeast Africa (Greef and Deuter, 1993). There are debatably 14-20 species that comprise the *Miscanthus* genus (Sun et al., 2010; Chou 2009;Lewandowski et. al., 2003). This number varies depending on the criteria used for classification and grouping of species. One species that has particular potential as a biomass feedstock is *Miscanthus* x *giganteus* GREEF et DEU (Lewandowski et. al., 2000). This sterile hybrid has been cultivated in Europe since the 1930s. Although used in landscaping, more recently it has been considered as a bioenergy feedstock, with field trials in Europe starting in 1983 (Lewandowski et. al., 2000). Not only has it proved highly productive under low or zero input conditions, but its sterility averts any significant risk of it becoming an invasive pest. It has been proposed that *M.* x *giganteus* is the offspring of a diploid *M. sinensis* (2n=2x=38) and a tetraploid *M. sacchariflorus* (2n=4x=76) (Lafferty and Lelley 1994; Jones and Walsh 2001; Clifton-Brown et al. 2008; Yu et al. 2009). Because *M.* x *giganteus* is a sterile triploid it must be propagated vegetatively by rhizomes and only clonal offspring are produced. However, since rhizomes cannot be distinguished among accessions, a molecular means to characterize these would be particularly valuable. Although *M. x giganteus* distributed throughout Europe and the USA is most likely from the single clone first brought to Denmark in 1932, it is possible that somatic mutations and epigenetic changes could have caused some divergence even within this cloned material.

Accessions of the parent species have also been trialed as potential bioenergy feedstocks. *Miscanthus sinensis* accessions were originally transported to the United States for planting in

landscapes, which has made it a prominent garden and landscaping ornamental across the country today. Yet, their long history as horticultural crops, often being passed from nursery to nursery, and garden to garden has made it difficult to determine the origins of many accessions, and to maintain accurate labeling, of *Miscanthus* currently distributed throughout the US (Hodkinson et al. 2002b). While these plants have not proved invasive in long-term trials in Europe, there is evidence that some accessions planted into gardens in the eastern USA have escaped (Raghu and Anderson 2006; Quinn et al. 2010). It will therefore be particularly important to be able to rapidly identify accessions with these invasive characteristics.

The classification and identification of *Miscanthus* varieties has relied heavily on phenotypic characteristics observed by those working in the nursery industry, most related to their ornamental traits. At the morphological level, studies have been conducted on *Miscanthus* to improve taxonomy within the genus. Most have focused on conserved floral features, including length of the inflorescence axis, length of the racemes, disposition of spikelets on the axis, nerves of glumes, dorsal hairs of glumes and the presence or absence of awns (Jones and Walsh, 2001). However, these cannot be applied to rhizomes or other material that is not flowering; they also require considerable experience with floral characters and are time consuming.

High Resolution Melting (HRM) is a molecular tool used for the detection of mutations, epigenetic change and polymorphisms (Reed et. al., 2007). This technique was developed by Idaho Technology in conjunction with the University of Utah (Liew et al., 2004) and is based on the fact that the temperature required to disassociate a double stranded DNA (dsDNA) fragment (i.e. melting point) is dependent on the sequence of nucleotides that make up that fragment. G:C bonds melt at a higher temperature than A:T, therefore melting temperature is dependent on G:C content. Arrangement of base pairs in the amplicon can also affect the melting curve profile. Thus, the melting curve can be used

to differentiate slightly differing sequences, making it a useful molecular marker and allowing rapid identification of variation between accessions, without the need for sequencing.

The HRM technique works as follows: DNA fragments of ~200bp are amplified with primers during polymerase chain reaction (PCR). The double stranded DNA fragments are then heated at a steady incline. The exact temperature at dissociation is detected using dyes that fluoresce only when the DNA is in its double stranded form. As temperature rises, fluorescence decreases as a result of the dissociation of the DNA from its double stranded form to single stranded. When the melting point is reached, there is a rapid decline in fluorescence which is readily seen in the melting curve profile. Based on the difference in peaks in the derivative melting curve for specific regions of DNA, variation within the genome is detected without the need for costly whole genome sequencing. The HRM platform is sensitive enough to have been used successfully to genotype plant DNA sequence polymorphisms in species as diverse as ryegrass and almond (Wu et al. 2008; Studer et al. 2009).

In this study, the HRM platform will be used to genotype 5 accessions originally identified as *M*. x *giganteus*, 3 as *M*. *sacchariflorus* accessions and 3 as *M*. *sinensis* accessions. This platform will be used to determine if interspecific relationships within the *Miscanthus* genus can be clearly delineated. This information will then be compared to morphological observations. The viability of HRM markers as a low-cost fingerprinting tool for the *Miscanthus* breeding community is demonstrated in this study.

Materials and Methods

Establishment of Common Garden

A common garden containing replicated plantings of several accessions of different perennial grass species and potential feedstocks was established at the University of Illinois Energy Farm just south of the site in Urbana, IL, USA (40°03’ 21.3”N, 88°12’3.4”W, 230m elevation). The soil type in this

area is Drummer soil and it is a deep, dark black topsoil that is common to this area (Alexander et al., 1974).

Potted plants of different accessions of *M. x giganteus, M. sacchariflorus and M. sinensis* were provided by Kurt Bluemel Nursery, Emerald Coast Growers and Mendel Biotechnology. 65 accessions of *Miscanthus* spp., including the 11 used here, and 15 accessions of other perennial grasses, were planted, 9 individuals per accession in a completely randomized design (CRD). Rows were at 0.6 m spacing with individuals within rows separated by 0.9m. After planting, a unique barcode was placed next to each individual plant to ensure unambiguous tracking of accessions.

Plant Material

Accessions of *Miscanthus* used in this study can be found in Table 2.1 and all but 2 of the accessions were from the above common garden experiment. The accessions used in this study were delivered with the following names: *M.* x *giganteus* 'Bixby', *M.* x *giganteus* 'Frank', *M.* x *giganteus* 'Gmax', *M.* x *giganteus* 'Illinois', *M.* x *giganteus* 'Kurt Bluemel', *M. sacchariflorus* 'Robustus', *M. sacchariflorus* 'Golf Course', *M. sinensis* 'Andante', *M. sinensis* 'Hercules', *M. sinensis* 'Sarrabande', *M. sinensis* 'Zebrinus'. *M.* x *giganteus* 'Frank' was grown in the Plant Biology Greenhouse in a plastic flat that is 11 inches long, 22 inches wide and 2" deep while *M.* x *giganteus* 'Bixby' only leaf tissue was sampled from an offsite location.

Morphology

Morphological characteristics were recorded for 10 of the accessions which include: growth habit, presence of flowering and date of flowering (Table 2.2). Morphological observations were not recorded for *M. x giganteus* 'Bixby', which was grown in Oklahoma. The growth habit was recorded as either Tufted (T) or Rhizomatous (R). Tufted were clump forming plants while rhizomatous plants had a spreading characteristic. The flowering date of plants growing at the Energy Farm was assessed via

biweekly visual observations beginning the second week of June. Flowering date was defined as the date when at least one panicle was fully emerged from the shoot.

CTAB Genomic DNA Isolation

Young leaf tissue was collected from 1 plant from each of the 11 *Miscanthus* accessions by cutting ~10 tillers from each plant and peeling back old leaves at the leaf sheath to expose the young un-emerged leaf tissue, which was cut, wrapped in foil and immediately plunged into liquid nitrogen. The frozen sample was subsequently ground to a fine powder in a pre-chilled mortar and then ca. 4 g was transferred to a 50ml polypropylene centrifuge tube (CLS430291, Corning Inc., Corning NY) and stored at -80 °C. Genomic DNA was isolated from all the accessions of *Miscanthus* using a modified cetyltrimethylammonium bromide (CTAB) extraction protocol (Doyle & Doyle, 1987) as described below.

20ml of CTAB extraction buffer (appendix) and 50µl of β-mercaptoethanol (βME) were added to the powdered tissue in the centrifuge tube and vortexed for 30s. The suspension was incubated at 65°C in a water bath for 1 hour and inverted at 30 min to ensure complete mixing. Samples were then cooled to room temperature. An equal volume of phenol:chloforom:isoamyl alcohol mixture (25:24:1) at pH 6.7 was added to each tube and inverted to ensure proper mixing. The samples were centrifuged at 7000-8000g at 10°C for 10 minutes. The aqueous top phase was transferred to a new 50ml tube and the lower phases discarded. The phenol:chloforom:isoamyl alcohol (25:24:1) addition step and centrifugation were repeated and the upper phase removed and mixed with equal volumes of chloroform:isoamyl alcohol (24:1). The samples were then inverted and centrifuged at 7000-8000g at 10°C for 10 minutes and the aqueous top phase was again transferred to a new tube where 0.7 volumes of 2-proponal was added to the tube. The tubes were then inverted gently ~5 times and stood for 5 minutes until a visible DNA precipitate was present in solution. The precipitate strands were wrapped around a Pasteur pipette tip and transferred to a 50 ml tube containing 20ml of 70% ethanol then centrifuged at 7500 g at 20°C for 10 minutes. The resulting pellet was air dried and then resuspended with 500 µl of 1X Tris-

EDTA buffer pH 8.0 with 2 µl of Ribonuclease A (RNase A**,** A7973, Promega Corp, Madison, WI). 5µl of the resuspended liquid was loaded onto a 1% agarose gel and run at 110V for 50min to assess the quality of the DNA and a further 1µl was used to quantify the amount of DNA spectrophotometrically (Nanodrop ND-1000, Thermo-Scientific, Wilmington, DE).

Primer Design & HRM setup

Primer design was based on transcriptome sequencing of two diploids, *M. sinensis* 'Gross Fontaine' and *M. sinensis* 'Undine', by targeting regions where single nucleotide variations were detected (SNVs). A total of 96 primer pairs were designed (Swaminathan et al. 2012), each amplifying a region of approximately 200bp containing an SNV (Table 2.3). All primers had a M13 tail added (M13for-21 tail GTAAAACGACGGCCAGT, M13rev-24 tail AACAGCTATGACCATG). These primers were designed to have an annealing temperature of ~55°C and a GC content of 45-55%.

PCR amplification and HRM analysis were carried out sequentially on a Real-Time PCR System (LightCycler 480, Roche Applied Science, Indianapolis, IN). RT-PCR used a 10µl total volume containing: 1µl of DNA (5ng/µl), 5µl of proprietary "LightCycler 480 HRM master mix" which contains a heat activated DNA polymerase ("hot start" PCR enzyme) and a novel proprietary saturating DNA dye (Roche Applied Science, Indianapolis, IN), 1µl of .5µM of each primer set, 1µl of 25mM $MgCl_2$ and 2µl of water. The program consisted of an initial denaturing step at 95°C for 10min followed by 35 cycles of 95°C for 10s, 55°C for 10s and 72°C for 15s, then a final extension step of 72°C for 2min. Melt curve analysis was conducted by raising the temperature from 72°C to 90°C with 0.1°C/s increase per acquisition step. All reactions were conducted in a 384 well plate format with the 11 accessions and 1 water control. 32 primer sets were used in each 384 well plate.

HRM Melt Curve Analysis

Proprietary software (The LightCycler® 480 Gene Scanning Software v1.50, Roche Applied Science, Indianapolis, IN) was used to analyze the raw melt curves. The software automatically uses a negative filter to detect samples with low fluorescence or that lack a clear melting curve. Raw melting data was normalized to the pre-melt (initial fluorescence) and post-melt (final fluorescence) signals of all the samples (Figure 2.1). The curves were then normalized further by temperature shift. The point where the entire dsDNA was completely denatured was set as the threshold (Figure 2.2). A default temperature shift threshold of 5% is automatically applied to all melt curves and was only changed if necessary for a specific primer set. Each primer subset was examined for variations in the melting curve profile which could then be used as a genetic marker.

Each primer set (1 primer pair, 11 accessions) was scored by first assessing default grouping patterns present in the melting curve profiles that were visualized after normalization standards had been applied. The accessions were scored based on a presence/absence system. If the accession was assigned to a specific group by the program then it would receive a score of 1 for presence. Any accession not belonging to that specific group was subsequently given a score of 0 for absent. Using Figure 2.4 as an example, all the accessions that were blue were assigned to group 1 and therefore would be scored as 1 which indicated presence of that marker. All other samples would have a 0 score for group 1. All samples marked as purple would have a score of 1 for group 2 and every other sample would be scored as 0 for group 2 and so forth. Scoring was completed on all subsets and cluster analysis was performed using NTSYSpc 2.1 software package (Exeter Software, Setauket, NY).

Cluster Analysis

A Jaccard coefficient of similarity was used along with the sequential, agglomerative, hierarchical, nested cluster analysis (SAHN) to compile an unweighted pair group method with arithmetic averaging (UPGMA) dendrogram. The Jaccard coefficient of similarity considers only shared

1s as contributing to the similarity of individuals and does not account for any shared 0s (Kosman and Leonard 2005).

Results

96 primers were tested on 11 *Miscanthus* accessions. Fifteen of the primer pairs used in analysis were non-informative (Table 2.3), i.e. there was either no amplification or the melt curves did not show any variation between amplicons (Figure 2.3). The remaining 81 primers were able to produce variations within the melt curves for the accessions yielding a total of 304 different melt profiles. HRM clearly separated all 11 accessions tested; however it also grouped accessions according to pre-analysis designation into the three species in most cases (Figure 2.4). The three species had less than 22% similarity to each other (Table2.4).

Four out of the five accessions that were labeled as being *M.* x *giganteus* grouped within one clade. *M.* x *giganteus* 'Frank' and *M.* x *giganteus* 'Illinois' had the highest similarity at 74%. *M.* x *giganteus* 'Gmax' had the lowest similarity (Table 2.4) but still grouped within the *M.* x *giganteus* clade along with *M.* x *giganteus* 'Bixby' (Figure 2.5). Surprisingly, *M*. x *giganteus* 'Kurt Bluemel' did not group within the *M.* x *giganteus* clade. Instead, it grouped with the *M. sacchariflorus* clade. *M*. x *giganteus* 'Kurt Bluemel' was most similar to *M. sacchariflorus* 'Robustus.' Also surprising was that *M. sinensis* 'Hercules' grouped with the *M*. *sacchariflorus* clade. Three of the four plants previously labeled *M. sinensis* grouped with the *M. sinensis* clade.

The morphological observations collected are documented in Table 2.2. All accessions originally identified as *M.* x *giganteus* and *M. sacchariflorus* had a rhizomatous growth habit, which is distinguished by a spreading growth pattern. Plants originally labeled as *M. sinensis* accessions, except for *M. sinensis* 'Hercules,' were tufted (clump forming) in their growth habits. *M. sinensis* 'Hercules' also

flowered in July along with the *M. sacchariflorus* accessions. Two *M.* x *giganteus* accessions and *M. sinensis* 'Zebrinus' flowered in October. *M.* x *giganteus* 'Kurt Bluemel' did not flower at all.

Discussion

HRM successfully separated all 11 accessions including accessions of *M. x giganteus* which most likely differ only in somatic mutations or epigenetic changes. It also grouped the accessions according to species, and identified two accessions that had been wrongly classified by their suppliers. All *M. sacchariflorus* grouped together with *M. sinensis* 'Hercules' which had phenotypic characteristics, such as early flowering and rhizomatous growth pattern, more closely related to the *M. sacchariflorus* species (Sun et al. 2010). This suggests that *M. sinensis* 'Hercules", as supplied, was previously mislabeled as *M. sinensis* and is in fact a *M. sacchariflorus.* All accessions originally labeled *M.* x *giganteus* were part of the same clade except for *M*. x *giganteus* 'Kurt Bluemel.' Although the developmental timing of *M*. x *giganteus* 'Kurt Bluemel ' (Table 2) did not show a clear identification with one species, its HRM grouping with the *M. sacchariflorus* clade (Figure 2.5) is confirmed by cell DNA content as determined by flow cytometry data and ploidy (Won Byong Chae, personal communication). The results suggest that *M. x giganteus* 'Kurt Bluemel' was incorrectly labeled by the supplier and is in fact a *M. sacchariflorus*. Even though the *M.* x *giganteus* 'Kurt Bluemel' accession did not flower during the field season it is known that flowering time varies substantially within species originating from a wide latitudinal range, as is the case for *M. sacchariflorus*. Therefore *M. x giganteus* 'Kurt Bluemel' is likely to originate from lower latitude (e.g. closer to the equator) than the other *M. sacchariflorus* accessions in the common garden, and would require a longer growing season to flower.

The HRM derived dendrogram suggests that the *M. sinensis* and *M. sacchariflorus* ornamental accessions sampled are highly diverse with a similarity ranging from 18%-50% between both species (Table 2.4) and not as closely related as previously assumed (Figure 2.5). This may be due to the fact that

the lines that have been introduced into the U.S. nursery industry were selected for aesthetic beauty and collected from a broad geographic range.

Two major conclusions may be drawn from this analysis. 1) While accessions can be reliably separated, at least to species level, by flower morphology, this technique is not suitable if plants do not flower at some locations or identification of rhizome or seed stock is necessary. HRM is shown to serve as a rapid and practical method to separate *Miscanthus* species and cultivars within species, as demonstrated. At a practical level, as new cultivars of *Miscanthus* are developed and commercialized, HRM could prove a valuable technique for protecting this intellectual property. 2) The large apparent genetic differences between cultivars suggest that the morphological diversity found in *Miscanthus* cultivars primarily originates from natural genetic variation. It is unlikely that *M.* x *giganteus* accessions tested in this study are from more than a single original collection but they are all distinguishable with HRM.

It is also important to discuss the time and cost associated with using HRM as a screening method for *Miscanthus*. HRM is a rapid method in regards to the amount of time needed to collect the samples and run the PCR and subsequent High Resolution Melting. Following genomic DNA isolation set up time for a single 384 well plate with 96 primers was ~30min. The actually run time on the LightCycler 480 machinery is 1hr and 10min. The analysis time adds an additional ~1hour. In total, a single run from plate setup to analysis takes approximately 3hrs. This is a quick turnaround compared to Simple Sequence Repeat (SSR) screening that is analyzed via gel electrophoresis. In the case of SSRs, the set up time and the time for PCR would be the same as HRM but the samples would then need to be loaded onto a 4% Super Fine Resolution (SFR) agarose gel and run for ~2hours for separation of size fragments. Even without considering analysis time, which takes multiple hours to compare the patterns of bands on the gel image, the time for SSR exceeds the time required for HRM from plate setup to analysis. This

calculation is based on four samples being analyzed per technique used against 96 primers. The use of capillary electrophoresis is comparable in time to HRM at all steps except for at the analysis which takes longer in the capillary electrophoresis. In addition to saving time, HRM is also cost effective. A breakdown of cost can be found in the appendix Table (6.2) assuming 96 plant samples were to be tested with 32 primer sets. The reason for testing 32 sample sets is that from this study it was found that the species level separation was identifiable even with a minimum of 32 primer sets (data not shown). As primer sets were added to the analysis the only change that occurred was in the similarity coefficient. Overall, a subset of 32 primers can successfully be used to identify species level differences in *Miscanthus* accessions tested with the HRM method. The cost breakdown per sample is forty-two cents per sample in HRM while it is fifty cents per sample for capillary electrophoresis. If HRM was being used on a large data set the cost savings would add up over the long run.

Table 2.1 *Miscanthus* accessions used in the High Resolution Melt (HRM) study. The species and cultivar name for each accession corresponds to the name given when the plant was initially obtained from the source. Each accession has a unique barcode beginning with "EF" if the plant is planted in the common garden at the Energy Farm (Urbana, IL). Barcode numbers that have a range represent accessions where an individual plant could no longer be distinguished from its adjacent replicates.

Table 1. *Miscanthus* Accessions used in High Resolution Melt Analysis

Species	Cultivar	Barcode	Location	Source
M. sacchariflorus	Golf Course	NA	Energy Farm	Unknown
	Robustus	EF05(59-61)	Energy Farm	Kurt Bluemel Nursery/Mendel Biotechnology
M. sinensis	Andante	EF0241	Energy Farm	Kurt Bluemel Nursery
	Hercules	EF03(01-03)	Energy Farm	Kurt Bluemel Nursery
	Sarabande	EF0238	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
	Zebrinus	EF0307	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
M. x *giganteus*	Bixby	N/A	Bixby, Oklahoma	Bixby, Oklahoma
	Frank	N/A	Greenhouse	Mississippi State
	G-Max	EF0198	Energy Farm	Unknown
	Illinois	EF03(85-87)	Energy Farm	University of Illinois
	Kurt Bluemel	EF04(6-9)	Energy Farm	Kurt Bluemel Nursery

Table 2.2 Morphological observations of accessions sampled in this study. Growth Habit was recorded as either Rhizomatous (R) =spreading nature or Tufted (T) =clump forming. Flowering dates were assessed on a biweekly basis starting from the 2nd week of June. Flowering date was defined as the date when at least one panicle was fully emerged from the shoot.

Table 2.2 Morphological Characteristics

Species	Cultivar	Growth Habit	Flowering Date
M. sacchariflorus	Golf Course	R	7/11/2011
	Robustus	R	7/11/2011
M. sinensis	Andante	T	8/23/2011
	Hercules	R	7/11/2011
	Sarrabande	T	9/13/2011
	Zebrinus	T	10/14/2011
M. x *giganteus*	Bixby	N/A	N/A
	Frank	R	N/A
	G-Max	R	10/14/2011
	Illinois	R	10/14/2011
	Kurt Bluemel	R	none

Table 2.3 List of the 96 primer sets designed for High Resolution Melting Analysis. Primers marked in grey were non informative and either had no amplification or showed melt curves that depicted no variation between amplicons.

Table 2.3 Primer Information			
PrimerID	**Forward Primer**	**Reverse Primer**	**Contig**
GFUN1	ACTACAAGGCTCTCGAAGTCATGAAGG	TTCATCGCTGAAGCCATCATTGAGCATG	GFUNContig15281_1286
GFUN2	AGCTGATTCTGTGCCTATTGGTGTACTG	AGTGTGATGAGGAAGTGCAGCATGC	GFUNContig9489_660
GFUN3	CCAAGATAATGACGAAGAACCGTCTGATTCG	CTGCACTTGATCATCAGAGGACGG	GFUNContig17916_723
GFUN4	GCAACTCTTCTGTCCTATAGGCACC	TTGAAGGAGTGCCAATGGCTCCTC	GFUNContig20159_1535
GFUN5	TGCTATGGTGTGGCCACACCATGT	ATGAAGAATGGTAGCTGGATCGGCAC	GFUNContig9757_126
GFUN6	GATGATCTCTGCTGCACAATGGTTGAAC	ATGTCACCTTGCTTACTGTGGCCTG	GFUNContig17937_2335
GFUN7	CACCAGCAAGAAGCACCAGCAAGA	CCTGTCCTTGAGACAATATCTGGTATGG	GFUNContig16626_851
GFUN8	CTGTTCGCCATGCCAGAGATCACT	AGCAGCTGGAATGTGCAGTTGGAAG	GFUNContig18335_922
GFUN9	AGTTATGCTGGCTATTGGCGACCAC	TGGAGTTCCTGAGACGACATGCAC	GFUNContig16666_456
GFUN10	GCCGTTCTAGTATCCGCATTGGCT	CCATACCATGCACACATGTCTAGAAGTG	GFUNContig12925_418
GFUN11	GGATAATGTGGCTTCTATGTCAGCAACG	GGCCAGTACACAGAGGCAGAACTTGA	GFUNContig14016_602
GFUN12	GGTTGTGGTGGACAGCAAGATCAATC	TTATCTCCACCAATCCTTCTGCCTCAAG	GFUNContig12428_768
GFUN13	GGACATGGATGAGTGGATGAGACAC	TGCAAGATGGACTCTTCTTCTACTCCTC	GFUNContig17257_1535
GFUN14	CTCTACGCGTATACGATCTTGGTGTC	CATTGAATGTGACAACAGTCGGCTTGATCTG	GFUNContig20378_1618
GFUN15	GGCTGATATAGCTGCATGCTGCTC	TTGCTGACCATGTGAGGAACAGTGAGT	GFUNContig15903_1035
GFUN16	CAATCACCATGCTCGGCTGGATTGCA	GGTCTGTCACTGGCTCGATCTCAATG	GFUNContig15694_992
GFUN17	ACGAGAGCCTGGAGAAGCTCAAGA	TTGAGGTCCGCGAAGCTGAAGAAG	GFUNContig16263_552
GFUN18	GTTGCAGTATTGTCTGAGCAAGTGTTGTTGG	AGATGTCATCACCGGACTGGCAATAC	GFUNContig19452_1146
GFUN19	GTGAGCTATATTGCCTTCACAAGCGAG	AGGCAGGAGTCGGAGCAATGCTTAAC	GFUNContig16706_375
GFUN20	GGAAGTTCCTAGCGACGGAAGCTTCT	CGTGCAGAACATCCTTGAGACGCT	GFUNContig17337_1261
GFUN21	AACACACATACTGATCGCTGCTTCTAGG	GAATGCACCAACAACAGATGTCTTCTCATGG	GFUNContig17769_257
GFUN22	CGATTCTTCTGCAGTACTTGCCTGC	AGAAGATAGACCTGAGCAGCAACGATG	GFUNContig19792_3700
GFUN23	ATAGAACTTCCATGTCCGTGCTCATGG	GACACAGCCATGGACATTCACATGAC	GFUNContig13472_113
GFUN24	GGTCAGACATGAAGTTGCTGATGAGG	GCACCACGCTTCGATTACATCTTGAC	GFUNContig20460_331
GFUN25	CCATTCCATCAGTCTGCTGCTGCT	TGTTGCTGTTGTAAGCCTGTGGCAG	GFUNContig11068_522
GFUN26	TGACATGCAACCTGTACCATGGAATTGC	GACCTTCTTGAAGTTAGGCTGGTTAACC	GFUNContig7223_314
GFUN27	TCCTTGGTCTCAATGTAGGATCCATCAG	AACATGCACTCAGGCCTTGGACTTC	GFUNContig19060_1027
GFUN28	AGCAGCTCCTCATGGAGCAAGTCA	AGAGAGTTGTATCGAGCACGTCTGG	GFUNContig11300_1325
GFUN29	GGTGCGCGGAATATATGTGATATCTCTG	TTGGAGTTGGACCTACCAGAAGCTC	GFUNk25ctg2201346_85
GFUN30	CCTTCATAGGCTTCTGCGTGATGC	CGTTAGTCAGCTGAAGGCAATATCTGAAGG	GFUNContig17267_487
GFUN31	GGCATCTTCATCAACAAGAGGAGCAC	CGACGTGACATAATGGCAGCAGGA	GFUNContig19900_2935
GFUN32	CCATGAGCTGTGTTAGCATGCATAGC	ACTATGCTGCAGGTCCTGGTCTCT	GFUNContig14220_720
GFUN33	GGCAGGAAGACATACATGTTGGCG	GATGGTGGCCAACGTTGTGCTCTACA	GFUNContig16447_152

Table 2.3 Continued

PrimerID	Forward Primer	Reverse Primer	Contig
GFUN34	TGCTCAACAAGCTGCAAGAGCTGTC	TTGATGCCAGAGCCAATCTCACAGAG	GFUNContig15542_2728
GFUN35	CCAGCATCTTGATCTGATGATACGACAG	AATGATCACTCAGGAGGTGAAGCTGC	GFUNContig19789_133
GFUN36	GGTGACCACTCAATAGAGGCCTCA	AGGTTCCAGTTGCGTTGAGTGTATTCTTGTG	GFUNContig17974_1001
GFUN37	GAGAGCAGCCACTGAGATTATATGTTCC	GCCAGATGTGTGGTGACCAATGTC	GFUNContig13068_479
GFUN38	GCACGGTAGTGCTCATCCTCAATG	GAGCTCGGTATCTCTAGGAAGCAG	GFUNContig2922_351
GFUN39	GACAGGAATTACAGGACTTGACAGTCTC	CACCAATCGAGACTCAGGAACGTTG	GFUNContig20540_4918
GFUN40	GCTGTCCTTGGAATTGCTCTTATTGCTATGG	AAGAGGAACTGCTCGTCTTATATTCTGCTCG	GFUNContig10260_226
GFUN41	GCTTGCTCCTCATCGATTAGCTCAC	ACATCCTTGCAACTGTGGCAGTTGG	GFUNContig17924_316
GFUN42	GATGAGCTGGAGAAGAACCTGGAG	TACCAGAGTGAGCTGCTTGATGTGTTG	GFUNContig17850_1447
GFUN43	CAAGCTCAAGTTCGTCGACACCTC	AACTTAGGCCTTGAGCTTGCCATAGAAC	GFUNContig16152_1214
GFUN44	GGCTGCCACTGTAATGATAGCCAG	ACATCAAGGCGTTCGGATCTGCGT	GFUNContig16696_1059
GFUN45	AGCTCACTTGTTCAATGCACCGATTAAGGAG	TTCCTCTTGAATGCTAGACCTGCAAGC	GFUNContig20063_1843
GFUN46	CCACAAGAATTGCTGCTGCTTCCAC	AATGGCTGAAGAAGGATGTGCGGC	GFUNContig16047_340
GFUN47	CAGCTATTGGCTTGGAATCGTATGATTGACG	GCTGTTCAAGCTTCCGTTCACATGAG	GFUNk40ctg491715_390
GFUN48	AAGCTCAGCCAGGAAGTTCTGGTATTC	TAAGAGTGATGTGCAGTGCAAGATCTGC	GFUNContig16715_972
GFUN49	ACCTCCTAAGGTCTGCCTCAGTGA	ACCGGATGCAGCGTAAGCACTACA	GFUNContig19880_1506
GFUN50	TTGTCGAATTCGAACTTGTTCGACAGTAGCC	GGCATCATCGCTCTAAGACTGCAAC	GFUNContig13605_64
GFUN51	CCTTCAGCACCTCCATTGACTGAAC	CTGGATACAGTGCAGGCTATGGAAG	GFUNContig16449_381
GFUN52	CCACTGTTGACATCTCAGTGACTGC	AACTAACCATTGCAGTACACGCTGCAG	GFUNContig473
GFUN53	AAGCCTTGCTTGCAAGAAGTGGCG	GGCATAAGCTGCCTCCAACTACATTG	GFUNContig18300_2073
GFUN54	CAACACGCCAATAAGCTCATCCACAAC	TTGACACGCAACGCCACTCTGACT	GFUNContig19643_242
GFUN55	TGGTTACTGCTCCAGGTGTTGGTTATG	GGCATCAGAATTGTTGACATCAGATGGC	GFUNContig15062_54
GFUN56	TTGCAGTATGCAGAACTGCTCTCGG	AAGGTGCAAGAGGCGCATCAGGATTG	GFUNContig16680_1380
GFUN57	TTCAGCTTCTCGTTCCTGGTGAAGG	CCGATCATCACGGTGAAGAAGATCG	GFUNContig20291_500
GFUN58	CCTCTACCAGAAGACCAAGGATGG	TTGTCGGTGCCTGCAACCTCAATG	GFUNContig18141_379
GFUN59	GGAGCACCATGAGATGGTAATCGTG	AGGCCTACGTGTCGGTCAACAAGA	GFUNContig19546_2836
GFUN60	CCACCTCAATTGTGAGCTACAACCAC	TGAGACCATGTCATCCACCACATTGC	GFUNContig19366_1718
GFUN61	TCCACCTTCTCTGGCTGATTCGCT	CTACTCCAAGTGAGGACGCTCCTACA	GFUNContig8054_340
GFUN62	GGTCAGATGGCAAGAACAGAATCCTC	TCCAACTCAAGCATGTCATCCATGCTC	GFUNContig14898_1144
GFUN63	ACATGTACAGAGCAAGGATCTGATCCAACG	CTATCAACTCTGCGAGAGAATTGCGG	GFUNContig7384_126
GFUN64	GTTCTGTTCACTGACTACACTAGAGTCC	TGGCTACTTCGCTTGGTCTCTCCT	GFUNContig12803_225
GFUN65	GAGCAGTAGGCGTTCACCAAGCAACT	ATCAACTGTTGCATGTTCGGCAAGCTC	GFUNk50ctg277349_159
GFUN66	CGTACTTACAGACGCACGGACATATAC	AGCTTCTAACTGATCAATGGAAGCTGCTGC	GFUNContig19989_1346
GFUN67	GGACATGCTGGAAGAGTACAAGCG	GCTCATGTGCTGAACATTCATGTTGCG	GFUNContig17297_1683
GFUN68	GCCGCAGTGGACACATTCTATCAC	TTGCTGCCTTCGGTGCATACAGAC	GFUNContig15462_364
GFUN69	AAGTTCTCCGGCAAGCACATGCCA	AACACTGACAGGCATGCCGGCAATGT	GFUNContig15573_959

Table 2.3 Continued

PrimerID	Forward Primer	Reverse Primer	Contig
GFUN70	AATGAAGGATGGCTGGAAGAGGACC	CAAGACCAGCTCTTCCGTGGAGAAGA	GFUNContig15308_282
GFUN71	GCAATGGAGATATCACAGAGGTTCGG	CAGGAGCCTGGAGAGAACTCATGT	GFUNContig15044_645
GFUN72	CGGAGTTGTGATGTAACCGATCTCAG	ACGGCTACAGCAACGTGGATAGCT	GFUNContig17006_576
GFUN73	GGCAAGCAAGGAACCTGTCAAGCA	GGAACAATGATGACAAGAGGCCACC	GFUNContig18697_1249
GFUN74	AGCAATTCCTCCAGCATTGTCACTGATAGG	ATGTACGGTGTTGCTGTGGCTGCT	GFUNContig13492_952
GFUN75	GATACAGACCTTGCTAGATACGCACG	AGAAGCGACATGAAGAAGTACATCGTGC	GFUNContig6509_318
GFUN76	GCCAGACCATATTACATGCCGGAC	TCGGCATGCTGGTAGACCTGTTGA	GFUNContig16774_1572
GFUN77	CTCAGGATGGTAGTACTCAAGATACCAC	GCCATACACGCATGCTAATGTAAGCC	GFUNContig19418_380
GFUN78	TTCCACTGCTCTCGGACGAATGCT	AATCCACAACCTCGAGATGAGGCAG	GFUNContig16807_560
GFUN79	CTCATGCGCTCGATCGTCGATATG	GTTGTCCATCGGAATTATTGGAAGCAGG	GFUNContig10699_258
GFUN80	TCTGCATTCACCTCAGTGCAGTTCTAC	ACATGAGGAGAGGAGAGGAGAGCA	GFUNContig18297_52
GFUN81	GCTGAACGGCAGTAGTCATTGTCC	ATGTCATCTTCACTGCGACACCACTTG	GFUNContig17265_575
GFUN82	GTCGAGTACTACCTGCACTACACG	ACTCCACCTTGAACCTCTGAATCATCATGTG	GFUNContig15694_635
GFUN83	CATTGTAAGACAAGTTAAGTCGACGGAGTCG	CGGCATAGAAGAGCTTGCCTTGGT	GFUNContig19043_388
GFUN84	TTCTTGTGAAGGCATGGTGTGATCAGAG	GTAAGTGACGATATCAGGCTCAACACC	GFUNContig15224_746
GFUN85	GGCGAGCAGTGTTAGTGCAGATTG	TTGATCCTCTGGCAGCTCTCCTCA	GFUNContig775_598
GFUN86	CAGAAGGCATCGGAGTCATCGAGA	ACAGCTCTACATGTCGCTGAGGATG	GFUNContig10791_1021
GFUN87	ATCGGTGCCTTCGACGATCAGGAACT	TCATGTGCACGTTCCGATTCAAGATGTG	GFUNContig17337_2234
GFUN88	CATGGCGTTGCACTTGTCCAGCATGT	GTTGCGTTCTGATCCAACCAGGATTC	GFUNContig16272_530
GFUN89	GGATGCTGATTCTCTTGGCCTCAC	CCATTGTCGGTAGTAACAGTCACATCC	GFUNContig13700_2721
GFUN90	AACGCCAACTACATTACGCCGACAG	TGGTATCAGAGAGAGCGGCATCAAG	GFUNContig19466_1847
GFUN91	TGGCTTAATTCCACCGTTACCGAGAG	CCTGACTGGTTCAGCTCTTCTTCC	GFUNContig19568_1644
GFUN92	CTCGAAGTCTGGTGCATCAATGAGG	ACTCGTTGCAAGGCTGCTGCATCT	GFUNContig19109_546
GFUN93	ACAACTGTGATTCTGATGCAACATTCCAGCC	CAGAAGAAGCACCTCTGGTTGTTCAG	GFUNk50ctg116835_233
GFUN94	CCAGCAACAAGCTTGTCGATGAGG	GGCCTTGAGGAGCACTTCGATATC	GFUNContig18923_254
GFUN95	TACATCAACGACCGGCTGGACAAG	CAGGAAGGACCAGATCCGGTAGTTGT	GFUNContig3532_69
GFUN96	AAGCCTTGCTGGCGGTGCATTACT	AGGCTCACTGCAACATGATCAAGGC	GFUNContig20076_2471

Table 2.4 Jaccard's genetic similarity coefficients between *Miscanthus* accessions screened by HRM Analysis.

		1	2	3	4	5	6	7	8	9	10
1	*Miscanthus x giganteus* 'Illinois'										
2	*Miscanthus x giganteus* 'G-Max'	0.33									
3	*Miscanthus x giganteus* 'Bixby'	0.61	0.28								
4	*Miscanthus x giganteus* 'Frank'	0.74	0.31	0.7							
5	*Miscanthus x giganteus* 'Kurt Bluemel'	0.06	0.09	0.13	0.12						
6	*Miscanthus sacchariflorus* 'Golf Course'	0.22	0.2	0.26	0.3	0.36					
7	*Miscanthus sinensis* 'Hercules'	0.14	0.13	0.17	0.22	0.37	0.47				
8	*Miscanthus sacchariflorus* 'Robustus'	0.12	0.1	0.16	0.17	0.38	0.33	0.33			
9	*Miscanthus sinensis* 'Andante'	0.18	0.37	0.18	0.22	0.21	0.25	0.23	0.23		
10	*Miscanthus sinensis* 'Zebrinus'	0.13	0.17	0.1	0.14	0.21	0.22	0.22	0.18	0.33	
11	*Miscanthus sinensis* 'Sarrabande'	0.13	0.22	0.14	0.16	0.2	0.22	0.22	0.2	0.51	0.42

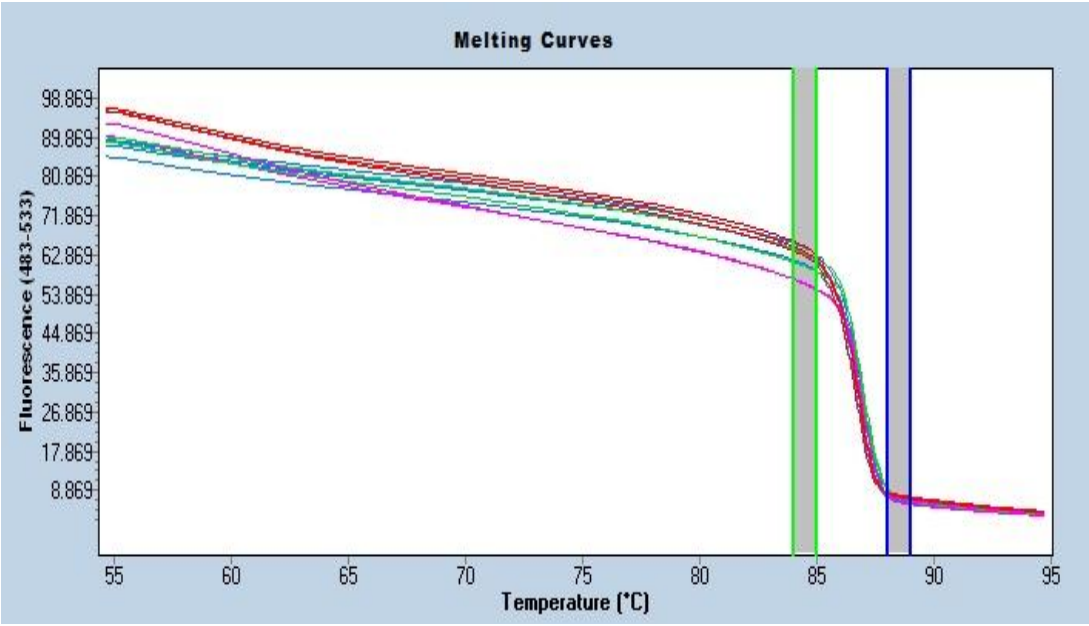


Figure 2.1 High Resolution raw Melt Curve depicting pre-melt and post-melt settings on non-normalized curve (Primer set GFUN87). This is the first normalization step applied to raw melt curves in the LightCycler 480 Gene Scanning Software. Pre-melt is depicted by the region between the green vertical lines and post-melt is depicted by the region between the blue vertical lines. Pre-melt is defined as the temperature before fluorescence values begin to sharply decline, DNA is still in double stranded form (i.e. 100% double stranded). Post-melt is defined as the temperature where all samples have begun a flat lined fluorescence; DNA is now in single stranded form (i.e. 100% single stranded).

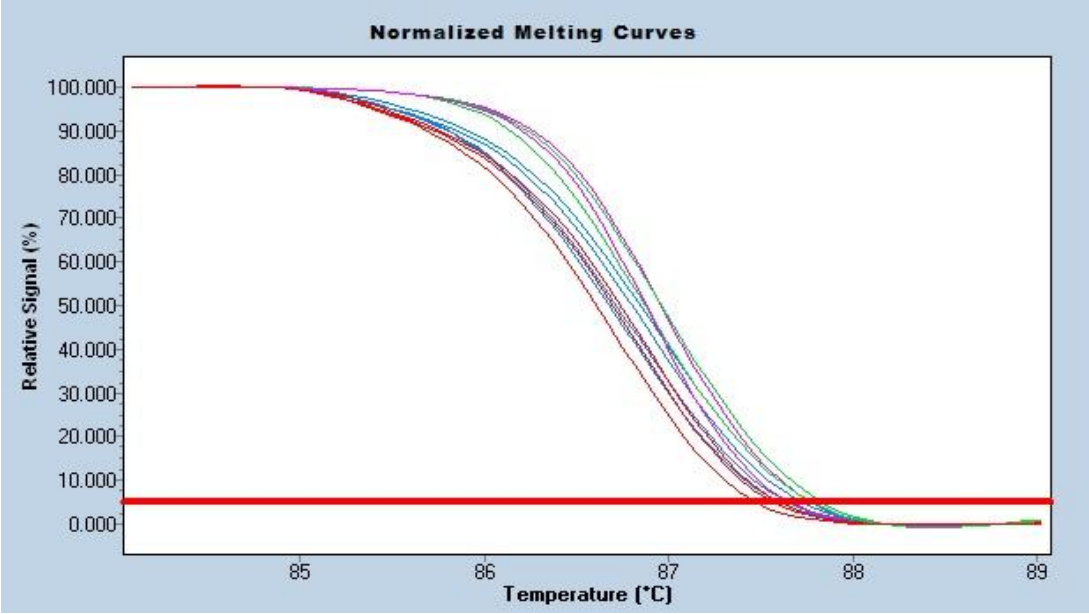


Figure 2.2 High Resolution Melt Curve depicting temperature shift normalization. This is the second normalization step applied to after normalization step in Fig 2.1 is completed. The red line represents the temperature shift normalization. A 5% threshold is the default setting of the Light Cycler 480 Gene Scanning software. This represents the position where all the dsDNA has been dissociated and its now single stranded. The normalization accounts for any temperature variation that may occur from well- to-well within the 384 well plate format.

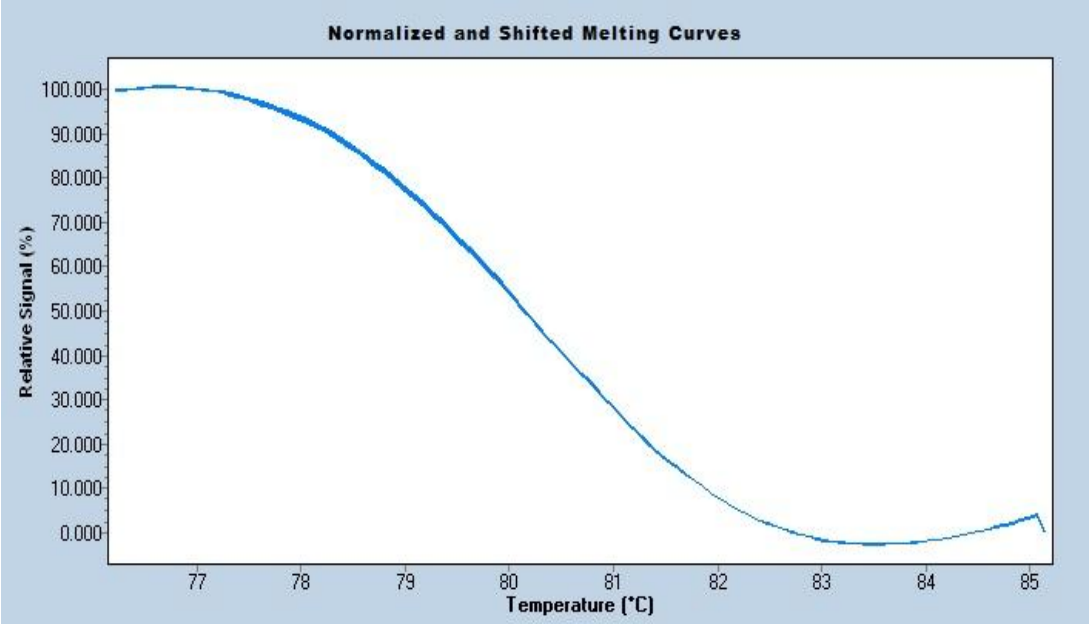
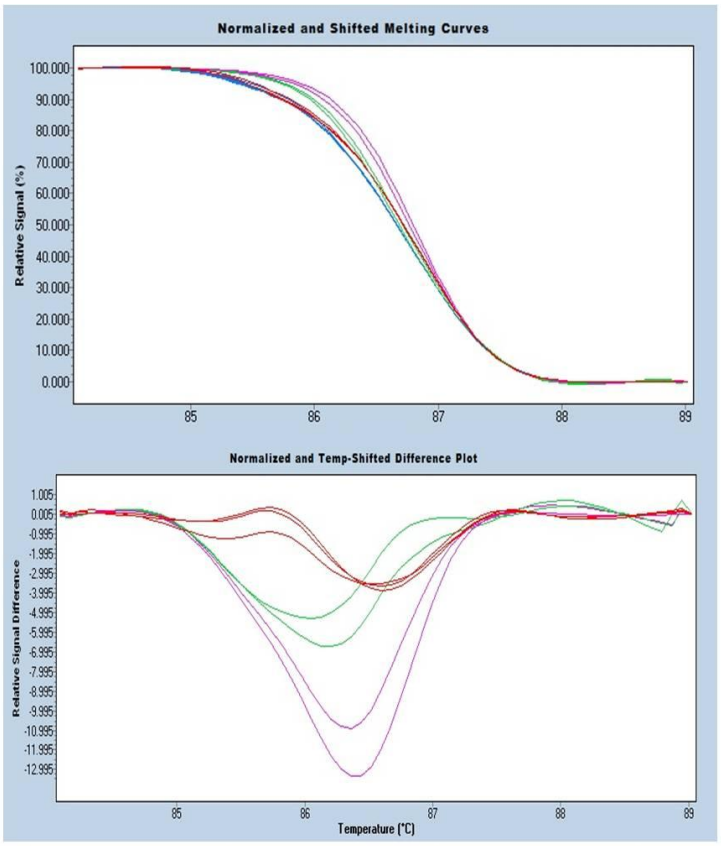


Figure 2.3 High Resolution Melt Curve depicting a primer that is non-informative melt curve. In total 15 primer sets were either non informative and had melt curves that showed no variation between amplicons or had no amplification at all. Primer sets that were found to be non-informative for this sample set are listed in the table and colored in grey.



	M .x g 'Iliinois'	M. x g 'Frank'	M. x g "Bixby"	M. x g 'Gmax'	M x g 'K.B.'	M. sacch 'Golf Course'	M. sacch. 'Robust'	M. sin. 'Hercules'	M. sin. 'Andante'	M. sin. 'Sarrabande'	M. sin. 'Zebrinus'
Primer 1 Blue	1	1	1	1	0	0	0	0	0	0	0
Primer1 Red	0	0	0	0	0	0	0	0	1	1	1
Primer1 Green	0	0	0	0	0	1	0	1	0	0	0
Primer 1 Purple	0	0	0	0	1	0	1	0	0	0	0

Figure 2.4 Normalized and shifted High Resolution Melt Curve and normalized and temperature shifted difference plot for primer GFUN87. Groups were colored by default within the LightCycler 480 Gene Scanning software. Attached table represents how presence/absence scoring was done in relation to melt curves. If all samples in blue represent a specific group based on the similarity in the melt curve they are given a score of 1 while all other samples are given a score of 0. All samples in red are a separate group and given a score of 1 while all other samples are given a score of 0 and so forth. The table represents how the scoring and identification of presence/absence was made. Completed tables were then put into NTSYSpc v. 2.1 for compiling the dendrogram.

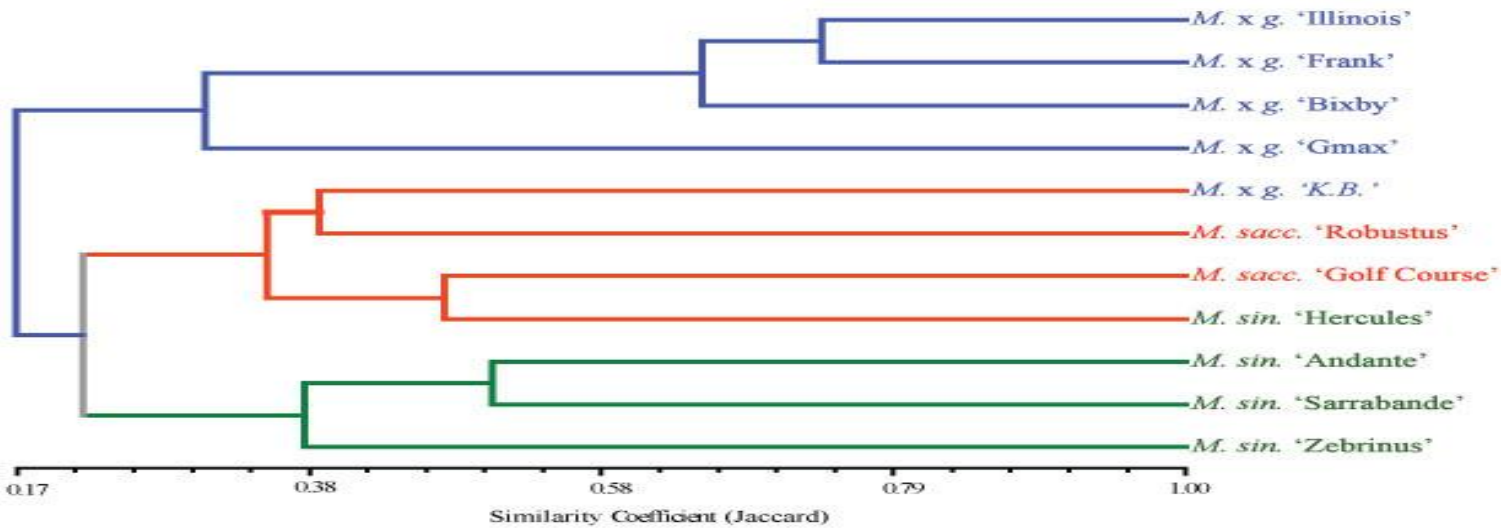


Figure 2.5. Phylogenetic Tree of *Miscanthus* accessions compiled from 81 primer sets used for High Resolution Melt Curve Analysis. A Jaccard coefficient of similarity was applied to the data. Cluster analysis using UPGMA-SAHN was able to distinguish three groups at the species level. The tree starts at 1.00 which would signify 100% similarity. The shorter the lines the more similar the accessions are to one another. The names of each sample are color coded based on the species classification from the source. Names in blue represent *M.* x *giganteus*, red= *M. sacchariflorus* and green= *M. sinensis*. The associating line on the tree is colored to represent where the accessions falls in relation to the three species. Both *M.* x *giganteus* 'Kurt Bluemel' and *M. sinensis* 'Hercules' group with *M. sacchariflorus* which shows that these lines were previously misidentified.

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Chapter 3: Both interspecific and intraspecific relatedness of ornamental *Miscanthus* accessions can be obtained by use of Fragment Analysis of Simple Sequence Repeats (SSRs)

Abstract

Simple Sequence Repeats (SSRs) also known microsatellites are repeating sequences of 2-6bp of DNA. The high level of polymorphisms in SSRs is largely due to slipping caused by mis-pairing of the repeating unit during DNA replication. This affects the number of repeat units makes making SSRs exceptional molecular marker, when trying to distinguish genetically distinct accessions. SSRs have been used in the past to assess variation in *Miscanthus* accessions but the number of SSRs available has been limited, as has the number of plants sampled. To assess diversity both within and between *Miscanthus* species, we used 48 fluorescently labeled primer pairs designed to amplify regions containing SSRs. These primers were originally designed against both genomic DNA and expressed sequence tags (ESTs) of sugarcane accessions. Since *Miscanthus* shares 98% nucleotide identity with sugarcane at the genic level, it was our expectation that a subset of these primers would be capable of amplifying the equivalent SSR in *Miscanthus* accessions. The 48 primers were tested on a total of 87 *Miscanthus* lines. 39 of the 48 primer sets amplified one to forty-eight fragments of DNA in *Miscanthus*. In all 723 amplicons of different lengths were identified as molecular markers and used to comprise a dendrogram of the percent similarity between the accessions. Based on the similarity between the accessions the dendrogram showed six distinct clades and clearly separates the species within the *Miscanthus* genus. The largest group was comprised of *M. sinensis* accessions. Within the *M. sinensis* group there is a range

of similarity between accessions. It appears that there is a high level of diversity within the accessions sampled and even accessions labeled as belonging to a different *Miscanthus* species can be found within this group.

Introduction

Simple sequence repeats (SSRs), also known as microsatellites, are short strings of repeats in the genomic DNA which typically consist of a tandemly repeated unit two to six nucleotides in length (Mahalakshmi, et al., 2002). Due to mis-pairing and slipping of repeating units during DNA replication SSRs are highly polymorphic. In these segments of DNA a number of allelic states with variation seen in the repeat unit are created. This feature makes SSRs good molecular markers and a tool for DNA fingerprinting. There are numerous detection methods for SSRs, all of which involve the resolution of amplified DNA fragments (amplicons), containing the SSR, by size. Traditional methods involve running the amplicons on high-resolution agarose or polyacrylamide gels and scoring the size differences by eye. More recently however, fluorescently tagged amplicons can be rapidly analyzed on modern capillary DNA analyzers where the size resolution is to a single base pair difference, i.e. one single nucleotide polymorphism (SNP). Use of capillary DNA analyzers is an advantage that allows for the detection of a large number of alleles. Since SSRs allow for the identification of many alleles at a single locus, this makes it particularly useful for typing polyploids. An additional advantage of SSRs is that they are cost effective, require nothing more than a PCR machine and a gel running apparatus, and require relatively little genomic DNA (Berry et al., 2002; Mahalakshmi et al., 2002). SSR fingerprinting, unlike high-resolution melt curve analysis, is also more forgiving of DNA quality and method of DNA extraction.

Molecular methods have already played an important part in classification and taxonomic grouping within *Miscanthus* and between closely related genera (Hodkinson, Chase, Lledó, et al. 2002). Sequencing of the intergenic spacer region of the plastid and nuclear ribosomal DNA has shown that *M.*

sinensis and *M. sacchariflorus* group more closely with *Saccharum officinarum* and *S. robustum* than with *Miscanthus* spp. from Africa, such as *M. junceus*, which clearly calls for a re-evaluation of generic divisions in this group (Hodkinson, Chase, Takahashi, et al. 2002; Hodkinson, Chase, Lledó, et al. 2002). Molecular studies of *Miscanthus* have been conducted using isozyme analysis to assess genetic diversity. Most taxonomic studies in *Miscanthus* published so far are based on morphological characteristics (Amalraj and Balasundaram 2005; Sun et al. 2010), variations in nuclear ribosomal DNA sequences (Hodkinson, Chase, Lledó, et al. 2002) and the study of some organelles DNA sequences (Cesare et al. 2010). There are few multi locus studies at the molecular level but they are restricted to a small number of DNA markers (Hernández et al. 2001; Hodkinson and Chase 2002; Ho et al. 2011). Because of the lack of markers most of these studies lacked the ability to differentiate between closely related varieties within a species although there was a clear separation at the species level between *M. sinensis* and *M. sacchariflorus*(Hodkinson et. al., 2002a; Hodkinson, M W Chase, Lledo, et al., 2002). Other species, like *M. transmorrisonesis* that were included in the study fell into clusters closer to *M. sinensis* but with unclear separation (Hodkinson and Chase 2002) .

Molecular studies of *Miscanthus* have also been conducted using isozyme analysis to assess genetic diversity (Von Wuhlish, et al., 1994) as well as amplified fragment length polymorphism (AFLP) (Greef et. al., 1997). These studies were limited in terms of the number of the number of cultivars sampled and that only 6 primers sets were used. Hodkinson et al in 1997 used DNA sequences form the internal transcribed spacer of nuclear ribosomoal DNA (nrITS) to distinguish inter species relationships within the *Miscanthus* genus but were unable to differentiate between cultivars or varieties(Hodkinson et al. 1997). Later work by this group used AFLPs and inter-simple sequence repeats (ISSR) for DNA fingerprinting (Hodkinson et al., 2002a). Once again these studies were limited in the number of actual primer pairs used to assess diversity within the *Miscanthus* genome and the reproducibility in the

testing platforms. A total of three AFLP primers and 2 ISSR primers which produced 26 markers were used to fingerprint 75 *Miscanthus* accessions (Hodkinson et al., 2002a).

More recent studies have used chloroplast microsatellites (cpSSRs) developed from the complete chloroplast genome of sugarcane to help in differentiating species within the *Miscanthus* genus (de Cesare et al, 2010). The six cpSSR markers developed for this study were highly polymorphic and were tested on 164 *Miscanthus* genotypes. Although sample size was robust the number of markers used still does not allow for clear differentiation within species. With the recent release of SSR markers from *Brachypodium distachyon researchers have begun to test them on M. sinensis accessions (Zhao et al., 2011). Out of the 57 SSR markers selected for testing on Miscanthus 86% of them were effective. The phylogenetic tree grouped the 21 Miscanthus sinensis accessions into 3 clusters that correlated with the geographical distribution and ecotype classification (Zhao et al, 2011). The use of SSR markers has become more prevalent in Miscanthus research but the studies have yet to get at the level of diversity within the ornamental accessions and the genus as a whole*

Until recently, the number of molecular markers available in *Miscanthus* for a global analysis of variation at the genomic DNA level was few making the analysis of variation among *Miscanthus* accessions a challenge. James et al. suggested that sugarcane SSRs can be used in *Miscanthus* (James et al. 2011) to assess the diversity among the *Miscanthus* ornamental collection available to us. More recently three groups published in 2012 (Kim et al. 2012; Ma et al. 2012; Swaminathan et al. 2012) greatly enhanced the number of genetic markers available in *Miscanthus sinensis* and *M. sacchariflorus*. Kim et al. constructed a genetic map for *Miscanthus sinensis* and *M. sacchariflorus* with 409 SSR markers, the second group used 3,745 SNP markers to construct a high resolution genetic map in *M. sinensis* (Ma et al. 2012) mined from genome sequencing and Swaminathan et al. used a combination of SSR and GoldenGate genotyping to construct a genetic map for *M. sinensis*. Thus, there is now

information at the genetic level that can be used to help substantiate the existing classifications and taxonomical grouping in place so far for *Miscanthus*. Here we used 39 *Saccharum* SSR primers to characterize the similarity among 87 *Miscanthus* accessions.

Methods

Plant Material

The plants listed in this study were provided by Kurt Bluemel Nursery (Baldwin, MD), Linda Kleiss Nursery (Tolono, IL), Emerald Coast Growers (Pensacola, FL) and collaborators at the University of Illinois (Table 3.1). Plants stated as being located at the Energy Farm were planted into a common garden in July of 2008. Details of planting and maintenance are given in Chapter 2. A further subset of accessions were sampled from an earlier common garden collection of ornamental accessions planted at the southern end of the University Illinois South Farms, at the site of the SoyFACE experiment (Savoy, IL), in 2002. These were provided by Linda Kleiss Nursery, (Tolono, IL). All plants used in this study were assigned unique barcodes (Bartender Label Design Software, Seagull Scientific, Bellevue, WA) to ensure that samples could be tracked unambiguously to an individual plant in the field. Barcode prefixes SF and EF indicate whether the plant was located at SoyFACE and the Energy Farm, respectively. Other non-ornamental accessions collected from the wild in Japan were provided by Dr. Ashley Spence and Dr. Erik Sachs, and were held in the greenhouses of the University of Illinois Plant Sciences Facility and had barcodes beginning with UI or PI. In total 87 *Miscanthus* (*M. sinensis* = 71 accessions, *M.* x *giganteus* =5 accessions, *M. sacchariflorus* = 4 accession, *M. transmorrisonesis*= 2 accessions, *M, floridulus* = 2 accessions, *M. oligostachyus* =1, *M. junceus* =1 accession and 1 unknown specie)and 3 sugarcane accessions (*Saccharum officinarum*, *S. robustum* and *S. spontaneum*) were used as positive controls. Positive controls are needed for the verification of negative amplification results. Since the primers were designed from *Saccharum* the controls should amplify if the reaction goes as planned.

Genomic DNA Extraction

DNA was extracted from fresh tissue from single plants to represent each genotype. A modified Cetyltrimethylammonium Bromide (CTAB) DNA extraction protocol was used and is described in detail in Chapter 2. After genomic DNA isolation 5µl of the resuspended liquid was loaded onto a 1% agarose gel and run at 110V for 50min to assess the quality of the DNA and a further 1µl was used to quantify the amount of DNA spectrophotometrically (Nanodrop ND-1000, Thermo-Scientific, Wilmington, DE).

Primers

The SSR primers used in this experiment were mined from expressed sequence tags (ESTs) and genomic sequences of *Saccharum* (Table 3.2, James et al, 2011). Sequences from *S. officinarum* 'LA Purple' and *S. robustum* 'Molakai' were assembled into contigs and were identified using the SSR finder software (http://www.Maizemap.org). SSRs that had flanking sequences greater than or equal to 20 base pairs were then selected for primer design (James et al. 2011). The molecular similarities between *M. sinensis, M. sacchariflorus* and *S. officinarum* (De Cesare et al. 2011) and their ability to hybridize(Chen et al. 1993) suggested that these SSR primers would also be effective in separating *Miscanthus* accessions. A selection of 48 primers that were highly polymorphic across *Saccharum* cultivars were chosen for use on the *Miscanthus* accessions. SSR primers having annealing temperatures >55°C and melting temperatures >60°C were selected. The primers were designed with a fluorescently tagged end so that diversity could be assessed with an automated capillary DNA sequence (ABI Prism 3730xl, Applied Biosystems, Carlsbad, California). The primers were fluorescently labeled at their 5' end with 1 of 4 dyes: blue *6 – Carboxyfluorescein* (6-FAM), red (ROX), yellow (TAMRA) or green (MAX) and purchased from Integrated DNA Technologies(Integrated DNA Technologies, Inc, San Jose, CA). A list of the primers with corresponding fluorescently labeled dye can be found in Table 3.2.

SSR Genotyping

Polymerase chain reactions (PCR) was conducted in plates of 96 with 10µl reactions in each well to which 1µl of DNA (5ng/µl), 5µl of proprietary colorless "GoTaq Hot Start Polymerase" which contains deoxyribonucleotide triphosphates (dNTPs), buffer and taq polymerase (M5001, Promega Corp, Madison, WI), 1µl of forward and reverse primer at .2µM, and 3µl of de-ionized distilled water. Because the primers are light sensitive due to their fluorescently tagged ends they were pipetted rapidly under minimal light. *Saccharum officinarum 'LA Purple'* (LAP), *Saccharum robustum* 'Molokai' and *Saccharum spontaneum* 'SES 208'were used as positive controls and water was the negative control. The PCR protocol was: 94°C, 5 min, 94°C for 30s, 65°C for 30s and 72°C for 45s (2 cycles) 94°C for 30s, 63°C for 30s and 72°C for 45s (2 cycles) 94°C for 30s, 61°C for 30s and 72°C for 45s (2 cycles) 94°C for 30s, 59°C for 30s and 72°C for 45s (30 cycles) and 2°C, 10 min. Samples were then transferred to 96 well plates after the PCR and submitted for capillary electrophoresis fragment analysis with an automated DNA sequencer (ABI Prism3730xl, Applied Biosystems, Carlsbad, CA) at the Roy J. Carver Biotechnology Center (University of Illinois, Urbana, IL). Samples were run according to manufacturer's protocol.

Statistical analysis

Fragment sizes (bp) of microsatellite markers were estimated on the capillary electrophoresis automated DNA sequencer (ABI Prism3730xl, Applied Biosystems). The fragments were analyzed with Genemarker® v.1.91 software (SoftGenetics LLC, State College, PA). The lengths of the SSR products were measured through comparison with the internal size standards run alongside the PCR amplified fragments during capillary electrophoresis. The calculation is based on equal migration of DNA fragments with the same length. A size standard (LIZ500Applied Biosystems, Carlsbad, CA) was included in each run to allow for accurate determination of fragment size. The amplified fragments were assigned "allele calls" for each microsatellite locus using genotype analysis software (Genemarker® v. 1.91, Softgenetics LLC, State College, PA). If the fragment size was present in an accession it was given a value

of 1 for presence otherwise it was scored 0 for absence. All fragments were used to generate a genetic similarity matrix using numerical taxonomy software (NTSYSpc v. 2.1, Exeter Software, Setauket, NY). Relationships among accessions were analyzed using Jaccard's similarity coefficient with the unweighted pair-group method average-UPGMA (Sokal 1966; Gurrutxaga et al. 2009).

Results

Genetic diversity analysis

A total of 48 SSR primers were tested on 90 accessions of *Miscanthus* and *Saccharum*. Nine primers did not reveal differences between accessions and were discarded from further analysis (Table 3.2, discarded primers in grey). The 39 primers that were used produced 723 SSR markers that were subsequently used in creating the dendrogram. The total number of independent fragment lengths per primer set can be found in Table 3.3 and is broken down at the species level. The number of fragments per primer set ranged from one to forty eight. In addition a full list of fragment sizes and distribution between species can be found as supplementary data.

At a similarity coefficient of <0.1 the dendrogram divides into five *Miscanthus* species and 1 *Saccharum* clade (Figure 3.1). The dendrogram created from the SSR data showed 6 distinct groups that correlate to previous relationships based on both phenotypic classification (Sun et al. 2010) as well as molecular, in the form of ITS and nrDNA information (Hodkinson et al. 2002a). The groups separated on the species level for *Miscanthus sinensis* (Green), *Miscanthus sacchariflorus* (Red)*, Miscanthus purparescens* (Light Blue)*, Miscanthus* x *giganteus* (Dark Blue) *Miscanthus junceus* (Black) and the *Saccharum* complex Molokai, LAP, SES208 (Pink). The *M.* x *giganteus and M. sinensis* clade had a similarity coefficient of 0.24. *M. junceus* had a low level of similarity (.08) to the other *Miscanthus* accessions. *M. purparescens* had the highest similarity to the *M. sacchariflorus* group at .10.

M. sinensis accessions collected from the wild at known locations in Japan were labeled in yellow, and named after the location of their collection. Even though these *M. sinensis* span almost 15° of latitude from the southern sub-tropical tip of Kyushu to the cold climate of the northern tip of Hokkaido, they group together, within the *M. sinensis* clade (Figure 3.1). *M. sinensis* "Hercules' grouped with the *M. sacchariflorus* and all the plant samples named *M*. x *giganteus* by their suppliers grouped within one clade including *M.* x *giganteus* 'Kurt Bluemel'. A complete list of all similarities can be found as supplementary data.

Discussion

The goal of this study was to assess the effectiveness of SSR primers derived from *Saccharum* could effectively separate *Miscanthus* accessions and whether both interspecific and intraspecific relationships could be distinguished. In total 87 accessions of *Miscanthus* were sampled for this study and 726 markers were identified. The SSR markers were able to distinguish 6 distinct clades at the species level which included five *Miscanthus* groups and one *Saccharum* group. The largest group consisted of *M. sinensis* accessions in which the highest level of similarity was observed between 'Rotor Pfeil' and 'Undine EF' at .85 similarity. Within the *M. sinensis* species the level of similarity had a broad range and from this study it appears that the majority of accessions are only at best 50 percent similar to one another. Embedded within the *M. sinensis* group was *M. transmorrisonesis* 'Evergreen Maiden Grass' as well as *M. floridulus w*hich puts in question whether these accessions have been misidentified or mislabeled. It is important to note that accessions with the same common name such as 'Rigolleto', 'Goliath' and 'Undine' to name a few had a low similarity to the plant sampled from a different source with the exact same name. This shows that even though *Miscanthus* accessions are widely distributed within the nursery industry there is no assurance that plants with the same name are actually genetically the same.

As seen from the results in Chapter 2, *M. sinensis* 'Hercules' grouped with the *M. sacchariflorus* clade. It was most similar to 'Kurt Bluemel *sacchariflorus*' and it can clearly be stated that this accession has been misidentified. However, *M.* x *giganteus* 'Kurt Bluemel' grouped with the remaining *M.* x *giganteus* which is opposite from the findings in Chapter 2, but had a low similarity of .22. One suggestion into why the grouping of this accession is different from the previous study can be due to the drawbacks of using SSR markers that are originally designed for another plant species. Even though SSR markers have proved to be transferrable (Cordeiro et al. 2001; Zhou et al. 2011) there are drawbacks and limitations (Wang and Barkley 2009). The first of which is null alleles which is the failure of an allele at a specific locus to amplify due to primer and template DNA mismatch. The primers may have difficulty annealing to the template DNA because of point mutations or insertions/deletions that may be present. This can lead to false exclusions of a specific fragment for a plant accession. In addition there are issues with stutter bands which are caused by DNA slippage. Extra bands are typically shorter and have weaker signal intensity. Care needs to be taken to eliminate these extra bands. For the *M.* x *giganteus* 'Kurt Bluemel' accession it seems as the former explanation may be true since this accession more often than not had 1 common SSR fragment to other *M*. x *giganteus* sampled even when multiple SSR fragments were common for remaining accessions.

Another finding to point out within the dendrogram was the relationship of the Japanese samples as related to the ornamental accessions sampled. Teshio, Uruyu and Sugadairu were more closely related to one another than Miyazaki. In additional the three Japanese samples were more closely related to each other than any of the ornamentals. Miyazaki had a .32 similarity to *M. sinensis* 'Little Kitten'. Even though these Japanese samples range from up to 15° latitude they have a higher level of similarity to one another than many of the ornamental accessions tested.

Ornamental accessions of *Miscanthus* were collected solely for their phenotypic characteristics therefore it has been assumed that the level of diversity within the United States of these plants would be low. However, this study shows that high levels of diversity lie both within and between species, and greatly exceed the diversity of *Miscanthus* collected from the length of Japan (Figure 3.1). The SSR primer sets used in this study were successful in divulging the interspecific relationships between *Miscanthus* accessions. At the intraspecific level there is a broad range of similarity between accessions but the majority of accessions are below .50 similarity.

In comparison to High Resolution Melt (HRM) the SSR study is also cost effective and requires minimal extra machinery for application and analysis if a gel based system is used. If a capillary DNA analyzer is used for obtaining fragment sizes then the cost of the fluorescently labeled primers and service will be comparable to that of HRM. However, there is an increase in time needed between sample submission to data analysis. Advantages to using SSR derived markers include the ease of adding samples to the analysis as well as the ease in which polyploids can be analyzed.

Acknowledgements

I would like to thank Dr. Ashley Spence for providing the Japanese accessions sampled in this study. In addition I would also like to thank Dr. Eric Sacks and his lab for giving me access to their collection of *Miscanthus* accessions. In addition I would like to thank Ornella Ngamboma for her assistance in scoring the SSR data.

Table 3.1 *Miscanthus* accessions used in this SSR study. The species and cultivar name for each accession corresponds to the name given when the plant was initially obtained from the source. Each accession has a unique barcode. If the barcode begins with "EF" the plant is found in the common garden at the Energy Farm (Urbana, IL) and "SF" for plants that can be found at SoyFACE (Savoy, IL). Remaining barcodes without an SF or EF prefix were held in the University of Illinois Plant Sciences facility. Barcode numbers that have a range represent accessions where an individual plant could no longer be distinguished from its adjacent replicates. Cultivar names that have "*" represent accessions that were also tested in Chapter 2.

Table 3.1 *Miscanthus* Accessions for SSR study

Species	Name	Barcode	Location	Source
Miscanthus	NG77-022	PI417947	Greenhouse	Eric Sacks
M. floridulus	US56-0022-03	PI230189	Greenhouse	Eric Sacks
	N/A	PI295762	Greenhouse	Eric Sacks
M. junceus	N/A	UI10-00003	Greenhouse	Eric Sacks
M. oligostachyus	Purpurascens	EF0456	Energy Farm	Kurt Bluemel Nursery
M. sacchariflorus	Golf Course*	NA	Energy Farm	Kurt Bluemel Nursery
	Kurt Bluemel	EF05(95-97)	Energy Farm	Kurt Bluemel Nursery
	Robustus*	EF05(59-61)	Energy Farm	Kurt Bluemel Nursery
	Silver Banner Grass	EF03(58-60)	Energy Farm	Kurt Bluemel Nursery
M. sinensis	Adagio	EF0011	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
	Allegro	EF029	Energy Farm	Kurt Bluemel Nursery
	Altweibersommer	EF0112	Energy Farm	Kurt Bluemel Nursery
	Andante*	EF0241	Energy Farm	Kurt Bluemel Nursery
	Arabesque	EF0613	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
	Autumn Light	EF0101	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
	Berlin	EF0655	Energy Farm	Kurt Bluemel Nursery
	Blondo	EF0259	Energy Farm	Kurt Bluemel Nursery
	Bluetenwunder	EF0310	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
	Dixieland	EF0356	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
	Ferner Osten	EF0527	Energy Farm	Kurt Bluemel Nursery
	Flamingo	EF0244	Energy Farm	Kurt Bluemel Nursery

Table 3.1 Continued

Giraffe	EF0421	Energy Farm	Kurt Bluemel Nursery
Gold Bar	EF0701	Energy Farm	Kurt Bluemel Nursery
Gold und Silber	EF0565	Energy Farm	Kurt Bluemel Nursery
Goldfeder	EF0106	Energy Farm	Kurt Bluemel Nursery
Goliath	SF16	SoyFACE	Linda Kleiss Nursery
Goliath	EF0172	Energy Farm	Kurt Bluemel Nursery
Gracillimus	EF0115	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
Graziella	EF0166	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
Grosse Fontaine	EF070	Energy Farm	Kurt Bluemel Nursery
Grosse Fontaine	SF5	SoyFACE	Linda Kleiss Nursery
Haiku	EF0403	Energy Farm	Kurt Bluemel Nursery
Helga Reich	EF0129	Energy Farm	Kurt Bluemel Nursery
Hercules*	EF03(01-03)	Energy Farm	Kurt Bluemel Nursery
Jelitto	SF144	SoyFACE	Jelitto Perrenial Seed
Jelitto	SF182	SoyFACE	Jelitto Perrenial Seed
Juli	EF035	Energy Farm	Kurt Bluemel Nursery
Kascade	EF041	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
Kirk Alexander	EF031	Energy Farm	Kurt Bluemel Nursery
Kleine Silberspinne	EF0250	Energy Farm	Kurt Bluemel Nursery
Little Kitten	EF0459	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
Little Nicky	EF0	Energy Farm	Kurt Bluemel Nursery
Little Nicky (syn. Hinjo)	UI10-00066	Greenhouse	Eric Sacks
Little Zebra	EF0139	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
Malepartus	EF0148	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
Minuette	EF0203	Energy Farm	Kurt Bluemel Nursery
Miyazaki	N/A	Greenhouse	Ashley Spence
Morning Light	EF0221	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
Mysterious Maiden	EF0103	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers

Table 3.1 Continued

Nippon	EF0088	Energy Farm	Kurt Bluemel Nursery
November Sunset	EF0145	Energy Farm	Kurt Bluemel Nursery
Positano	EF0217	Energy Farm	Kurt Bluemel Nursery
Puenktchen	EF0314	Energy Farm	Kurt Bluemel Nursery
Red Tango	EF0109	Energy Farm	Kurt Bluemel Nursery
Rigoletto	UI10-00079	Greenhouse	Eric Sacks
Rigoletto	EF0319	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
Roland	UI10-00080	Greenhouse	Eric Sacks
Roter Pfeil	EF0484	Energy Farm	Kurt Bluemel Nursery
Rotsilber	EF0457	Energy Farm	Kurt Bluemel Nursery
Sagadairu	N/A	Greenhouse	Ashley Spence
Sarabande*	EF0238	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
Silberfeder	EF0235	Energy Farm	Kurt Bluemel Nursery
Silberspinne	SF27	SoyFACE	Linda Kleiss Nursery
Silberspinne	EF019	Energy Farm	Kurt Bluemel Nursery
Silberturm	SF2	SoyFACE	Linda Kleiss Nursery
Sirene	EF0154	Energy Farm	Kurt Bluemel Nursery
Stardust	EF0517	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
Strictus	EF0214	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
Super Stripe	EF0199	Energy Farm	Kurt Bluemel Nursery
Teshio	N/A	Greenhouse	Ashley Spence
Undine	SF20	SoyFACE	Linda Kleiss Nursery
Undine	EF0604	Energy Farm	Kurt Bluemel Nursery
Uruyu	N/A	Greenhouse	Ashley Spence
US47-0011	CANE9233	Greenhouse	Eric Sacks
US64-0004-02	PI294602	Greenhouse	Eric Sacks
Variegatus	EF0016	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
Wetterfahne	UI10-00099	Greenhouse	Eric Sacks

Table 3.1 Continued

	White Kaskade	SF23	SoyFACE	Linda Kleiss Nursery
	Zebrinus	SF54	SoyFACE	Linda Kleiss Nursery
	Zebrinus*	EF0307	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
	Zwergzebra	EF0298	Energy Farm	Kurt Bluemel Nursery
M. sinensis var. condensatus	Caberet	EF0062	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
	Cosmopolitan	EF0476	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
M. transmorrisonensis	Evergreen Maiden Grass	EF0143	Energy Farm	Kurt Bluemel Nursery
	N/A	UI10-00106	Greenhouse	Eric Sacks
M. x *giganteus*	Cleveland	N/A	Greenhouse	N/A
	G-Max*	EF0198	Energy Farm	Kurt Bluemel Nursery
	Illinois *	EF03(85-87)	Energy Farm	Kurt Bluemel Nursery
	Kurt Bluemel*	EF0046	Energy Farm	Kurt Bluemel Nursery
	Ohio	N/A	Greenhouse	N/A

Table 3.2 Primer Information for 48 primer sets tested for Simple Sequence Repeat (SSR) analysis. Primers in grey showed no amplification in *Miscanthus*

Primer ID	REPEAT UNIT	Forward Primer	Reverse Primer	Fluorescent Label	Contig Name
Mis.fluor.1		CATATATTGACCTATGTGTG	TAGCTTCGTTCCATCTCCAT		6-FAM
Mis.fluor.2		TAACAAATCCAGCAGCAGCA	CTTTCTCACTGCCTGCAAGA		6-FAM
Mis.fluor.3		TGCAAAGCTAGAGGGGAAGA	GAAGGGAAGGGGAAGTGGT		6-FAM
Mis.fluor.4		TCATCTGGTCCTGTGGAACAT	TGGGCTCAGGAATTGACTCT		6-FAM
Mis.fluor.5		TGACGATGATGTTGATGATGA	TAAGCACGCAGCTTGTTGTT		6-FAM
Mis.fluor.6	TTATATTATATTATATTATA	TACGCGATTGTGTAAAGTACACCG	TAGCTAGCTCTCTCCTCATCGCTC	6-FAM	LAP_newbler_Contig133257A
Mis.fluor.7	ATATAATATAATATAATATA	AGCATCAAGCACAATCCTCATTCT	TCAGGTACACTACTTGGTCTGTTTGTG	6-FAM	LAP_newbler_Contig20806A
Mis.fluor.8	AATATAATATAATATAATAT	GACGAATTGACCGCCTACCTTTAT	ACACTCACTCAAGTGCCTTGCTTC	6-FAM	LAP_newbler_Contig95697A
Mis.fluor.9	TTCTATTCTATTCTA	CGAATTGGTCAAGACTCTCCTGTT	AGCCTGAAGCAAATTCAATGAAAC	6-FAM	LAP_newbler_Contig157447A
Mis.fluor.10	ATATTATATT	CACGTACATTGCTAGCTGGAACC	ATTTGATCGTACTCGGAAGCGTAG	6-FAM	LAP_newbler_Contig154154A
Mis.fluor.11	ATTATTATTATT	ATCCGCTTCGACCTCTACATCAC	TGACAGGAGATGAAATGCATCGTA	6-FAM	LAP_newbler_Contig17562A
Mis.fluor.13	AAATAAATAAAT	CGCGCAACTTTCTTTCTTTTT	GGTCCATAGCCACTGCAATAAA	6-FAM	LAP_newbler_Contig79943A
Mis.fluor.14	ATTATTATTATT	AAAGTTGGGTGAACAAATAAAATAAAAA	ATCGGGGAGCCTAAGTCATTT	ROX	LAP_newbler_Contig94759A
Mis.fluor.15	AACAACAAC	CAACCTGAGCATACAGGCTAGACA	TGAACTGTGCATCAATGGTAATGA	ROX	LAP_newbler_Contig119056A
Mis.fluor.16	AGATC	CAAGCAAAGTTGCGTTTACTCTCT	CCATGCTATCATTAGTGCAGCTC	ROX	LAP_newbler_Contig101072A
Mis.fluor.17	GGACG	TAGCTTACTTTGACGGTGCTCGAT	ATGTATGATGGTGCCTGGTGC	ROX	LAP_newbler_Contig142521A
Mis.fluor.18	AGAGA	ATGTCCAGCAAGGAGGGAAAG	TCTCGATCAGGAGAAGAAGCCTTA	ROX	LAP_newbler_Contig27119A
Mis.fluor.19	AATCA	TAGAAGCACGTGTTCGCGATG	GCTGCTAGCGATCGAGCTGAC	ROX	LAP_newbler_Contig42620A
Mis.fluor.20					ROX
Mis.fluor.21	GCCGCC	CTTGCCTGCTTCGGCATCTT	GACGGTCTCACTCTCACCATCATC	ROX	LAP_newbler_Contig18364A
Mis.fluor.22	GCTGTT	GTTGTCCTTCCCTTTGGGTTG	GATCTATCAGAGAAGTCCCAGCGA	ROX	LAP_newbler_Contig71161A
Mis.fluor.23	CCTGGA	CAAACACCGTCGTGTTACTCCTC	TCAGGACTTTCTTCGTCAGGATTC	ROX	LAP_newbler_Contig88886A

Table 3.2 Continued

Primer ID	REPEAT UNIT	Forward Primer	Reverse Primer	Fluorescent Label	Contig Name
Mis.fluor.24	TGTC	GTTTTCTTCACCCACAGCATTGAC	CAACCAACTATCGCGGTTGC	ROX	LAP_newbler_Contig31149A
Mis.fluor.25	AATA	AAGATCTCACATGGTTATGTTTTGACA	GGCGAGACAGAGTCATTTTTCTTT	TAMRA	LAP_newbler_Contig93903A
Mis.fluor.26	CCGTC	AAACAGAAGTCTACGTGGAGGTGG	GAAATAATGGAGGGAGGGAGGAT	TAMRA	LAP_newbler_Contig105624A
Mis.fluor.27	GAGAG	GGGGATATATAAATGAGGATGGCG	CCTGTCCTGACTCCTCTTCTGTTC	TAMRA	LAP_newbler_Contig112030A
Mis.fluor.28	CTGAT	TATATGGCACGGTGCAGTAACATT	TATATAGGATATCCGGCCGTGTGA	TAMRA	LAP_newbler_Contig121789A
Mis.fluor.29	GATCG	ACGCACTTCAGACCTCAGTCAGTT	GAGAGACCACCCGATCCCAG	TAMRA	LAP_newbler_Contig123547A
Mis.fluor.30	GGGAA	GTTATAGGCCGGGATAAACAATGG	ATGTTTACACATATGCCCTCGCTC	TAMRA	LAP_newbler_Contig126858A
Mis.fluor.31	TCATG	GCCTAGTCTAGCCGGACAGTATGA	GTTGTGTTGAATTAAGTTGCACGG	TAMRA	LAP_newbler_Contig13731A
Mis.fluor.32	TGCGT	AGCAGTGCAGGTTGTAGCAGC	TCCATCCATCTTCTTCTCCGATTA	TAMRA	LAP_newbler_Contig21025A
Mis.fluor.33	GAGAG	GCGAGATCTCAGTTCGTTGGTAAT	TTTCTCAGTTCTTATGTTTAAGCCAGC	TAMRA	LAP_newbler_Contig56434A
Mis.fluor.34	AGGGG	GGGGTTTAGGGCTTTGGAAGA	CCGCTCCTCCTGTTACTTTTCTTT	TAMRA	LAP_newbler_Contig95735A
Mis.fluor.35	TGCTCC	CAACAAGTTGCTGTGTTGACGTT	ATATCACATCGGACTATCGGAGGA	TAMRA	LAP_newbler_Contig03035A
Mis.fluor.36	ACGAAG	GAAGGAGAGATCATGTCTTGGCTC	CTGTTCTACTTGCAGCTTCCTTCC	TAMRA	LAP_newbler_Contig08720A
Mis.fluor.37	GTCT	TCAAAACTGAATGCAGGCAATAGA	TACATAGCCTGAAAGCAACGGGTA	MAX	LAP_newbler_Contig102859A
Mis.fluor.38	ATTA	GACCGAAAAGAAAACCACCAAAAT	TTCAGAAAATTAAGGCCACGTGAT	MAX	LAP_newbler_Contig112357A
Mis.fluor.39	CTTCGT	TCAGCTCTTTCCAGCATTTGTACC	CAAGTCGTTGCTGGCGAAAG	MAX	LAP_newbler_Contig118099A
Mis.fluor.40	TGATGG	TGTTGTATGGAGTGAGGTGAGGAA	TGTCCAAATTTTAAGCAAAGCACA	MAX	LAP_newbler_Contig123337A
Mis.fluor.41	TGTTGT	GATGCGCAGTTGTTCTCTCATTAT	CAAATATCTCCAGGAACAGCATGA	MAX	LAP_newbler_Contig126978A
Mis.fluor.42	TGTGTG	ATTTTGAAATAAGAAAGACGGCCA	ACACACAAACACACTCATCATTCG	MAX	LAP_newbler_Contig146010b
Mis.fluor.43	GCACCG	TGGACATTTACATCCACTTTGCAG	AGAGAAACAGGAGAGGCACTAGCA	MAX	LAP_newbler_Contig171628A
Mis.fluor.44	GAGAGA	AACGTGTCGTAAGGTTTGGTTGTT	TGCTGCAGGTCCGTACTATACATC	MAX	LAP_newbler_Contig187434A

Table 3.2 Continued

Primer ID	REPEAT UNIT	Forward Primer	Reverse Primer	Fluorescent Label	Contig Name
Mis.fluor.45	CGTGAT	AGAGGAGATGTTGGAAGGTACACG	TAGTTGGCTCTTGCACACCTGTAA	MAX	LAP_newbler_Contig21729A
Mis.fluor.46	CCACGG	GACCAGGGCAATAAGCACAAC	ATATCGAGATGCCTACGAGAAAGG	MAX	LAP_newbler_Contig22378A
Mis.fluor.47	TATC	ATGGATTTGGCTAGTTTGCATTGT	GGTCTGAGGTTGGGTAGGAGTTTT	MAX	LAP_newbler_Contig23054b
Mis.fluor.48	CTCTCT	TGGAATTAGTCTTTTCAACCAACCA	GGTAAAGACCCAAATTACTGGTGATG	MAX	LAP_newbler_Contig24672A

	Table 3.3 Total number of independent fragment lengths per primer set					
	Saccharum	*M. sacchariflorus*	*M. sinensis*	*M.* x *giganteus*	other *Miscanthus* species	ALL *Miscanthus* sp
Mis.fluor.1	3	5	24	3	8	28
Mis.fluor.3	2	2	8	3	4	11
Mis.fluor.4	6	0	7	5	4	10
Mis.fluor.5	5	5	14	6	9	19
Mis.fluor.6	4	3	22	8	4	29
Mis.fluor.7	6	2	9	4	3	13
Mis.fluor.8	3	2	7	3	2	13
Mis.fluor.9	7	1	5	3	0	10
Mis.fluor.10	3	2	38	3	7	40
Mis.fluor.11	6	0	3	2	0	10
Mis.fluor.12	0	0	1	0	0	1
Mis.fluor.13	10	1	17	3	5	26
Mis.fluor.14	11	5	17	2	7	34
Mis.fluor.15	0	0	4	1	0	14
Mis.fluor.17	4	6	31	3	17	48
Mis.fluor.19	4	1	2	1	0	5
Mis.fluor.21	0	0	5	2	2	4
Mis.fluor.22	5	9	20	5	7	25
Mis.fluor.24	0	0	6	1	5	7
Mis.fluor.25	6	3	19	3	4	22
Mis.fluor.26	7	4	16	3	16	34
Mis.fluor.27	0	0	7	0	0	7
Mis.fluor.28	12	3	26	8	14	40
Mis.fluor.29	7	2	17	3	7	21
Mis.fluor.30	8	8	21	5	13	30
Mis.fluor.31	6	1	5	3	3	13

Table 3.3 Continued

Mis.fluor.32	4	4	8	3	3	11
Mis.fluor.33	4	4	15	7	6	22
Mis.fluor.34	12	5	18	10	5	31
Mis.fluor.35	10	0	13	2	3	23
Mis.fluor.36	7	2	7	1	2	11
Mis.fluor.37	6	0	23	8	4	31
Mis.fluor.38	8	3	2	0	3	11
Mis.fluor.40	10	3	19	4	13	21
Mis.fluor.41	10	2	11	7	3	20
Mis.fluor.42	9	3	15	2	6	24
Mis.fluor.43	4	3	10	2	6	10
Mis.fluor.46	2	2	8	3	0	10
Mis.fluor.47	2	0	6	2	1	7
Mis.fluor.48	0	1	1	0	1	1

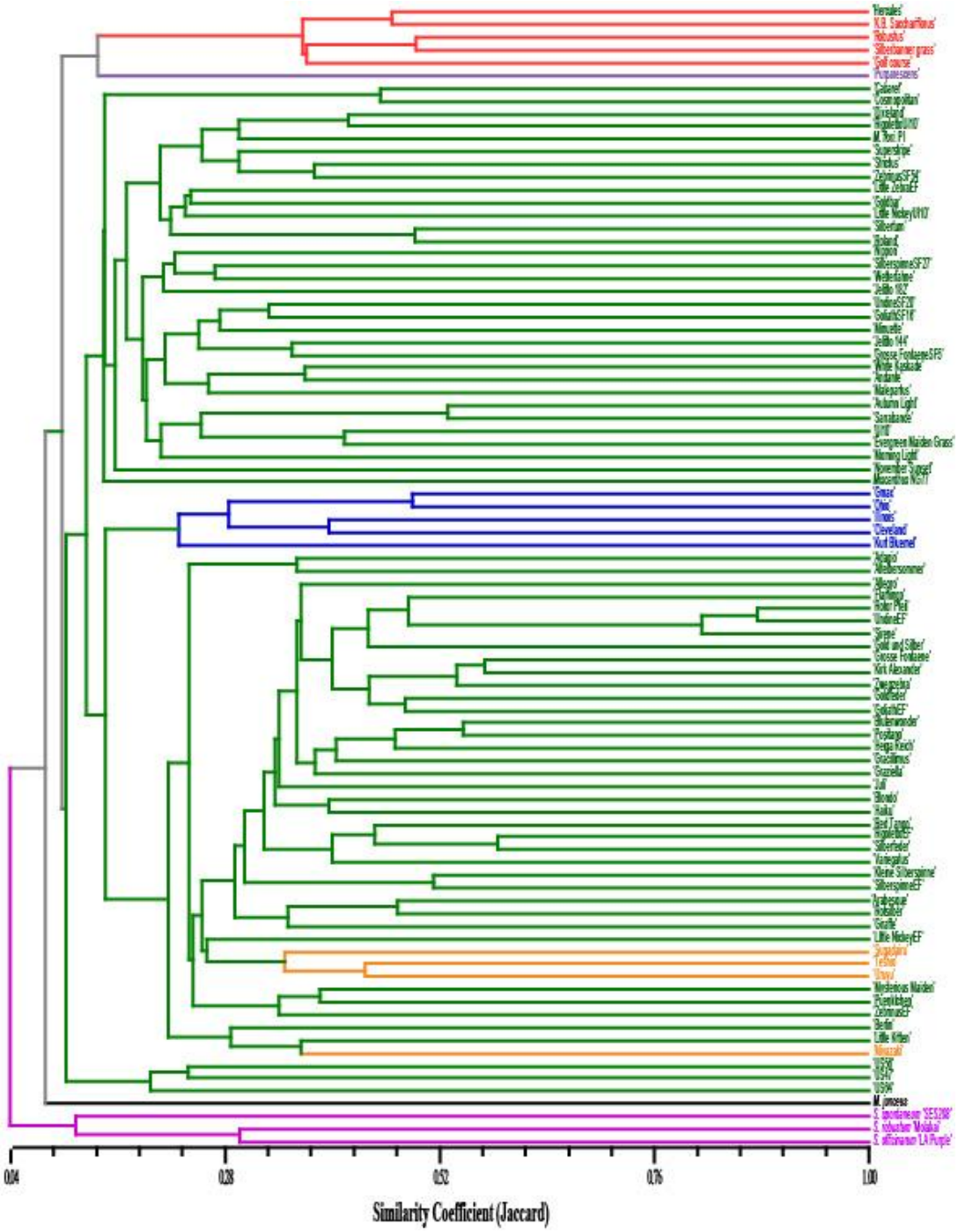

Figure 3.1 Phylogenetic Tree of *Miscanthus* accessions compiled from 39 primer sets used for Simple Sequence Repeat marker detection. A Jaccard coefficient of similarity was applied to the data. Cluster analysis using UPGMA-SAHN was able to distinguish three groups at the species level. The tree starts at 1.00 which would signify 100% similarity. The shorter the lines the more similar the accessions are to one another. The names of each sample are color coded based on the species classification from the source. Names in blue represent *M.* x *giganteus*, red= *M. sacchariflorus,* light blue=*M. purparescens,* orange=Japanese accessions, pink = *Saccharum,* black =*M. junceus* and green= *M. sinensis*. The associating line on the tree is colored to represent where the accessions falls in relation to the three species.

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Chapter 4: High-throughput SNP genotyping can assess the level of diversity within both ornamental *Miscanthus* accessions and seed from an open-pollinated stand

Abstract

Single nucleotide polymorphisms (SNPs) are the most abundant variation throughout the genome of plants or humans. SNPs have been used as molecular markers for association mapping, marker assisted breeding, fingerprinting of cultivars as well as in the construction of high-density genetic maps. SNPs were selected from transcriptome data of two *M. sinensis* accessions, 'Gross Fontaine' and 'Undine' and used to generate a Illumina GoldenGate pre-optimized assay, i.e. a procedure that uses a discriminatory DNA polymerase and ligase to interrogate 1,536 SNP loci. This assay was used to assess the genetic diversity of 398 *Miscanthus* accessions, of which 240 accessions belonged to a *M. sinensis* population with unknown parentage that was purchased from Jelitto Perennial Seed. The remaining samples were either provided by collaborators or part of two ornamental gardens established at the University of Illinois. Allelic information was generated for 803 of 1,536 SNP loci. A dendrogram of dissimilarity was computed with the allelic distribution which showed distinct clades at the species level. The largest group was comprised of *M. sinensis* accessions in which the Jelitto population was part of. Each accession from the Jelitto population had an independent SNP profile that was not matched to any of the known ornamental accessions. *M. sacchariflorus, M.* x *giganteus*, *M. purparescens* and *M. oligostachyus* all had distinct groups and were .24 dissimilar from the major *M. sinensis* group. In addition, within both *M. sacchariflorus* and *M.* x *giganteus* with the exception of *M.* x *g.* 'Kurt Bluemel'

there was 0 dissimilarity suggesting that all the accessions are identical. However, since the GoldenGate array was designed for diploid models there may be a bias against non *M. sinensis* samples. PCA analysis showed that the *M. sinensis* from the ornamental population and *M. sinensis* from the Jelitto population were evenly distributed but did distinguish *M. sacchariflorus*, *M. purparescens* and *M. oligostachyus* as being distinct. In addition, there was the presence of overlapping samples in the PCA which implies that samples with different names have been misidentified.

Introduction

Single Nucleotide Polymorphisms are ideal for the construction of high resolution genetic maps, investigation of population evolutionary history and discovery of marker-trait associations in association mapping experiments (Hyten et al., 2008). The Illumina BeadArray genotyping platform in conjunction with the GoldenGate assay is able to genotype up to 1,536 polymorphic sites in up to 384 individuals in a single reaction (Oliphant et al. 2002). This system uses three assay oligonucleotides designed for each SNP locus. The Illumina GoldenGate assay (Illumina Inc., San Diego, CA) utilizes two allele specific oligos (ASOs) to differentiate between the allelic states of the SNP locus. The third oligo, which is known as the locus specific oligo (LSO), hybridizes between 1-20 bp downstream of the ASO site (Yan et al. 2009). The hybridization process allows for the ASOs to bind to the genomic DNA and occurs before any amplification steps thereby reducing the occurrence of amplification bias. Allele specific extension and ligation reactions of the ASOs and LSO joins information about the genotype present at the SNP site to the address sequence of the specific locus sampled(Fan et al. 2003). This forms a template for PCR in which three universal primers P1, P2 and P3 are used. The P1 and P2 primers are labeled with Cy3 and Cy5 dyes, respectively and amplify the product depending on the allele present. The sample is then hybridized to the array and then the fluorescence signal is analyzed for genotype clusters using the Genome Studio Software (Illumina Inc, San Diego, CA)

Recently a high resolution and comprehensive structural genetic map of the *M. sinensis* genome has been obtained by two independent studies (Ma et al. 2012, Swaminathan et al. 2012). Mapping of the 19 chromosomes were obtained from single nucleotide polymorphisms (SNP) and simple sequence repeat (SSR) derived markers from which linkage groups were assembled. Prior to this random amplified polymorphic DNA (RAPD) markers had suggested 28 linkage groups (Atienza et al. 2002), while an analysis of an interspecific cross between *M. sinensis* and *M. sacchariflorus* with SSRs had suggested 23 and 40 linkage groups for the two species (Atienza et al. 2002, Zhang et al. 2012).

The two studies that successfully derived the 19 linkage groups of *M. sinensis* did so by significantly different approaches. Swaminathan et al. (2012) derived SNP markers from transcriptome data derived from two *Miscanthus sinensis* accessions used to produce a mapping population from which they produced a 1536 SNP array. Ma et al. (2012) directly sequenced a *M. sinensis* mapping population to obtain SNP information for their genetic map. The 1,536 GoldenGate SNP array of Swaminathan et al. (2012) provided a unique opportunity to assess the genetic diversity of *Miscanthus* accessions available at the University of Illinois.

Customized GoldenGate assays have been used in a number of crops including maize, soybean, barley and polyploid wheat for measurement of genetic variation between members of the species (genotyping) (Rostoks et al. 2006; Hyten et al. 2008; Zhang et al. 2009; Akhunov et al. 2009; Yan et al. 2009). In soybean, a GoldenGate custom oligo pool assay (OPA) which contained 384 SNPs was designed to compile a high density consensus linkage map and estimate allele frequencies (Hyten et al. 2008). This was done by genotyping three recombinant inbred line (RIL) populations. In addition to the initial 384 SNP assay two additional 1,536 assays were created for creation of a universal SoyOPA linkage panel that could be used for Quantitative Trait Loci (QTL) mapping. A QTL is a genomic region (locus) that contributes to phenotypic variations. Ultimately, QTL analysis is a statistical method that links

phenotypic data (trait measurements) to genotypic data (molecular markers). A custom OPA was also developed for barley for estimation of linkage disequilibrium as well as marker trait associations. A 1,536 maize SNP OPA has been used to genotype recombinant inbred lines (RILs) and create a linkage map (Yan et al. 2009). This assay was also validated for use on diverse inbred maize germplasm showing that custom assays can be used successfully to genotype a wide array of maize germplasm. What is important to note about all these plants that have been genotyped with the GoldenGate assay is that, the platform is capable of handling both diploid (soybean, maize) (Hyten et al. 2008; Yan et al. 2009) and polyploids (wheat)(Akhunov et al. 2009). Also, the GoldenGate assay can be used successfully for SNP genotyping in highly duplicated plant genomes like soybean and maize (Hyten et al. 2008).

The Illumina BeadArray platform and GoldenGate SNP assays provide a fast and reliable method for the large-scale acquisition of SNP genotype data. Together with other genomic tools developed for *Miscanthus* the GoldenGate SNP array developed for *M. sinensis* opens new possibilities for analysis of genetic variation across accessions. Here the GoldenGate SNP array is tested as a means to resolve both inter and intra-specific relationships across 394 *Miscanthus* accessions. Since the *Miscanthus* accessions ranged from diploid to tetraploid it was also our goal to test the range of this assay and its ability to predict the ploidy of a given accession. In addition, previous studies have shown that *Miscanthus* has a high level of genome duplication (Ma et al. 2012, Swaminathan et al. 2012) which could limit the effectiveness of this platform for genotyping.

To test the feasibility of the GoldenGate SNP array for screening genetic diversity and identification of ploidy variation between accessions a large sampling of *Miscanthus* plants were tested. Seed was purchased from Jelitto Perennial Seed (Louisville, KY) which was derived from open crossing of *M. sinensis* ornamental accessions and sown at the SoyFACE field site (Savoy, IL). The Jelitto plants serve as a single population with unknown parentage. This sampling in addition to the ornamental plants

previously tested in Chapter 3 serve as the basis for this diversity analysis. The opportunity to resolve differences in the Jelitto population relative to known (named) ornamental accessions is an objective. However, the overall goal of this study is to use high throughput SNP genotyping to assess the diversity of both the ornamental *Miscanthus* accessions (Chapter 3) and the 243 plants from the Jelitto population and obtain a unique fingerprinting profile for each accession to allow future QTL analysis to be done. QTL analysis can explain the genetic basis of variation between traits that may improve *Miscanthus* as a biomass feedstock. Trait such as flowering time, plant height or tiller number to name a few may be able to be traced to specific genes. The relationship between phenotype and genotype can only be assessed if this GoldenGate assay is successful for SNP genotyping this diverse set of *Miscanthus* accessions. The assessment of population structure within *M. sinensis* and trait clustering is outside the scope of this current work but the findings here will aid in future analysis.

METHODS

Plant Material

In 2005 seed from an open-pollinated stand of a wide range of *M. sinensis* accessions were obtained from Jelitto Perennial Seeds and sown at the SoyFACE research facility site (Savoy, IL). The accessions sown were from three different seed packs named *Miscanthus sinensis* 'Early Hybrids', 'Late Hybrids' and 'New Hybrids' (Jelitto Perennial Seeds, Louisville, KY). A total of 243 plants were raised from individual seeds. In the summer of 2011 each plant was given an individual barcode number for tracking (Bartender Label Design Software, Seagull Scientific, Bellevue, WA). In addition to these 243 lines, the study included the 87 *Miscanthus* accessions that were sampled previously (Table 3.1), accessions from collaborators at the University of Illinois and two *M. sinensis* double haploid lines (Swaminathan et al. 2012). Since the double haploid plants are homozygous at every locus they were used to distinguish bi-

allelic markers from the presence of variant paralogs. A complete table of accessions used in this high-throughput genotyping study can be found in Table 4.1.

DNA extraction

Genomic DNA was isolated using a PureGene 96 well genomic DNA isolation protocol provided from Qiagen (Qiagen, Valencia, CA). This protocol allows for 96 samples to be isolated at a time and was repeated until all 240 Jelitto accessions were processed. Ten disks of 6.5mm diameter were punched from young leaves of each plant in the Jelitto population and placed into a pre labeled well. After samples are collected the caps are placed onto the wells and are stored at -80°C. Frozen samples were placed in a high-throughput homogenizer for 30 seconds at 1500rpm (GenoGrinder, Spex Sample Prep, Metuchen, NJ). After the initial grinding, samples were placed at 80°C for 2minutes, followed by homogenization for another 30 seconds. Samples were centrifuged for 1minute at 8000g to remove remaining tissue fragments. 300µL of a cell lysis buffer (Qiagen, Valencia, CA) and RNAse (Promega) at 3µg/ml were added to each well and samples were vortexed for 1min. Samples were incubated at 65°C 1hr. After incubation the samples are centrifuged at 8000g for 1 minute and samples were then placed on ice. 100µL of pre chilled Protein Precipitate was added and then samples were vortexed for 1 minute and then incubated for 1 hour on ice. Meanwhile, 200 µL of isopropanol is added to each well of a new 96 well plate. After incubation, samples were centrifuged for 30minutes at 8000g. 200µl of supernatant is then transferred to the corresponding prefilled wells containing isopropanol. The samples were vortexed for 1 minute and then centrifuged for 30 minutes at 8000g. The supernatant was then discarded by inverting samples and blotting wells on paper towel. Pellets were then washed with 300µl of 70% EtOH and vortexed for 1 minute. Samples were then centrifuged for 30 minutes at 8000g after which supernatant was discarded. Pellets were allowed to air dry overnight and then resuspended with 200µl of DNA Hydration Solution (Qiagen, Valencia, CA). After genomic DNA isolation 5µl of each sample was loaded onto a 1% agarose gel and run at 110V for 50min to assess the quality of the DNA and 1µl of

sample was quantified using a spectrophotometer (Nanodrop ND-1000, Thermo-Scientific, Wilmington, DE).

A modified Cetyltrimethylammonium Bromide (CTAB) DNA extraction protocol was used for the remaining samples and is described in detail in Chapter 2. After genomic DNA isolation 5µl of the resuspended liquid was loaded onto a 1% agarose gel and run at 110V for 50min to assess the quality of the DNA and a further 1µl was used to quantify the amount of DNA spectrophotometrically (Nanodrop ND-1000, Thermo-Scientific, Wilmington, DE).

GoldenGate sample submission & Data analysis

Genomic DNA samples were diluted to a concentration ranging from 50ng/ul-200ng/ul and a total of 20ul/per sample were analyzed for SNP detection using the Illumina Bead Array (Illumina Inc., San Diego, CA) at the University of Illinois Keck Center for Functional Genomics (University of Illinois). The individual accessions were grouped into clusters based on their allelic variations for specific loci identified by analysis of the Bead Array. The analysis software (Genome Studio, Illumina Inc., San Diego, CA) uses the fluorescence intensity of the Cy3 and Cy5 dye channels to define a locus as either homozygous or heterozygous genotypes. These two signals are specific to one allele of a bi-allelic marker. Cy3 produces a fluorescent signal when the allele specific oligo (ASO) binds to the A allele and Cy5 produces a fluorescent signal when the ASO binds to B allele. A genotype that is heterozygous at a locus will have both the Cy3 and Cy5 channels displayed and that will represent AB allele. For diploid genotypes up to three genotypes can be expected for each SNP locus, which include up to two homozygous clusters (AA, BB) and one heterozygous cluster (AB). In this analysis these three possibilities are referred to a "genotype calls". Because there were also triploid and tetraploid accessions the number of clusters increased at some loci. In the case of tetraploid up to five genotypes can be expected (AAAA, AAAB, AABB, ABBB, BBBB) while in triploids the expectation would be four genotypes (AAA, AAB, ABB, BBB) However, for this study at any locus the clusters are limited to the three possibilities above that fit the

diploid model. Triploid and tetraploid genotypes often fell outside these three clusters and were recorded as missing for that locus.

Because of an ancient duplication in the *Miscanthus* genome in which an earlier tetraploidisation, indicated by comparison with the *Sorghum bicolor* genome, has become diploidised (Swaminathan et al. 2012) the position of the allele calls in the case of the diploid lines tended to be skewed to either the Cy3 (left) or Cy5 (right) position and is depicted in Figure 4.1 panels B and C. The paralogs are non-segregating variants at a specific locus. For example, if locus 1 is a fixed paralog (AA) and locus 2 is segregating as either AA, AB or BB, it will cause skewed genotype cluster towards the Cy3 channel because of the non segregating variant (AA). To identify such duplications the *M. sinensis* double haploid accessions were included in the assay. The double haploids are homozygous at every locus allowing distinction of genotype clusters for loci which were skewed. Because of the presence of fixed paralogs manual genotype cluster calls were made. The genotype analysis software (Genome Studio, Illumina, San Diego, CA) is ideal for diploid models without the presence of non segregating alleles. The algorithms within the software are looking for the fluorescence distribution of the Cy3 and Cy5 probes to distinguish genotypes. When clusters are skewed toward one channel because of the presence of the fixed loci the analysis software cannot determine genotype calls. Therefore, this analysis could not be automated by the software. All, 1,536 SNP loci were analyzed using the Genome Studio genotyping software (Illumina, San Diego, CA). A maximum of three genotypes clusters were selected for any locus even if more clusters were present. Using the Genome Studio software each SNP loci was evaluated for overall signal intensity and dispersal area of clusters. Loci where accessions had low signal intensities (below .3 threshold) were excluded from further analysis. Also, loci where individual clusters could not be distinguished were also excluded from further analysis

Principle Component Analysis (PCA)

The PCA analysis is one of the most widely used and well known of the standard multivariate methods. It takes the data matrix of a certain number of samples by the number of variables and summarizes it by a principal component axes that is a linear combination of the original variable. The overall objective of a PCA is to rotate the axes to new positions that are ordered such that principal axis 1 has the highest variance and axis 2 has the next highest variance(Jolliffe 2002). The principle axes are uncorrelated however. A Principle Component Analysis (PCA) is a common technique for finding patterns in data and highlighting the similarities and differences (Smith 2002). R statistical code was used to create and run the PCA analysis on the samples. Two PCA analyses were conducted on the GoldenGate data. The first PCA included all accessions except for *M.* x *giganteus* and looked at whether there was any species level variation that could be accounted for. The second PCA looked at a subset of samples which only included *M. sinensis* ornamental accessions and looked at whether variegation in leaf blades contributed to any variation

Dendrogram

All the genotype calls made within the Genome Studio software (Illumina Inc., San Diego, CA) were exported and converted to allele calls as follows: 0 for AA homozygous, 1(AB) for heterozygous and 2 for (BB) for homozygous at each loci. Nei's genetic distances were computed using multivariate statistics and data analysis software (Numerical Taxonomy System v 2.2 (NTSYSpc) Exeter Software, Setauket, NY). Nei's genetic distance assumes differences arise due to mutation and genetic drift and is commonly used to compute genetic distance when allelic information is known (Nei 1972, 1974). An Unweighted Pair Group Method using Arithmetic Means (UPGMA) was used for hierarchical clustering to develop a dendrogram (SAHN) within the NTSYSpc software (Exeter Software, Setauket, NY).

Results

Out of the 1,536 SNP loci tested 803 loci showed clear separation of the accessions into 2 to 3 clusters. The 803 loci were the markers chosen for further analysis. All cluster calls were made manually and a detailed spreadsheet of all genotype calls can be found in a supplemental file. A dendrogram was constructed with the allele calls made (Figure 4.2). The largest group within the tree is comprised of *M. sinensis* accessions. Within that major group the level of dissimilarity ranges from 0 to .12. The zero represents accessions as being found to be identical at the 803 loci sampled. Accessions that had the same common name but were provided from different sources such as 'Grosse Fontaene' were 100% similar to one another. *M. sinensis* 'Andante' and 'White Kaskade' from different sources also had a 100% similarity. However, most of the *M. sinensis* had some level of dissimilarity (Figure 4.3, Figure 4.4). The Jelitto population of *M. sinensis* was not distinguishable from the ornamental accessions with known common names. *M.* x *giganteus*, *M. sacchariflorus*, *M. purparescens* and *M. oligostachyus* are .25 dissimilar from the major *M. sinensis* group. However, each one of these species was a uniquely identifiable group on the dendrogram. Within the *M.* x *giganteus* and the *M. sacchariflorus* group there was 0 dissimilarity except for *M.* x g 'Kurt Bluemel' which was .02 dissimilar from the *M. sacchariflorus* group.

In the PCA analysis (Figure4.3) there is a clear group of *Miscanthus sacchariflorus* accessions that separate from the *M. sinensis* group. In general all the *M. sinensis* accessions were in one broad group that included the ornamental accessions as well as the Jelitto population. Both *M. purparescens* and *M. oligostachyus* were distinct in their position on the PCA. The *M. floridulus* samples grouped closely with two *M. sinensis* accessions 'Gracillimus' and 'US56-0022'. The PCA that was limited to *M. sinensis* ornamental accessions (Figure 4.4) shows a clear overlap of some samples that have different names. You can clearly see that sample *M. sinensis* Undine EF is clearly different from the accession with the same name from SoyFACE. The presence of overlapping samples means that every SNP loci was

identical meaning there is likelihood accessions have been misidentified or mislabeled from the source. Also within Figure 4.4, there seems to be some correlation between variegated accessions where they are making two clear groups. Both the PCA and dendrogram are capable of depicting the GoldenGate data in an easily interpreted format that shows that *Miscanthus* accessions can be separated at the species level and overall similarity.

In addition to these two ways to visualize and interpret the data the Genome Studio analysis software allows for the distinction between ploidy levels especially in the case of *M.* x *giganteus.* In Figure 4.1 the position of *M.* x *giganteus* in relation to other species sampled helps distinguish its ploidy level as being different from other samples. Overall, *M.* x *giganteus* had a lower number SNP loci for genotyping because the samples tended to make their own cluster which were outside of/or more than the 3 maximum groups allowed for diploid species.

Discussion

The objective of this study was to assess if SNP genotyping using the GoldenGate assay (Illumina, San Diego, CA) could be used to assess the genetic diversity of both ornamental *Miscanthus* accessions and plants from the Jelitto population. In addition, it was our goal to obtain a unique fingerprint profile for each accession that can be used in future studies for possible QTL mapping. We can confidently state that the GoldenGate assay is a successful tool for genotyping *Miscanthus* accessions. In total 398 accessions spanning 7 species of *Miscanthus* were tested and 803 SNP markers were identified.

The Illumina BeadArray platform with the GoldenGate SNP assay provided a very clear separation of *M. sinensis* (Green), *M. sacchariflorus* (Red), *M. x giganteus* (Blue), *M. junceus* (Black) , *M. purpurascens* (Purple) and *M. oligostachyus* (Light Blue) on the dendrogram (Figure 4.2). It however, did not distinguish *M. floridulus* (Pink) from *M. sinensis* as seen in both the dendrogram (Figure 4.2) and the PCA (Figure 4.3) proving its efficacy at the species level. Across *M. sinensis* variation is detected between

accessions (Figure 4.2) but overall dissimilarity is less than 12%. By contrast, *M.* x *giganteus* no variation as seen in the dendrogram and has 0% dissimilarity (Figure 4.2). This is consistent with the fact that only one collection of *M. x giganteus* has been documented (Stewart et al. 2009) suggesting that despite different cultivar names probably all currently available accessions of this sterile triploid are derived from the same clone and that any variants result from somatic mutations or epigenetic changes.

Genotype calls made using the Genome Studio software (Illumina, San Diego, CA) were made in regards to all lines being scored as a diploid which means a maximum of three genotypes (AA, AB, BB) could be selected for each locus . There were times when *Miscanthus* x *giganteus* accessions did not group within these three genotype clusters because of it being triploid. A triploid plant can have one of four genotypes present as stated previously. Because of the presence of additional genotypes that were not called, *M.* x *giganteus* lacked genotypic data for an average of 100 loci (data not shown). This means that *M.* x *giganteus* had only 703 SNPs out of the 803 used on the entire data set. For comparison *M. sinensis* accessions averaged 5 loci of missing genotype data, *M. floridulus* had an average of 68 and *M. sacchariflorus* had an average of 11. This resulted in *M.* x *giganteus* having too much missing data to be used in the PCA analysis. However, because all the *M.* x *giganteus* samples had distinct clustering at the loci sampled we can say that the GoldenGate array was able to not only detect but also isolate this species and all accessions sampled showed little variation. In addition because *M. x giganteus* had distinct clustering that was outside of the three clusters/groups expected for a diploid we were able to distinguish it from *M. sinensis* in regards to ploidy level.

Even though the *M.* x *giganteus* accessions had different common names there grouping on the dendrogram (Figure 4.2, Blue) suggests they are all derived from the same clone. However, there is also the chance that all the *M.* x *giganteus* had 100% similarity due to ascertainment bias. Since the GoldenGate assay was designed specifically for single nucleotide variations between two *M. sinensis*

accessions (Swaminathan et al. 2012)the probes may have had only a certain level of specificity for the non diploid accessions sampled. Similarly, the *M. sacchariflorus* accessions sampled showed little variation between the samples (Figure 4.2, Figure 4.3). Once again, this may be that all *M. sacchariflorus* accessions sampled are actually more similar than previously thought or that there is once again a bias in the platform since it was designed specifically for *M. sinensis* variations.

With the high level of duplication within the *Miscanthus* genome (Ma et al. 2012) as evidenced by the clear shift in cluster positions due to a fixed paralogs the GoldenGate assay it is still capable of being used for fingerprinting studies and diversity analysis. It is important to note that while only 803 SNP markers out of 1,536 were successfully used for genotyping these accessions this number exceeds the number of usable SNP markers that were applied for the *Miscanthus sinensis* genetic map (Swaminathan et al. 2012).

Previous research at the taxonomical level as well as molecular level supports that *M. sacchariflorus* is distinct from *M. sinensis* and other *Miscanthus* species (Hodkinson, Chase, Takahashi, et al. 2002; Sun et al. 2010; Cesare et al. 2010). This was also evident in Chapters 2 and 3 where both HRM and SSR analysis was able to distinguish *Miscanthus* at the species level. Looking at Figure 4.3 there is a slight spread on the PC1 axis for the *M. sacchariflorus* accessions which could suggest that these samples are all originated from a similar location (Clifton-Brown et al. 2008). The misidentified *M.* x *giganteus* 'Kurt Bluemel' (identified as tetraploid *M. sacchariflorus* in Chapter 2) only shows 2% dissimilarity even though the other *M. sacchariflorus* are diploid (Figure 4.2) . Even though there is a small level of variation seen in both the dendrogram and PCA analysis the high level of similarity may be attributed to some ascertainment bias within the GoldenGate assay. Both Chapters 2 and 3 showed that all the M. *sacchariflorus* samples were distinct which is opposite from the findings here.

M. oligostachyus has been confirmed as its own species from ITS studies where it was placed into a new monophyletic group (Hodkinson, Chase, Lledó, et al. 2002). This finding was substantiated SNP genotyping within this study. Based on morphological assessments *M. purpurascens* could not be clearly separated from *M. sinensis* (Sun et al. 2010), however this SNP analysis shows a clear differentiation (Figure 4.2, Figure 4.3). *M. transmorisonesis* has been variously classified as a distinct species and as a subspecies or variety of *M. sinensis* (Chou 2009; Sun et al. 2010; Ho et al. 2011). As found with the SSR analysis in Chapter 3, this SNP analysis also suggests that *M. transmorisonensis* is not genetically distinct from the *M. sinensis* accessions (Figure 4.2). Since other species clearly separate in this analysis, this provides strong evidence that *M. transmorisonensis* is in fact a form of *M. sinensis.* This reiterates that samples names are arbitrary at the molecular level and for breeding purposes.

The Jelitto population proved as diverse as the separate collection of ornamental *M. sinensis* suggesting that the open-crossing parent population represented most of the diversity currently available (Figure 4.5). The Jelitto population is at most 19% dissimilar with the exception of 5 *Miscanthus* accessions while the ornamental accessions are at best 18% dissimilar (Figure 4.6). Within the Jelitto population there were only a few instances when the branch length was at 0 representing 100% similarity. This is important because it means that 99% of plants within this population are independent genotypes that can be looked into further for breeding purposes. This was not however the case with the ornamental accessions in both Figure 4.2 and Figure 4.6. In this instance there are accessions with different common names that had 0% dissimilarity which indicates that mislabeling and misidentification have occurred.

As seen in Chapter 3 with the SSR study, the accessions with origins to Japan once again were closely related. This was particularly evident with Uruyu, Teshio and Sugaidaru while Miyazaki once again was the least similar of all four accessions. This analysis by GoldenGate genotyping substantiates the claim

that the Japanese samples have less genetic diversity than the samples within the U.S. even though they were collected from varying latitudes. Overall, all the figures depict that there is a level genetic diversity between the *Miscanthus* accessions that can be detected with this method. In additional the Jelitto population is as diverse as the ornamental accessions collected from nurseries and are independent genotypes that are not identical to any known *M. sinensis* accessions sampled in this study. This assay can be used for fingerprinting large sample sizes and provides information for multiple loci and has worked to clearly identify and genotype samples with unknown names and origin.

Acknowledgements

I would like to thank Dr. Pat Brown for providing Sorghum accessions for analysis and his assistance with writing the R code used in the PCA analysis. I would like to thank Dr. Eric Sacks and his lab, Dr. Ashley Spence, Won Byoung-Chae and Dr. Kasia Glowacka for the *Miscanthus* accessions provided and Brandon James for providing maize sample for this study.

Table 4.1. *Miscanthus* Accessions

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
1	Haploid1	Poland	Kasia Glowacka
2	Haploid2	Greenhouse	Eric Sacks
3	*M. Junceus* UI10-00003	Greenhouse	Eric Sacks
4	*M.* NG77-022 PI417947	Greenhouse	Eric Sacks
5	*M.* US-56-0022-03 PI230189	Greenhouse	Eric Sacks
6	M x *giganteus* Illinois EF0385	Energy Farm	Kurt Bluemel Nursery
7	M x *giganteus* Kurt Bluemel EF0046	Energy Farm	Kurt Bluemel Nursery
8	M x *giganteus* Mxg	Energy Farm	N/A
9	*M.*sacchariflorisGolf Course	Energy Farm	N/A
10	*M.*sacchariflorisHercules EF0121	Energy Farm	Kurt Bluemel Nursery
11	*M.*sacchariflorisHercules EF0301-EF0303	Energy Farm	Kurt Bluemel Nursery
12	*M.*sacchariflorisKurt Bluemel Saccharifloris EF0450	Energy Farm	Kurt Bluemel Nursery
13	*M.*sacchariflorisRobustus EF0130	Energy Farm	Kurt Bluemel Nursery
14	*M.*sacchariflorisRobustus EF0559-EF0561	Energy Farm	Kurt Bluemel Nursery
15	*M.*sacchariflorisSilver Banner Grass EF0358	Energy Farm	Kurt Bluemel Nursery
16	*M. sinensis* Adagio EF0010	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
17	*M. sinensis* Allegro EF0029	Energy Farm	Kurt Bluemel Nursery
18	*M. sinensis* Altweibersommer EF0112b	Energy Farm	Kurt Bluemel Nursery
19	*M. sinensis* Andante EF0241	Energy Farm	Kurt Bluemel Nursery

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
21	*M. sinensis* Autumn Lights EF0101	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
22	*M. sinensis* Autumn Lights EF082	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
23	*M. sinensis* Berlin EF0655	Energy Farm	Kurt Bluemel Nursery
24	*M. sinensis* Blondo EF0259-1	Energy Farm	Kurt Bluemel Nursery
25	*M. sinensis* Blondo EF0259-2	Energy Farm	Kurt Bluemel Nursery
26	*M. sinensis* Cabaret EF0062	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
27	*M. sinensis* Dixie Land EF0394	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
28	*M. sinensis* Dixieland EF0356	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
29	*M. sinensis* Flamingo EF0244	Energy Farm	Kurt Bluemel Nursery
30	*M. sinensis* Giraffe EF0286	Energy Farm	Kurt Bluemel Nursery
31	*M. sinensis* Gold Bar EF0424	Energy Farm	Kurt Bluemel Nursery
32	*M. sinensis* Gold Bar EF0701	Energy Farm	Kurt Bluemel Nursery
33	*M. sinensis* Gold und Silber EF0085	Energy Farm	Kurt Bluemel Nursery
34	*M. sinensis* Goldfeder EF0106	Energy Farm	Kurt Bluemel Nursery
35	*M. sinensis* Goliath EF0172	Energy Farm	Kurt Bluemel Nursery
36	*M. sinensis* Graziella EF0166	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
37	*M. sinensis* Grosse Fontaine EF0070	Energy Farm	Kurt Bluemel
38	*M. sinensis* Grosse Fontaine SF5	Energy Farm	Linda Kleiss Nursery
39	*M. sinensis* Heiga Reich EF0127b	Energy Farm	Kurt Bluemel Nursery

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
40	*M. sinensis* Jelitto J144	SoyFACE	Jelitto Perennial Seed
41	*M. sinensis* Jelitto J182	SoyFACE	Jelitto Perennial Seed
42	*M. sinensis* Juli EF0034	Energy Farm	Kurt Bluemel Nursery
43	*M. sinensis* Kaskade EF0040	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
44	*M. sinensis* Kirk Alexander EF0031	Energy Farm	Kurt Bluemel Nursery
45	*M. sinensis* Kleine Silberspinne EF0250	Energy Farm	Kurt Bluemel Nursery
46	*M. sinensis* Little Kitten 0013	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
47	*M. sinensis* Little Nickey (syn. Hinjo)UI10-00066	Energy Farm	Eric Sacks
48	*M. sinensis* Little Zebra EF0139-1	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
49	*M. sinensis* Little Zebra EF0139-2	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
50	*M. sinensis* Malepartus EF0148	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
51	*M. sinensis* Malepartus EF148	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
52	*M. sinensis* Minuett EF0202	Energy Farm	Kurt Bluemel Nursery
53	*M. sinensis* Minuette EF0203	Energy Farm	Kurt Bluemel Nursery
54	*M. sinensis* Mysterious Maiden EF0103	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
55	*M. sinensis* Nippon EF0088-1	Energy Farm	Kurt Bluemel Nursery
56	*M. sinensis* Nippon EF0088-2	Energy Farm	Kurt Bluemel Nursery
57	*M. sinensis* November Sunset EF0145-1	Energy Farm	Kurt Bluemel Nursery
58	*M. sinensis* November Sunset EF0145-2	Energy Farm	Kurt Bluemel Nursery

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
59	*M. sinensis* November Sunset EF0229	Energy Farm	Kurt Bluemel Nursery
60	*M. sinensis* PI	Greenhouse	Eric Sacks
61	*M. sinensis* Positano EF0217	Energy Farm	Kurt Bluemel Nursery
62	*M.oligostachyus* Purparescens EF0456	Energy Farm	Kurt Bluemel Nursery
63	*M.oligostachyus* Purpurascens EF0256	Energy Farm	Kurt Bluemel Nursery
64	*M. sinensis* Red Tango EF0109b	Energy Farm	Kurt Bluemel Nursery
65	*M. sinensis* Rigoletto UI10-00079	Greenhouse	Eric Sacks
66	*M. sinensis* Roland UI10-00080	Greenhouse	Eric Sacks
67	*M. sinensis* Roter Pfeil EF0484a	Energy Farm	Kurt Bluemel Nursery
68	*M. sinensis* Roter Pfeil EF0484b	Energy Farm	Kurt Bluemel Nursery
69	*M. sinensis* Rotsilber EF0390	Energy Farm	Kurt Bluemel Nursery
70	*M. sinensis* Sarabande EF0238-1	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
71	*M. sinensis* Sarabande EF0238-2	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
72	*M. sinensis* Silber Banner Grass EF0358-EF0360	Energy Farm	Kurt Bluemel Nursery
73	*M. sinensis* Silberfeder EF0235	Energy Farm	Kurt Bluemel Nursery
74	*M. sinensis* Silberspinne EF0019	Energy Farm	Kurt Bluemel Nursery
75	*M. sinensis* Silbertum SF2	SoyFACE	Linda Kleiss Nursery
76	*M. sinensis* Sirene EF0156	Energy Farm	Kurt Bluemel Nursery
77	*M. sinensis* Stardust EF0511	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
78	*M. sinensis* Strictus EF0157	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
79	*M. sinensis* Strictus EF0214	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
80	*M. sinensis* Super Stripe EF0199	Energy Farm	Kurt Bluemel Nursery
81	*M. sinensis* Super Stripe EF0370	Energy Farm	Kurt Bluemel Nursery
82	*M. sinensis* Undine EF0604	Energy Farm	Kurt Bluemel Nursery
83	*M. sinensis* Undine SF20	SoyFACE	Linda Kleiss Nursery
84	*M. sinensis* US-47-0011 CANE9233	Greenhouse	Eric Sacks
85	*M. sinensis* US-64-0004-02 PI294602	Greenhouse	Eric Sacks
86	*M. sinensis* var. condensatus Cabaret EF0061	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
87	*M. sinensis* var. condensatus Cosmopolitan EF0438	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
88	*M. sinensis* var. condensatus Cosmopolitan EF0476	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
89	*M. sinensis* Variegatus EF0016	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
90	*M. sinensis* Wetterfahne UI10-00099	Greenhouse	Eric Sacks
91	*M. sinensis* White Kaskade SF23	SoyFACE	Linda Kleiss Nursery
92	*M. sinensis* Zebrinus SF54	SoyFACE	Linda Kleiss Nursery
93	*M. sinensis* Zwergzebra EF0298b	Energy Farm	Kurt Bluemel Nursery
94	*M.transmorisonensis* Evergreen Maiden Grass EF0143-1	Energy Farm	Kurt Bluemel Nursery
95	*M.transmorisonensis* Evergreen Maiden Grass EF0143-2	Energy Farm	Kurt Bluemel Nursery
96	*M.transmorisonensis* Transmorrisonensis UI10-00106	Greenhouse	Eric Sacks

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
97	Parent_of_Haploid1	Poland	Kasia Glowacka
98	Parent_of_Haploid2	Poland	Kasia Glowacka
99	J2	SoyFACE	Jelitto Perennial Seed
100	J3	SoyFACE	Jelitto Perennial Seed
101	J4	SoyFACE	Jelitto Perennial Seed
102	J5	SoyFACE	Jelitto Perennial Seed
103	J6	SoyFACE	Jelitto Perennial Seed
104	J7	SoyFACE	Jelitto Perennial Seed
105	J8	SoyFACE	Jelitto Perennial Seed
106	J9	SoyFACE	Jelitto Perennial Seed
107	J10	SoyFACE	Jelitto Perennial Seed
108	J11	SoyFACE	Jelitto Perennial Seed
109	J12	SoyFACE	Jelitto Perennial Seed
110	J14	SoyFACE	Jelitto Perennial Seed
111	J15	SoyFACE	Jelitto Perennial Seed
112	J16	SoyFACE	Jelitto Perennial Seed
113	J18	SoyFACE	Jelitto Perennial Seed
114	J19	SoyFACE	Jelitto Perennial Seed
115	J20	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
116	J21	SoyFACE	Jelitto Perennial Seed
117	J22	SoyFACE	Jelitto Perennial Seed
118	J23	SoyFACE	Jelitto Perennial Seed
119	J25	SoyFACE	Jelitto Perennial Seed
120	J26	SoyFACE	Jelitto Perennial Seed
121	J27	SoyFACE	Jelitto Perennial Seed
122	J28	SoyFACE	Jelitto Perennial Seed
123	J30	SoyFACE	Jelitto Perennial Seed
124	J31	SoyFACE	Jelitto Perennial Seed
125	J32	SoyFACE	Jelitto Perennial Seed
126	J33	SoyFACE	Jelitto Perennial Seed
127	J34	SoyFACE	Jelitto Perennial Seed
128	J35	SoyFACE	Jelitto Perennial Seed
129	J36	SoyFACE	Jelitto Perennial Seed
130	J37	SoyFACE	Jelitto Perennial Seed
131	J38	SoyFACE	Jelitto Perennial Seed
132	J39	SoyFACE	Jelitto Perennial Seed
133	J40	SoyFACE	Jelitto Perennial Seed
134	J41	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
135	J42	SoyFACE	Jelitto Perennial Seed
136	J43	SoyFACE	Jelitto Perennial Seed
137	J44	SoyFACE	Jelitto Perennial Seed
138	J45	SoyFACE	Jelitto Perennial Seed
139	J46	SoyFACE	Jelitto Perennial Seed
140	J48	SoyFACE	Jelitto Perennial Seed
141	J49	SoyFACE	Jelitto Perennial Seed
142	J50	SoyFACE	Jelitto Perennial Seed
143	J51	SoyFACE	Jelitto Perennial Seed
144	J52	SoyFACE	Jelitto Perennial Seed
145	J53	SoyFACE	Jelitto Perennial Seed
146	J54	SoyFACE	Jelitto Perennial Seed
147	J55	SoyFACE	Jelitto Perennial Seed
148	J56	SoyFACE	Jelitto Perennial Seed
149	J57	SoyFACE	Jelitto Perennial Seed
150	J58	SoyFACE	Jelitto Perennial Seed
151	J59	SoyFACE	Jelitto Perennial Seed
152	J60	SoyFACE	Jelitto Perennial Seed
153	J62	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
154	J63	SoyFACE	Jelitto Perennial Seed
155	J64	SoyFACE	Jelitto Perennial Seed
156	J65	SoyFACE	Jelitto Perennial Seed
157	J66	SoyFACE	Jelitto Perennial Seed
158	J67	SoyFACE	Jelitto Perennial Seed
159	J68	SoyFACE	Jelitto Perennial Seed
160	J71	SoyFACE	Jelitto Perennial Seed
161	J72	SoyFACE	Jelitto Perennial Seed
162	J73	SoyFACE	Jelitto Perennial Seed
163	J74	SoyFACE	Jelitto Perennial Seed
164	J76	SoyFACE	Jelitto Perennial Seed
165	J77	SoyFACE	Jelitto Perennial Seed
166	J78	SoyFACE	Jelitto Perennial Seed
167	J79	SoyFACE	Jelitto Perennial Seed
168	J80	SoyFACE	Jelitto Perennial Seed
169	J81	SoyFACE	Jelitto Perennial Seed
170	J82	SoyFACE	Jelitto Perennial Seed
171	J83	SoyFACE	Jelitto Perennial Seed
172	J84	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
173	J85	SoyFACE	Jelitto Perennial Seed
174	J87	SoyFACE	Jelitto Perennial Seed
175	J88	SoyFACE	Jelitto Perennial Seed
176	J89	SoyFACE	Jelitto Perennial Seed
177	J91	SoyFACE	Jelitto Perennial Seed
178	J92	SoyFACE	Jelitto Perennial Seed
179	J93	SoyFACE	Jelitto Perennial Seed
180	J94	SoyFACE	Jelitto Perennial Seed
181	J95	SoyFACE	Jelitto Perennial Seed
182	J96	SoyFACE	Jelitto Perennial Seed
183	J97	SoyFACE	Jelitto Perennial Seed
184	J98	SoyFACE	Jelitto Perennial Seed
185	J99	SoyFACE	Jelitto Perennial Seed
186	J100	SoyFACE	Jelitto Perennial Seed
187	J101	SoyFACE	Jelitto Perennial Seed
188	J102	SoyFACE	Jelitto Perennial Seed
189	J103	SoyFACE	Jelitto Perennial Seed
190	J104	SoyFACE	Jelitto Perennial Seed
191	J105	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
192	J107	SoyFACE	Jelitto Perennial Seed
193	J108	SoyFACE	Jelitto Perennial Seed
194	J109	SoyFACE	Jelitto Perennial Seed
195	J110	SoyFACE	Jelitto Perennial Seed
196	J111	SoyFACE	Jelitto Perennial Seed
197	J113	SoyFACE	Jelitto Perennial Seed
198	J115	SoyFACE	Jelitto Perennial Seed
199	J116	SoyFACE	Jelitto Perennial Seed
200	J117	SoyFACE	Jelitto Perennial Seed
201	J118	SoyFACE	Jelitto Perennial Seed
202	J119	SoyFACE	Jelitto Perennial Seed
203	J120	SoyFACE	Jelitto Perennial Seed
204	J121	SoyFACE	Jelitto Perennial Seed
205	J122	SoyFACE	Jelitto Perennial Seed
206	J123	SoyFACE	Jelitto Perennial Seed
207	J124	SoyFACE	Jelitto Perennial Seed
208	J125	SoyFACE	Jelitto Perennial Seed
209	J126	SoyFACE	Jelitto Perennial Seed
210	J128	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
211	J129	SoyFACE	Jelitto Perennial Seed
212	J130	SoyFACE	Jelitto Perennial Seed
213	J131	SoyFACE	Jelitto Perennial Seed
214	J132	SoyFACE	Jelitto Perennial Seed
215	J133	SoyFACE	Jelitto Perennial Seed
216	J134	SoyFACE	Jelitto Perennial Seed
217	J135	SoyFACE	Jelitto Perennial Seed
218	J136	SoyFACE	Jelitto Perennial Seed
219	J137	SoyFACE	Jelitto Perennial Seed
220	J138	SoyFACE	Jelitto Perennial Seed
221	J139	SoyFACE	Jelitto Perennial Seed
222	J140	SoyFACE	Jelitto Perennial Seed
223	J141	SoyFACE	Jelitto Perennial Seed
224	J142	SoyFACE	Jelitto Perennial Seed
225	J143	SoyFACE	Jelitto Perennial Seed
226	J144	SoyFACE	Jelitto Perennial Seed
227	J145	SoyFACE	Jelitto Perennial Seed
228	J146	SoyFACE	Jelitto Perennial Seed
229	J147	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
230	J148	SoyFACE	Jelitto Perennial Seed
231	J149	SoyFACE	Jelitto Perennial Seed
232	J150	SoyFACE	Jelitto Perennial Seed
233	J151	SoyFACE	Jelitto Perennial Seed
234	J152	SoyFACE	Jelitto Perennial Seed
235	J153	SoyFACE	Jelitto Perennial Seed
236	J154	SoyFACE	Jelitto Perennial Seed
237	J155	SoyFACE	Jelitto Perennial Seed
238	J159	SoyFACE	Jelitto Perennial Seed
239	J160	SoyFACE	Jelitto Perennial Seed
240	J161	SoyFACE	Jelitto Perennial Seed
241	J162	SoyFACE	Jelitto Perennial Seed
242	J163	SoyFACE	Jelitto Perennial Seed
243	J164	SoyFACE	Jelitto Perennial Seed
244	J166	SoyFACE	Jelitto Perennial Seed
245	J168	SoyFACE	Jelitto Perennial Seed
246	J169	SoyFACE	Jelitto Perennial Seed
247	J170	SoyFACE	Jelitto Perennial Seed
248	J171	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
249	J172	SoyFACE	Jelitto Perennial Seed
250	J173	SoyFACE	Jelitto Perennial Seed
251	J174	SoyFACE	Jelitto Perennial Seed
252	J175	SoyFACE	Jelitto Perennial Seed
253	J177	SoyFACE	Jelitto Perennial Seed
254	J178	SoyFACE	Jelitto Perennial Seed
255	J180	SoyFACE	Jelitto Perennial Seed
256	J181	SoyFACE	Jelitto Perennial Seed
257	J182	SoyFACE	Jelitto Perennial Seed
258	J183	SoyFACE	Jelitto Perennial Seed
259	J184	SoyFACE	Jelitto Perennial Seed
260	J185	SoyFACE	Jelitto Perennial Seed
261	J186	SoyFACE	Jelitto Perennial Seed
262	J187	SoyFACE	Jelitto Perennial Seed
263	J188	SoyFACE	Jelitto Perennial Seed
264	J189	SoyFACE	Jelitto Perennial Seed
265	J192	SoyFACE	Jelitto Perennial Seed
266	J193	SoyFACE	Jelitto Perennial Seed
267	J194	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
268	J195	SoyFACE	Jelitto Perennial Seed
269	J196	SoyFACE	Jelitto Perennial Seed
270	J198	SoyFACE	Jelitto Perennial Seed
271	J199	SoyFACE	Jelitto Perennial Seed
272	J200	SoyFACE	Jelitto Perennial Seed
273	J201	SoyFACE	Jelitto Perennial Seed
274	J202	SoyFACE	Jelitto Perennial Seed
275	J203	SoyFACE	Jelitto Perennial Seed
276	J204	SoyFACE	Jelitto Perennial Seed
277	J205	SoyFACE	Jelitto Perennial Seed
278	J208	SoyFACE	Jelitto Perennial Seed
279	J209	SoyFACE	Jelitto Perennial Seed
280	J210	SoyFACE	Jelitto Perennial Seed
281	J211	SoyFACE	Jelitto Perennial Seed
282	J212	SoyFACE	Jelitto Perennial Seed
283	J215	SoyFACE	Jelitto Perennial Seed
284	J216	SoyFACE	Jelitto Perennial Seed
285	J217	SoyFACE	Jelitto Perennial Seed
286	J219	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
287	J220	SoyFACE	Jelitto Perennial Seed
288	J223	SoyFACE	Jelitto Perennial Seed
289	J224	SoyFACE	Jelitto Perennial Seed
290	J225	SoyFACE	Jelitto Perennial Seed
291	J226	SoyFACE	Jelitto Perennial Seed
292	J227	SoyFACE	Jelitto Perennial Seed
293	J229	SoyFACE	Jelitto Perennial Seed
294	J230	SoyFACE	Jelitto Perennial Seed
295	J231	SoyFACE	Jelitto Perennial Seed
296	J232	SoyFACE	Jelitto Perennial Seed
297	J233	SoyFACE	Jelitto Perennial Seed
298	J234	SoyFACE	Jelitto Perennial Seed
299	J235	SoyFACE	Jelitto Perennial Seed
300	J236	SoyFACE	Jelitto Perennial Seed
301	J237	SoyFACE	Jelitto Perennial Seed
302	J238	SoyFACE	Jelitto Perennial Seed
303	J240	SoyFACE	Jelitto Perennial Seed
304	J241	SoyFACE	Jelitto Perennial Seed
305	J243	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
306	J244	SoyFACE	Jelitto Perennial Seed
307	J245	SoyFACE	Jelitto Perennial Seed
308	J246	SoyFACE	Jelitto Perennial Seed
309	J249	SoyFACE	Jelitto Perennial Seed
310	J250	SoyFACE	Jelitto Perennial Seed
311	J252	SoyFACE	Jelitto Perennial Seed
312	J253	SoyFACE	Jelitto Perennial Seed
313	J254	SoyFACE	Jelitto Perennial Seed
314	J255	SoyFACE	Jelitto Perennial Seed
315	J256	SoyFACE	Jelitto Perennial Seed
316	J257	SoyFACE	Jelitto Perennial Seed
317	J258	SoyFACE	Jelitto Perennial Seed
318	J260	SoyFACE	Jelitto Perennial Seed
319	J261	SoyFACE	Jelitto Perennial Seed
320	J262	SoyFACE	Jelitto Perennial Seed
321	J264	SoyFACE	Jelitto Perennial Seed
322	J265	SoyFACE	Jelitto Perennial Seed
323	J268	SoyFACE	Jelitto Perennial Seed
324	J269	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
325	J270	SoyFACE	Jelitto Perennial Seed
326	J271	SoyFACE	Jelitto Perennial Seed
327	J272	SoyFACE	Jelitto Perennial Seed
328	J273	SoyFACE	Jelitto Perennial Seed
329	J274	SoyFACE	Jelitto Perennial Seed
330	J275	SoyFACE	Jelitto Perennial Seed
331	J276	SoyFACE	Jelitto Perennial Seed
332	J277	SoyFACE	Jelitto Perennial Seed
333	J278	SoyFACE	Jelitto Perennial Seed
334	J279	SoyFACE	Jelitto Perennial Seed
335	J280	SoyFACE	Jelitto Perennial Seed
336	J281	SoyFACE	Jelitto Perennial Seed
337	J282	SoyFACE	Jelitto Perennial Seed
338	J283	SoyFACE	Jelitto Perennial Seed
339	J284	SoyFACE	Jelitto Perennial Seed
340	J285	SoyFACE	Jelitto Perennial Seed
341	J286	SoyFACE	Jelitto Perennial Seed
342	J34-2	SoyFACE	Jelitto Perennial Seed
343	J46-2	SoyFACE	Jelitto Perennial Seed

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
344	J95-2	SoyFACE	Jelitto Perennial Seed
345	*M.* x *giganteus* Bluestem	Greenhouse	Eric Sacks
346	*M.* x *giganteus* Freedom	Greenhouse	Eric Sacks
347	M x *giganteus* Gilded Tower	Greenhouse	Eric Sacks
348	*M.* x *giganteus* Hortico	Greenhouse	Eric Sacks
349	*M.* x *giganteus* Illinois	Greenhouse	Eric Sacks
350	*M.* x *giganteus* Longs Garden	Greenhouse	Eric Sacks
351	*M.* x *giganteus* Walla Walla	Greenhouse	Eric Sacks
352	*M. floridulus* Flower Factory	Greenhouse	Eric Sacks
353	*M. floridulus* Greenlee	Greenhouse	Eric Sacks
354	*M. floridulus* PI295762	Greenhouse	Eric Sacks
355	*M. floridulus* US56-0022-03	Greenhouse	Eric Sacks
356	*M. junceus*	Greenhouse	Eric Sacks
357	*M.* oligostachyus Bluemel	Greenhouse	Eric Sacks
358	*M. sacchariflorus* Earthly Pursuits	Greenhouse	Eric Sacks
359	*M. sacchariflorus* R2	Greenhouse	Eric Sacks
360	*M. sacchariflorus* Robustus-Earthly Pursuits	Greenhouse	Eric Sacks
361	*M. sacchariflorus* Triple Brook Farm	Greenhouse	Eric Sacks
362	*M. sinensis* Ben Rotkopf	Greenhouse	Eric Sacks

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
363	*M. sinensis* Bodacious Ben	Greenhouse	Eric Sacks
364	*M. sinensis* Border Bandit	Greenhouse	Eric Sacks
365	*M. sinensis* Burgander	Greenhouse	Eric Sacks
366	*M. sinensis* Emmanuel LePage	Greenhouse	Eric Sacks
367	*M. sinensis* Gracillimus	Greenhouse	Eric Sacks
368	*M. sinensis* Huron Blush	Greenhouse	Eric Sacks
369	*M. floridulus* PI295762-2	Greenhouse	Eric Sacks
370	*M. Junceus* UI10-00003-2	Greenhouse	Eric Sacks
371	*M.* sacchariflorisSilver Banner Grass EF0358-2	Energy Farm	Kurt Bluemel Nursery
372	*M. sinensis* Adagio EF0010-2	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
373	*M. sinensis* Blondo EF0259-1-2	Energy Farm	Kurt Bluemel Nursery
374	*M. sinensis* Graziella EF0166-2	Energy Farm	Kurt Bluemel Nursery/Emerald Coast Growers
375	*M. sinensis* Juli EF0034 -2	Energy Farm	Kurt Bluemel Nursery
376	Maize B73	N/A	Brandon James
377	*M. sinensis* Miyazaki	Greenhouse	Ashley Spence
378	Sorghum 73	N/A	Pat Brown
379	Sorghum 85	N/A	Pat Brown
380	Sorghum 87	N/A	Pat Brown
381	Sorghum 98	N/A	Pat Brown

Table 4.1 Continued

Phylogenetic Tree Reference Number	Name of Accession	Location	Source
382	*M. sinensis* Sugadairu	Greenhouse	Ashley Spence
383	*M. sinensis* Teshio	Greenhouse	Ashley Spence
384	UI10-00002	Greenhouse	Eric Sacks
385	UI10-00057-2	Greenhouse	Eric Sacks
386	UI10-00058	Greenhouse	Eric Sacks
387	UI10-00063	Greenhouse	Eric Sacks
388	UI10-00068	Greenhouse	Eric Sacks
389	UI10-00076	Greenhouse	Eric Sacks
390	UI10-00080	Greenhouse	Eric Sacks
391	UI10-00086	Greenhouse	Eric Sacks
392	UI10-00088	Greenhouse	Eric Sacks
393	UI10-00095	Greenhouse	Eric Sacks
394	Undine6	Greenhouse	Won Byoung Chae
395	Unparent5	Greenhouse	Won Byoung Chae
396	Unparent7	Greenhouse	Won Byoung Chae
397	Unparent8	Greenhouse	Won Byoung Chae
398	M *sinensis* Uruyu	Greenhouse	Ashley Spence

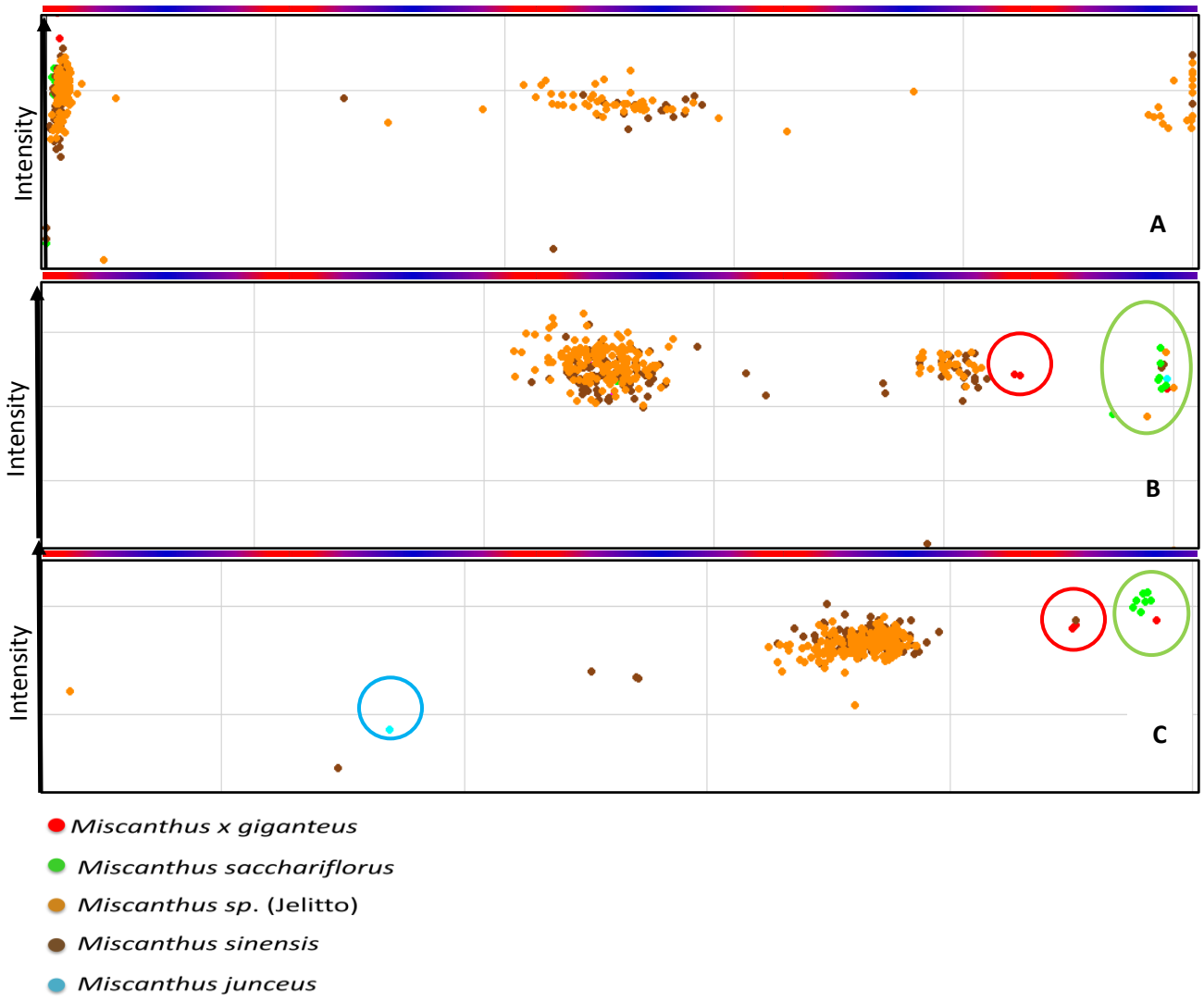

Figure 4.1 Genotype Calling for *Miscanthus* GoldenGate array. X axis is the intensity of the combined Cy3 and Cy5 signals while Y axis is read as Red= homozygous for allele 1, Blue=homozygous for allele 2, and Purple =heterozygous. *Miscanthus* accessions are color coded for easy detection. Panel A depicts a clear 3 cluster distribution which is typical to a diploid with no parlogs sampled. Panels B and C are skewed which represents the presence of a fixed loci. The circled samples represent clusters that are separating at the species level. You can clearly see that *M.* x *giganteus* and *M. sacchariflorus* are distinguishable from the *M. sinensis*. Both the ornamental accessions and the Jelitto population are indistinguishable from one another. *M. junceus* is clearly distinguishable from all other species sampled on the assay. This clustering is representative of what occurred at most SNP loci for *M. junceus*.

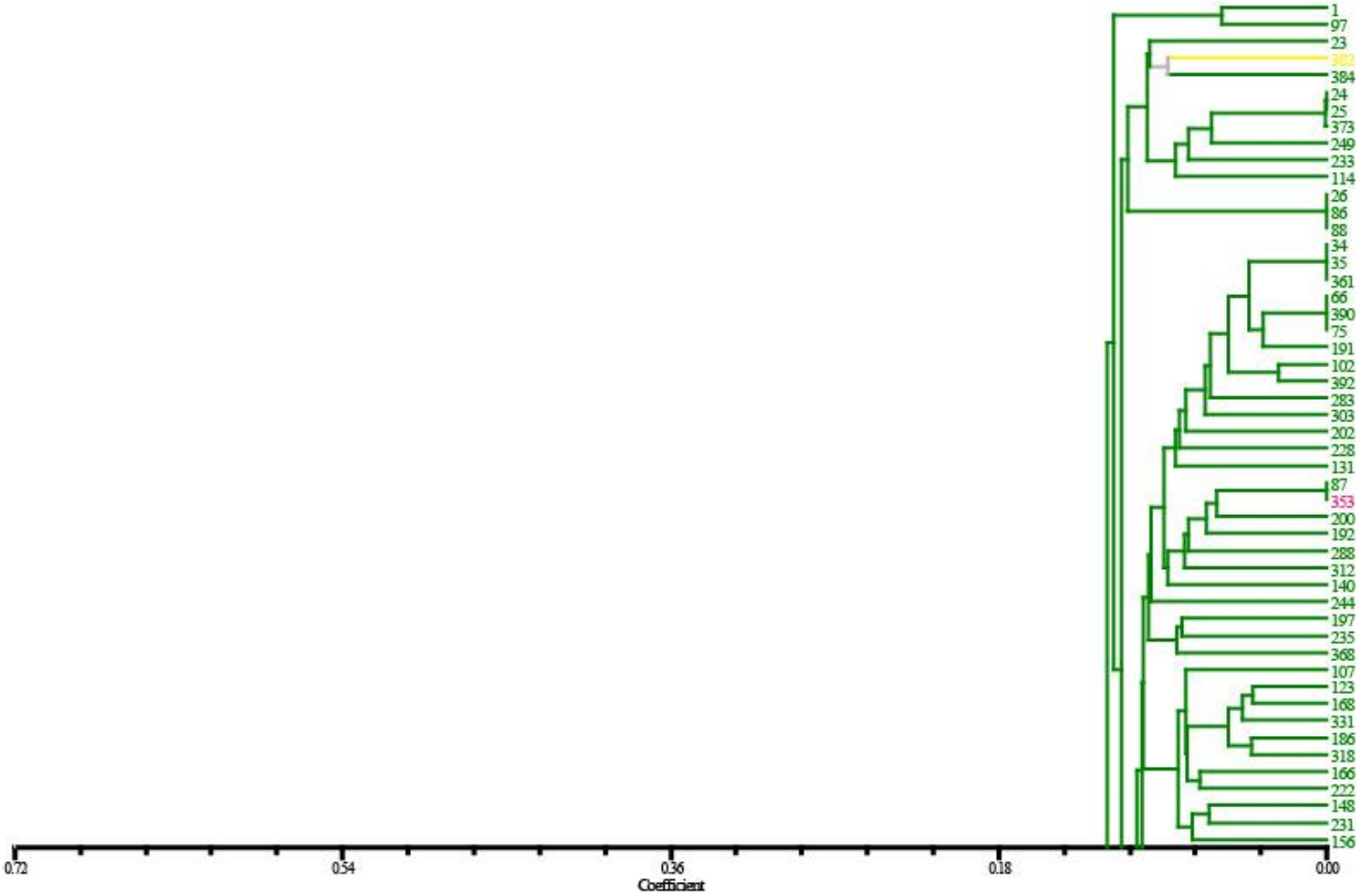
1
97
23
382
384
24
25
373
249
233
114
26
86
88
34
35
361
66
390
75
191
102
392
283
303
202
228
131
87
353
200
192
288
312
140
244
197
235
368
107
123
168
331
186
318
166
222
148
231
156
0.72
0.54
0.36
0.18
0.00
Coefficient

Figure 4.2

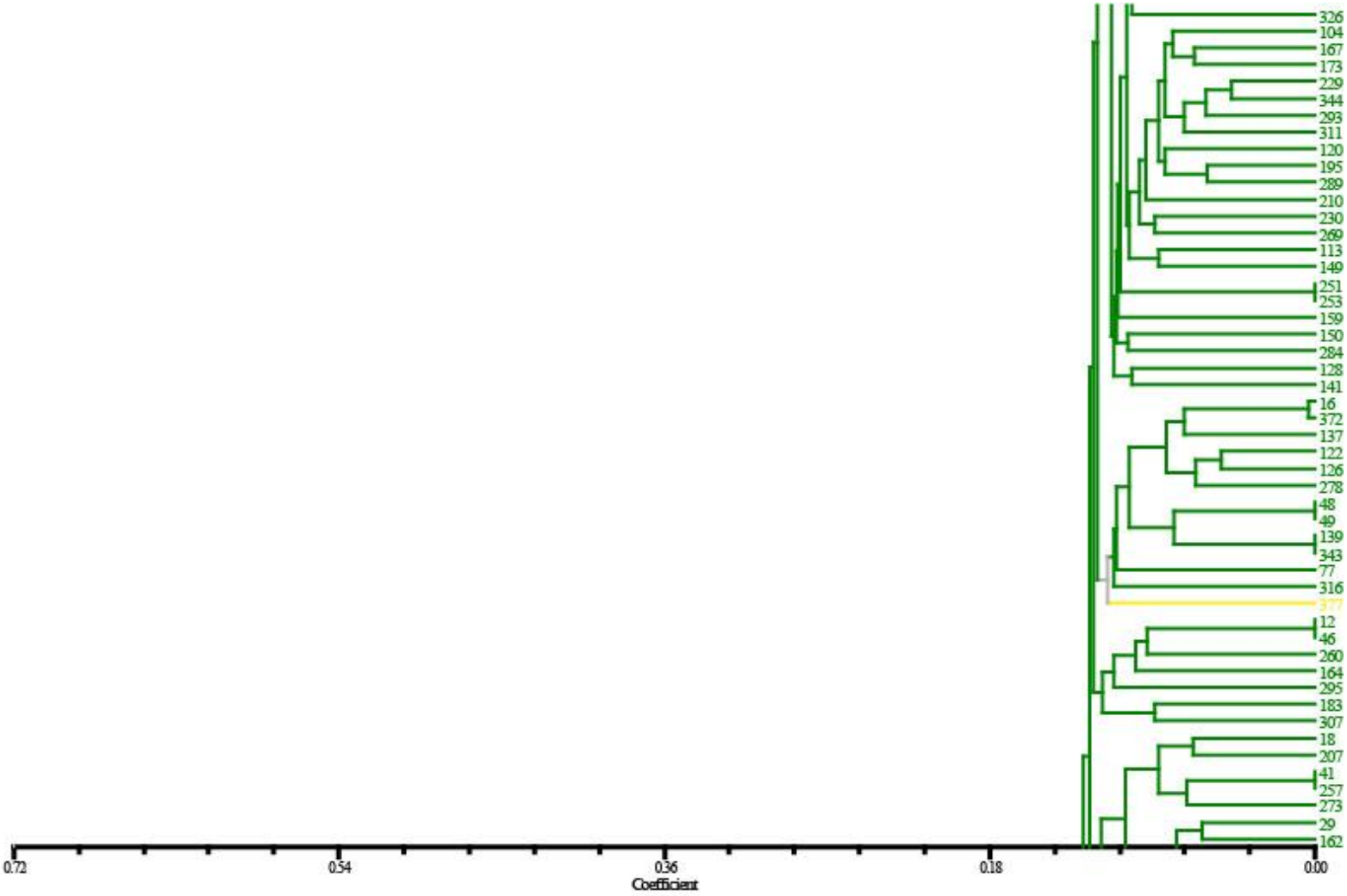

Figure 4.2 Continued

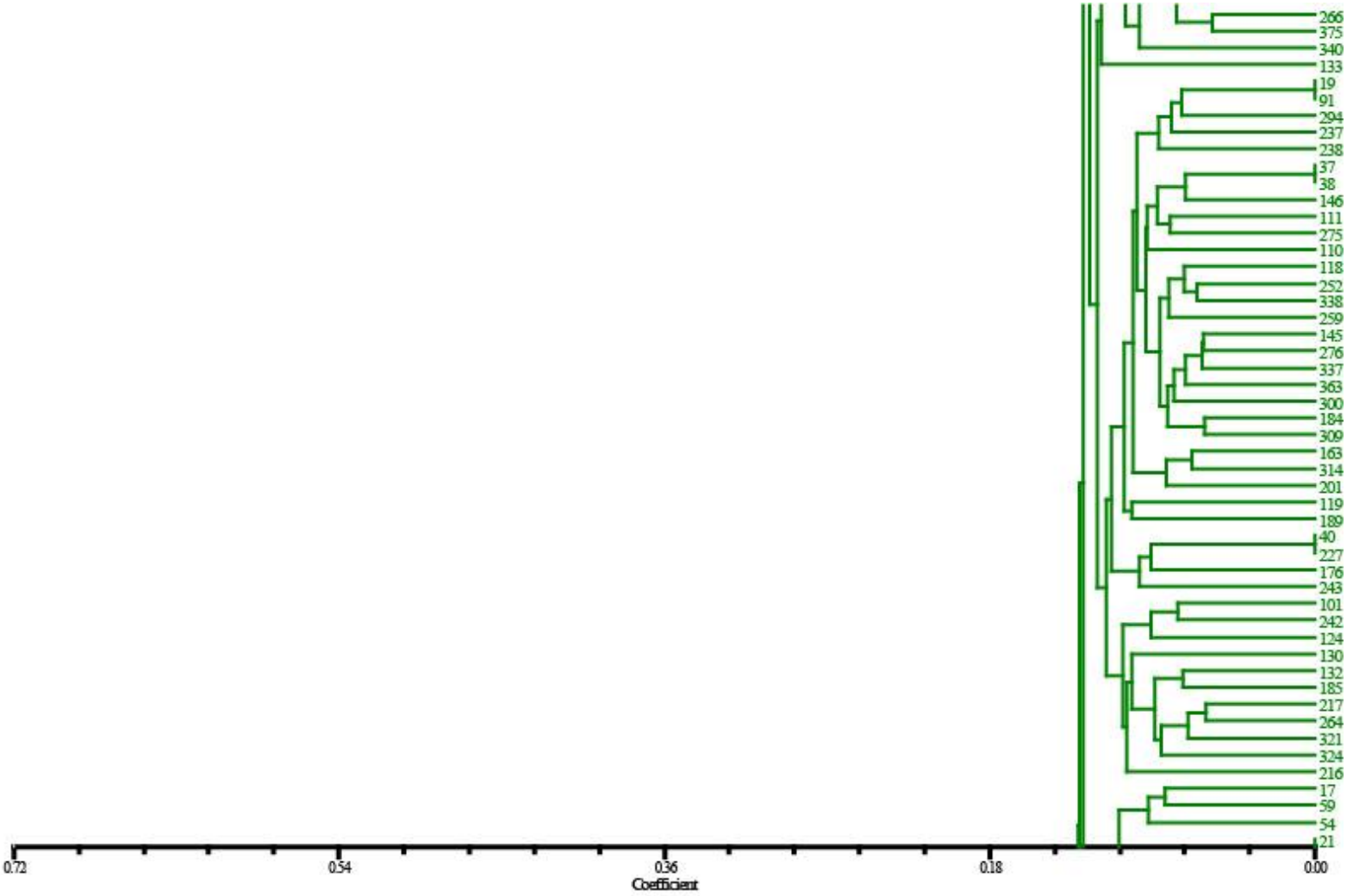

Figure 4.2 Continued

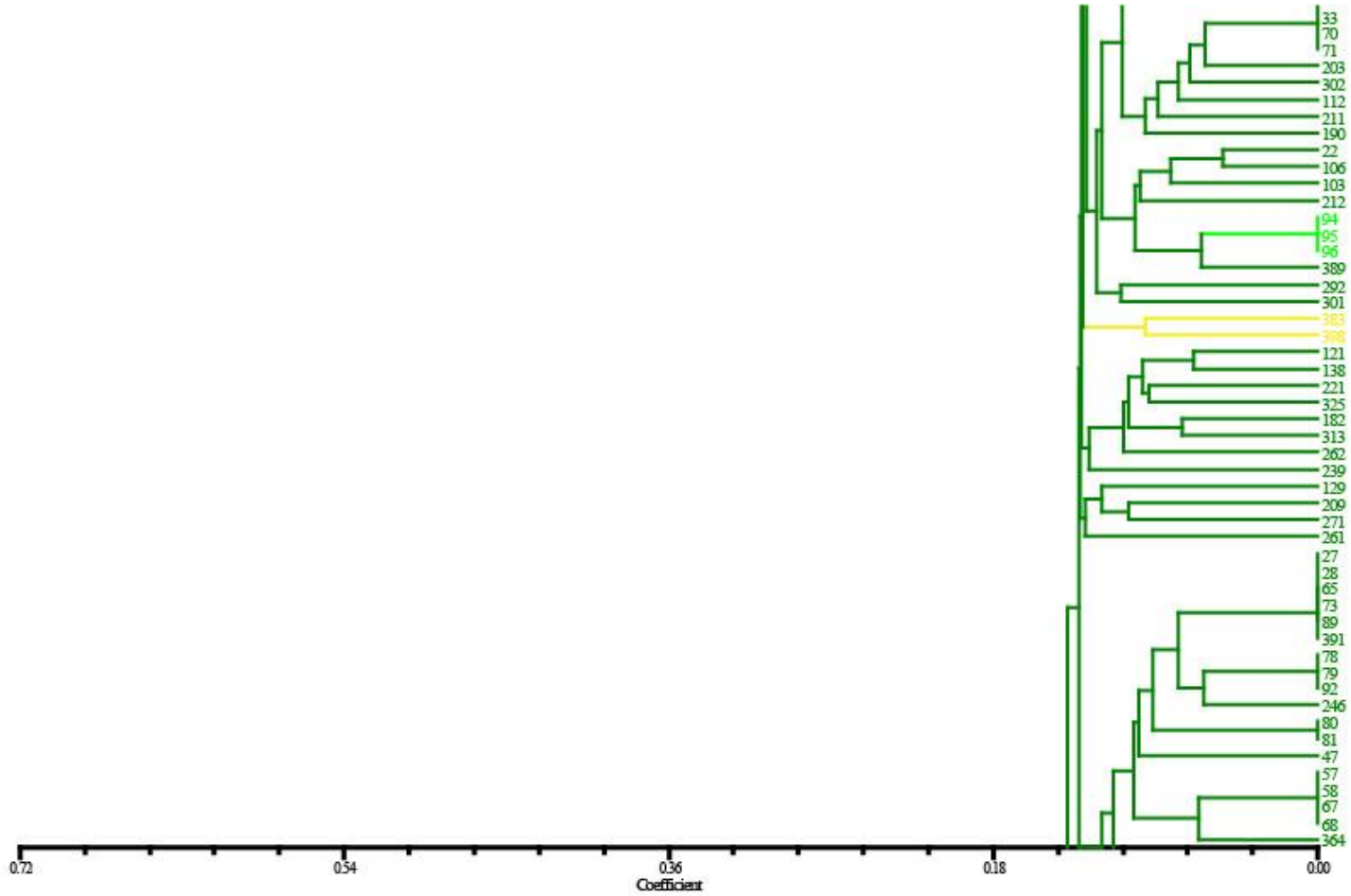

Figure 4.2 Continued

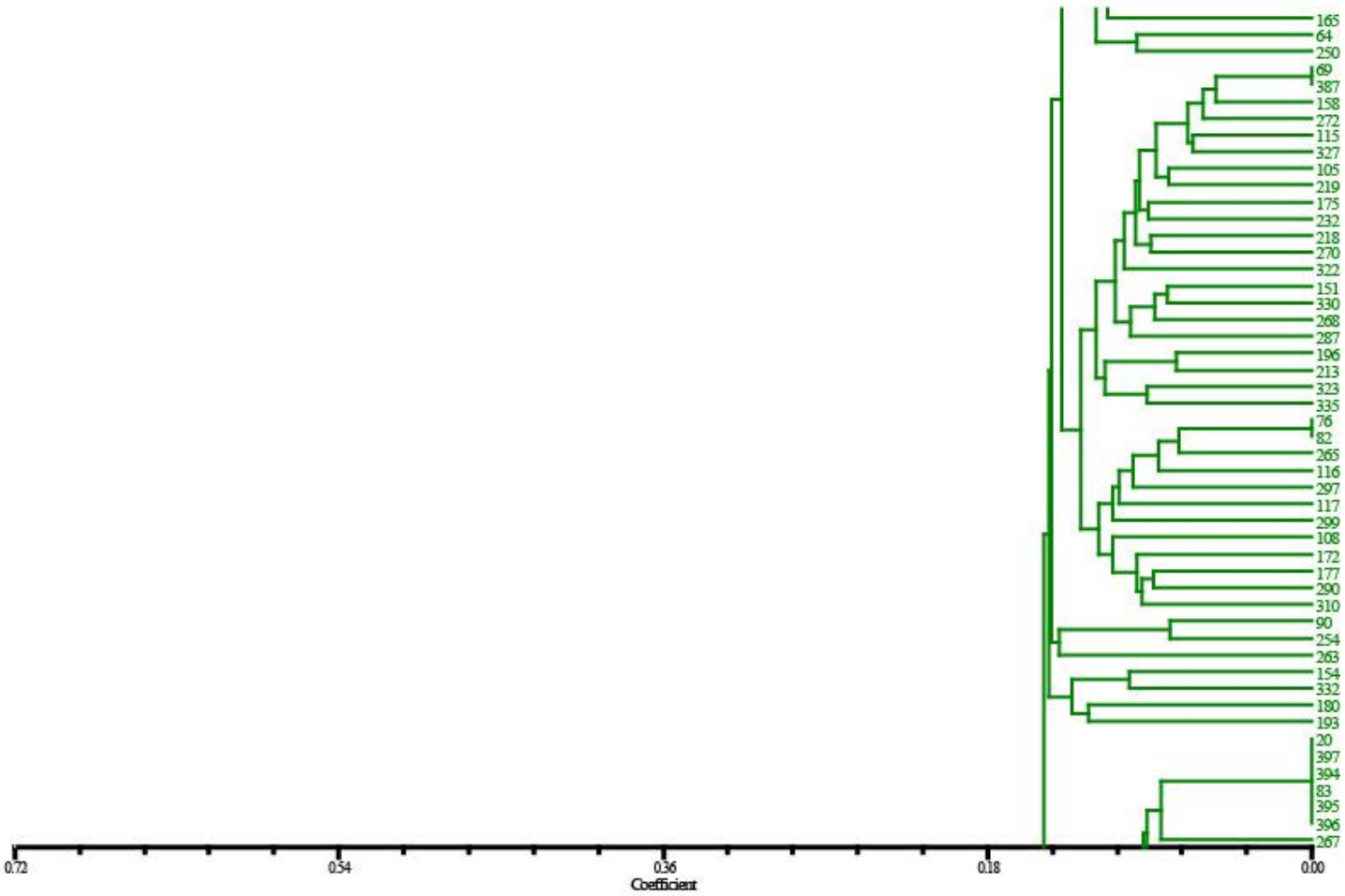

Figure 4.2 Continued

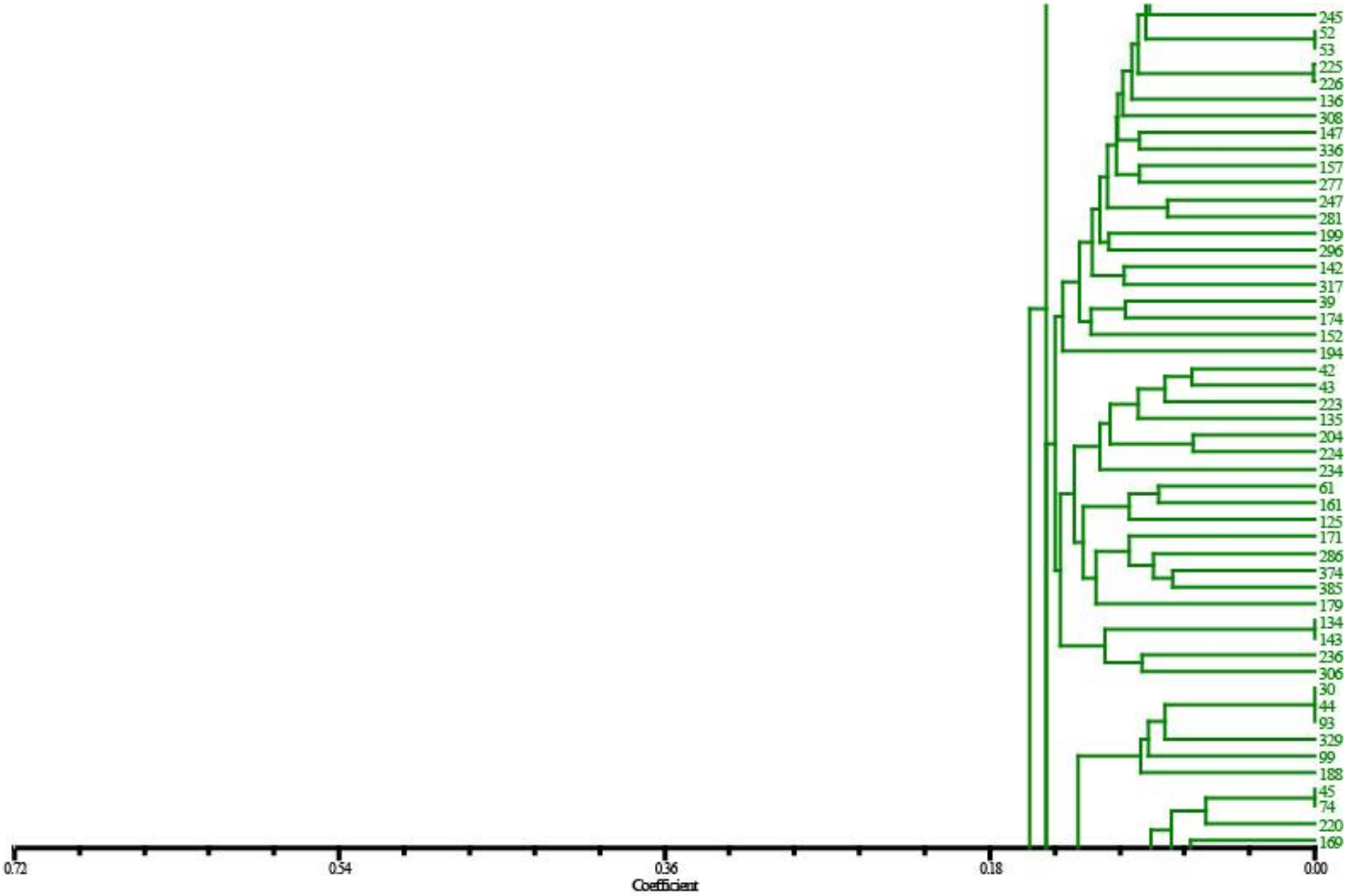

Figure 4.2 Continued

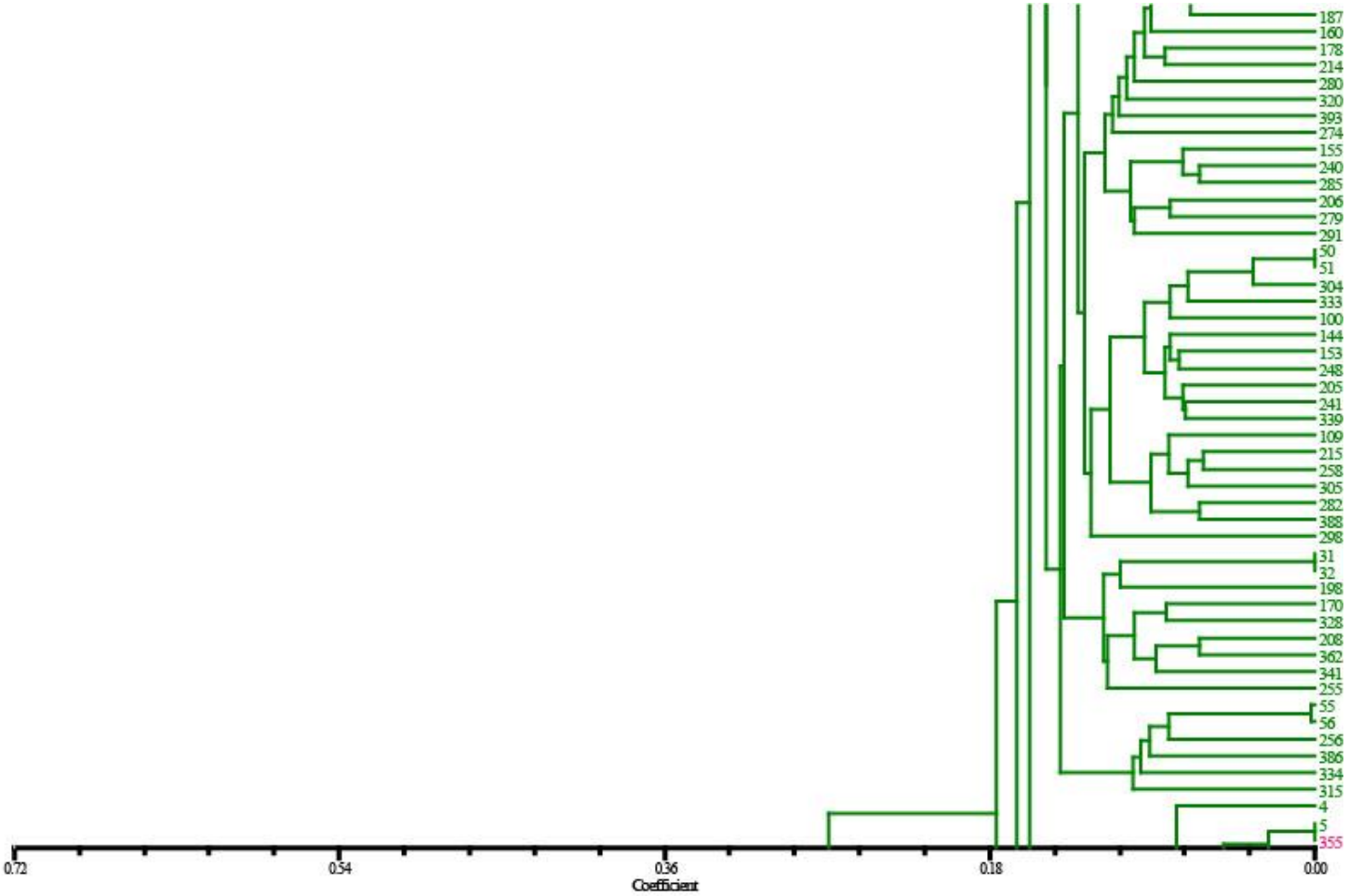

Figure 4.2 Continued

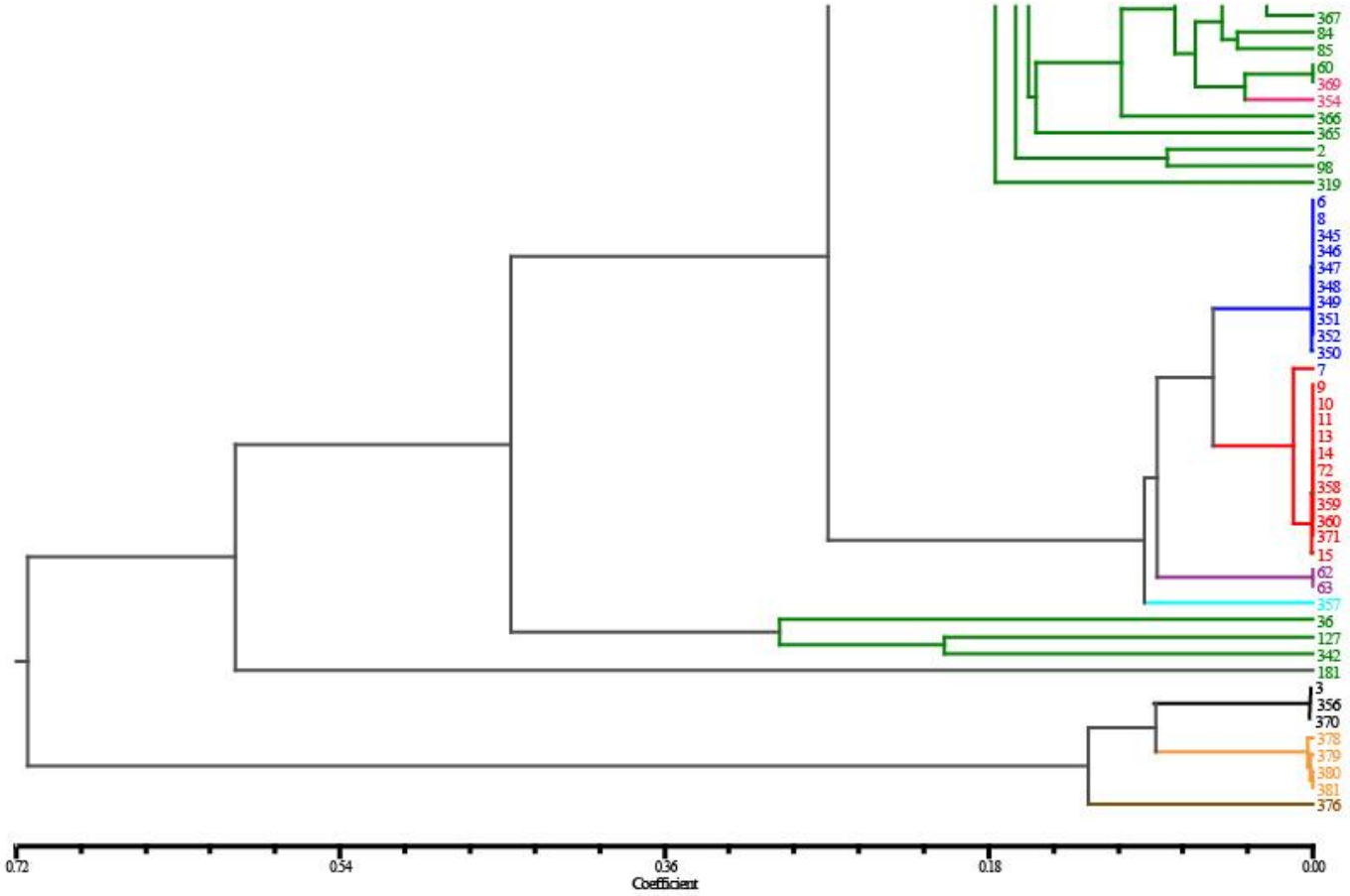

Figure 4.2 Continued Phylogenetic Tree of 398 *Miscanthus* Accessions compiled from GoldenGate genotyping data. A Nei's coefficient of dissimilarity was applied to the data. Cluster analysis using UPGMA-SAHN was able to distinguish groups at the species level. The tree starts at 0 which represents the lowest amount of dissimilarity (i.e. 100% similarity). The shorter the lines the more similar the accessions are to one another. Lines in Green = *M. sinensis*, Pink = *M. floridulus*, Blue=*M. x giganteus*, Red = *M. sacchariflorus*, Purple = *M. purparescens*, Light Blue = *M. oligostachyus*, Black=*M. junceus,* Japanese accessions = Yellow. Groups outside of *Miscanthus* include Brown=maize, Orange=*Sorghum*

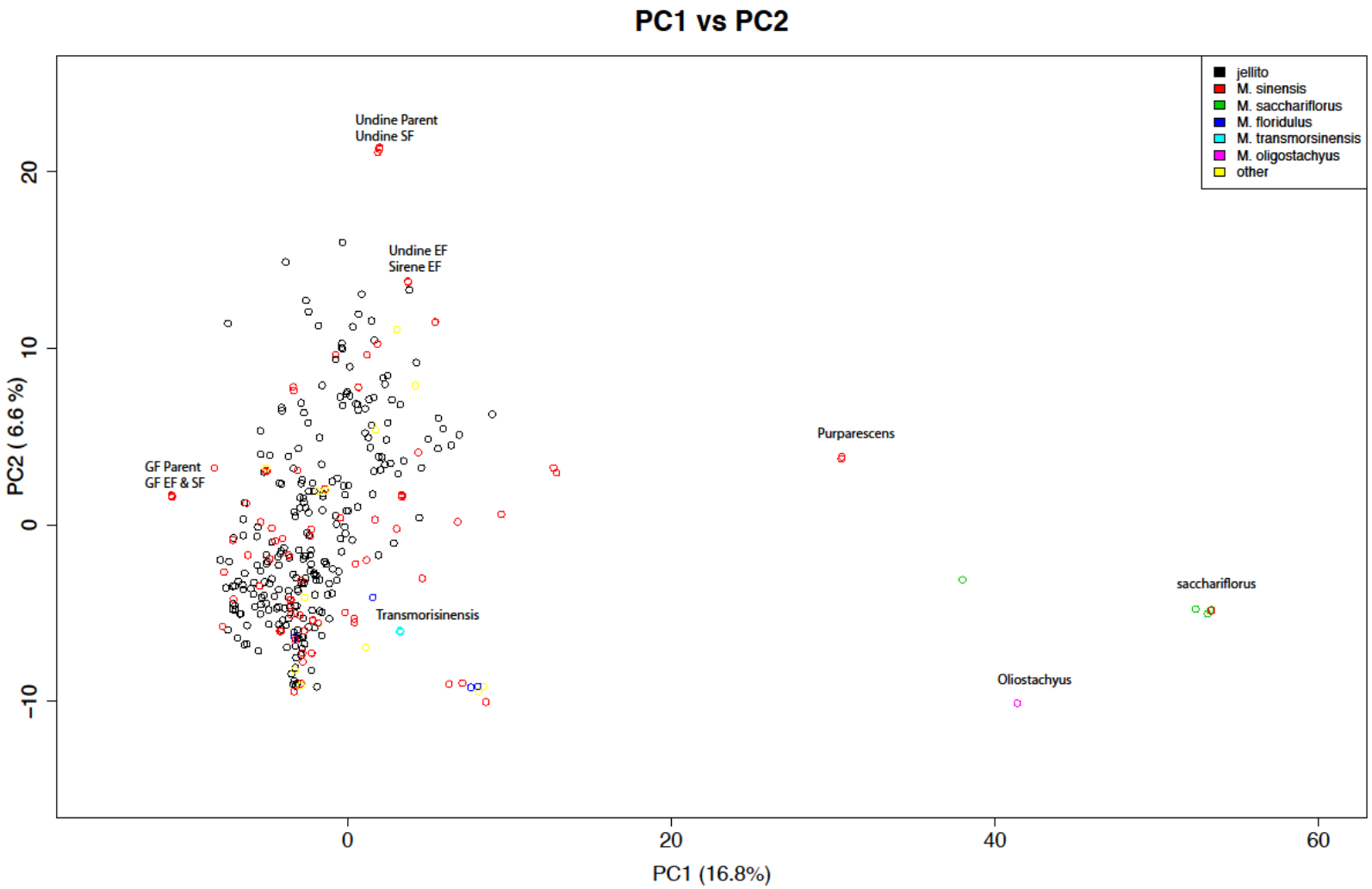

Figure 4.3 Principle Component Analysis of *Miscanthus* Accessions. Samples are color coded by species. All plants from the Jelitto population are colored in Black. It is clear from the PCA that the ornamental accessions and Jelitto population are not displaying any large variance from one another. To the right however, there is clear distinction between the *M. sacchariflorus*, *M. purparescens* and *M. oligostachyus* species sampled. *M. transmorrisonesis* is not clearly indistinguishable from the *M. sinensis* accessions.

Resul

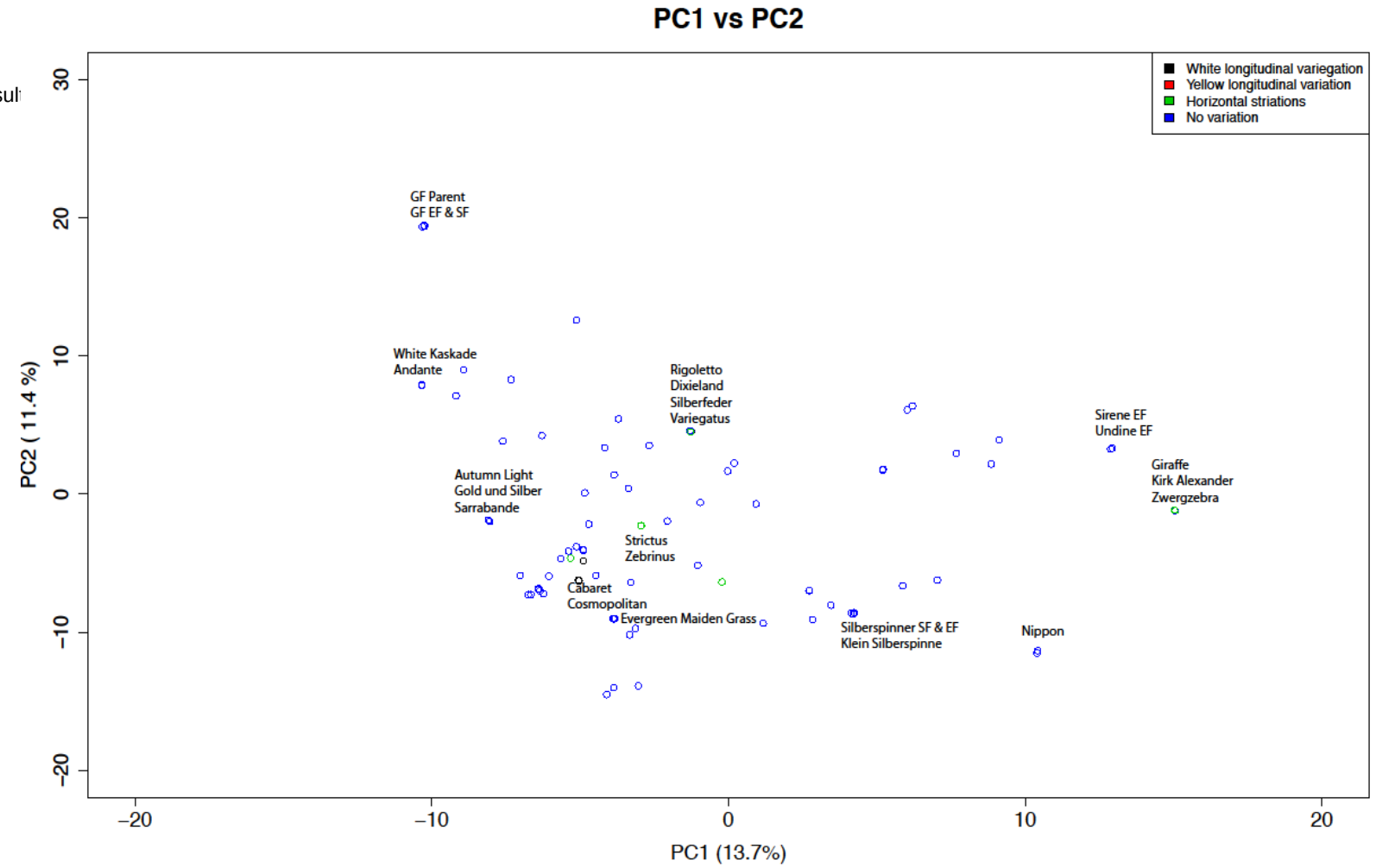

Figure 4.4 Principle Component Analysis of *M. sinensis* plants from Energy Farm. Plants are color coded for presence and type of variegation observed in the field. There is clear overlap between accessions that were given different common names. This suggests that these samples even though having different common name are actually the same.

Figure 4.5 Phylogenetic Tree of 251 *Miscanthus* Accessions from the Jelitto population compiled from GoldenGate genotyping data. A Nei's coefficient of dissimilarity was applied to the data. Cluster analysis using UPGMA-SAHN was used. The tree starts at 0 which represents the lowest amount of dissimilarity (i.e. 100% similarity). The shorter the lines the more similar the accessions are to one another. Lines in Green = *M. sinensis* while groups outside of *Miscanthus* include Brown=maize, Orange=*Sorghum*

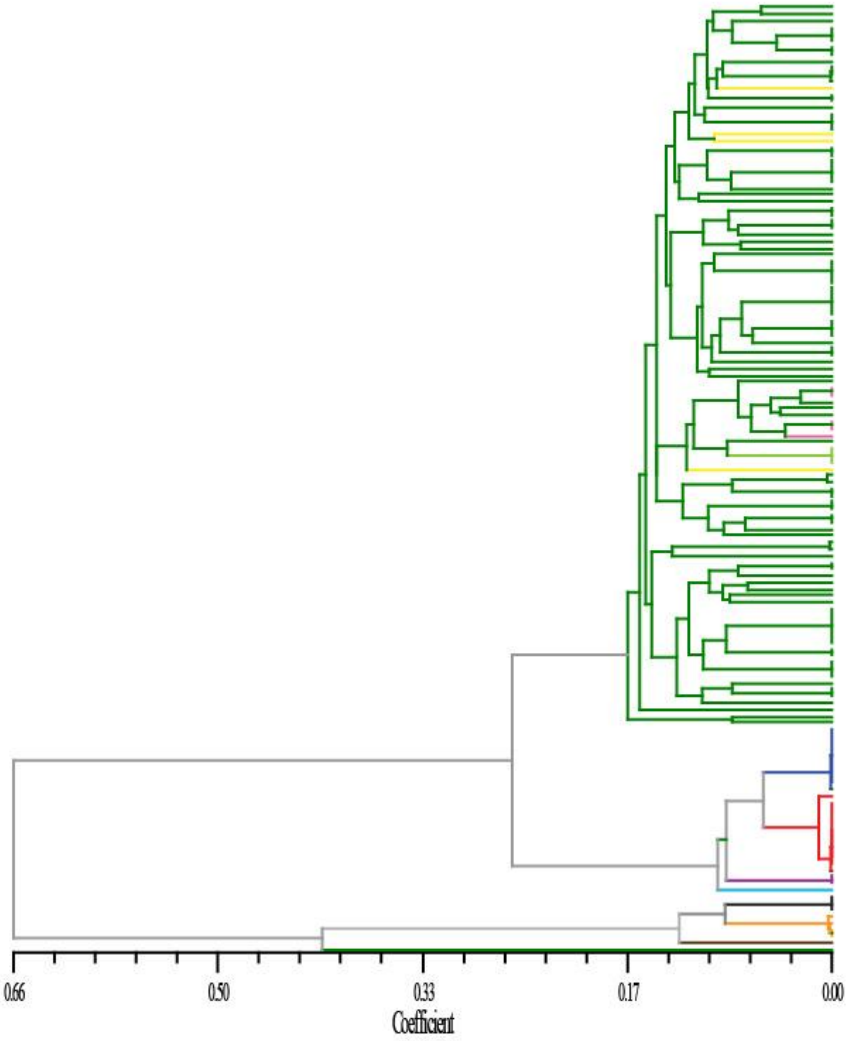

Figure 4.6 Phylogenetic Tree of 137 *Miscanthus* Ornamental Accessions compiled from GoldenGate genotyping data. A Nei's coefficient of dissimilarity was applied to the data. Cluster analysis using UPGMA-SAHN was used. The tree starts at 0 which represents the lowest amount of dissimilarity (i.e. 100% similarity). The shorter the lines the more similar the accessions are to one another. Lines in Green = *M. sinensis*, Pink = *M. floridulus*, Blue=*M. x giganteus*, Red = *M. sacchariflorus*, Purple = *M. purparescens*, Light Blue = *M. oligostachyus*, Black=*M. junceus,* Japanese accessions = Yellow. Groups outside of *Miscanthus* include Brown=maize, Orange=*Sorghum*

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Chapter 5: Concluding Remarks

This study used three emergent methods of marker based genotyping to re-examine inter-specific relations in the genus *Miscanthus* (Hernández et al. 2001; Hodkinson and Chase 2002; Ho et al. 2011), and identify effective methods for separating and analyzing intra-specific variation. Specifically, this study used molecular marker methods to understand the variability within *Miscanthus* resources already within the United States that might be used in improving *Miscanthus* as a bioenergy feedstock. *Miscanthus* has long been important to the nursery industry and is currently being considered for its potential as a bioenergy feedstock (Jones and Walsh 2001; Clifton-Brown 2002; Stewart et al. 2009), with a particular focus on the putative sterile inter-specific hybrid *M. x giganteus* (Heaton et al. 2008; Anderson et al. 2011). Despite previous taxonomical studies, there remains an overall lack of consensus on species delineation (Hodkinson and Chase 2002; Amalraj and Balasundaram 2005; Clifton-Brown et al. 2008; Sun et al. 2010), and over the diversity and similarity of the ornamental cultivars that are already available. Molecular approaches have improved this situation(Hodkinson, Chase, Takahashi, et al. 2002; Hodkinson, Chase, Lledó, et al. 2002; Hung et al. 2008; Zhou et al. 2011), but with methods allowing the use of a much wider number of markers and more rapidly, there are now new opportunities which have been investigated here.

Chapter 2 showed that High Resolution Melting (HRM) platform could effectively separate species of *Miscanthus* and separate accessions within each species, including variants of *M. x giganteus* which were likely derived from the same vegetative clone and could only differ via somatic mutations and/or epigenetic differences. Although 96 primer set were used; only 32 primers were be utilized at a time. Analysis of the finalized tree showed that 32 primers would be sufficient to achieve the separation. This means that the species level relationship could ultimately be determined with a very minimal set of primer sets. At this level HRM was effective in identifying two accessions that had incorrectly been

provided as a different species. Because HRM does not require sequencing it is both quick and low in cost, and would provide a practical method for checking the authenticity of supplied rhizomes, at least at the species level, and could also be an effective way of identifying infraction in the marketing of germplasm that is protected under plant breeders rights or utility patents. Even with its exceptional success in identifying mislabeled/misidentified accessions as shown in Chapter 2, such as *M.* x *giganteus* 'Kurt Bluemel' and *M. sinensis* 'Hercules,' there are drawbacks. First if the DNA samples used are highly methylated or extracted by different methods it could affect the melting profiles. The latter can of course be overcome by using an identical procedure for DNA isolation and critically a consistent buffer for resuspension(Reed et al. 2007; Wu et al. 2008; Studer et al. 2009). Secondly, additional samples cannot be analyzed for comparative purposes at a later date since normalization has to be on a plate by plate basis. Despite these weaknesses, HRM analysis is still a quick and cost effective manner of screening *Miscanthus* accessions for intraspecific and interspecific relationships.

In Chapter 3, the number of accessions sampled was increased 8-fold from the study in Chapter 2 and Simple Sequence Repeats (SSRs) primers were used for assessing genetic diversity. SSRs from sugarcane were used to separate *Miscanthus* accessions to assess inter and intra specific relationships. A total of 48 SSR primer sets were used with *Miscanthus* accessions as well as sugarcane control lines. Genotyping *Miscanthus* with SSR markers is not a new technique (Hernández et al. 2001; Hodkinson and Chase 2002; Hung et al. 2008; Ho et al. 2011), but this study generated significantly more markers and sampled more accessions within one experiment than had been conducted previously. The SSR markers separated all accessions and distinguished accessions at the species level. Critically it showed that the diversity in the ornamental accessions already present in the USA, surprisingly far exceeded the diversity of *M. sinensis* recently collected from the length of Japan. This suggests that the ornamental accessions are not a narrow selection from a few collections from the potential germplasm, but likely represent

multiple collections across the wild germplasm in E. Asia. The SSR analysis also confirmed findings from Chapter 2 that *M. sinensis* 'Hercules' had been mislabeled and is actually *M. sacchariflorus*.

There were drawbacks to the SSR platform. Because the SSR primers were developed from sugarcane the success of amplification in *Miscanthus* was decreased and this could be solved by using *Miscanthus* derived SSR primers; 7 primer sets failed to amplify any *Miscanthus* accessions. Also, the efficiency of the PCR assay can affect the ability to detect alleles. If a particular locus fails to amplify because of PCR error it may appear to be homozygous where it is really heterozygous. Unfortunately, there is no way to clearly distinguish between PCR efficiency and actual allele calls within this study. This may have accounted for the less clear separation of *M.* x *giganteus* 'Kurt Bluemel', than observed in the HRM analysis. It may also explain why closely related accessions, such as those of *M. x giganteus* showed almost the same similarity as different species.

The ease of use of SSR markers makes it a platform that can still be used to assess diversity and relationships within *Miscanthus*. One important attribute of this platform that makes it more feasible than HRM is that it can be performed by simple gel scoring, so while it may be more labor intensive it does not require additional machinery (Wang and Barkley 2009). Another advantage of SSR markers is that additional samples can be added into the analysis at a later time.

Both HRM and SSR are PCR based platforms that can lead to errors in. For many primer sets there is a possibility to amplify four variants of the target DNA. There are two possible variants because of the genome duplication within *Miscanthus* (Ma et al. 2012; Swaminathan et al. 2012) and the remaining two variants are due to the alleles having a high level of heterozygosity. If for instance one variant is initially amplified in the PCR reaction the likihood of the other variants to subsequently be amplified decreases at an exponential rate. Therefore it is important to note that because of the duplication within *Miscanthus* the results of both HRM and SSR could change in regards to the branch

lengths seen on the dendrograms. The long branch lengths seen in both Figure 2.5 and Figure 3.1 could be due to the error prone nature of PCR based genotyping methods and if these experiments were repeated by other laboratories slight differences may be seen. However, the overall grouping at the species level will not change which makes both methods acceptable for distinguishing these major differences. There is most likely a level of error/noise that can be factored into both methods but further investigation would have to be done to accurately calculate this.

In Chapter 4, high throughput genotyping using a GoldenGate SNP assay (Illumina Inc., San Diego, CA) was conducted on over 300 *Miscanthus* accessions. This study included a population of *M. sinensis* plants obtained from Jelitto Perennial Seed (Louisville, KY) as seed which were derived from open crossing of a collection of accessions with different flowering times. These plants were compared with the ornamental accessions examined in the preceding chapters. The GoldenGate assay successfully separated and fingerprinted each of the over 300 *Miscanthus* accessions analyzed. This assay provided genotyping information at 803 loci and achieved the most robust genotyping of *Miscanthus* ornamental accessions to date. Most importantly, in the longer term morphological and phenotypic traits can potentially be linked to specific SNPs or genes (Hyten et al. 2008; Yan et al. 2009). Currently, only species level differences and presence of leaf variegation within *M. sinensis* accessions were explored but other phenotypic traits could be included such as flowering time, plant height, or even biomass to find correlations to certain genes.

There are disadvantages to using the GoldenGate assay. The design of the assay used was ideal for examining relationships within *M. sinensis*. But since it was designed against single nucleotide variation between two *M. sinensis* accessions (Swaminathan et al. 2012) there was bias in the assay against other species, which may explain why it was poor at separating *M.* x *sacchariflorus* accessions. However, since *M. x giganteus* contains a *M. sinensis* genome, in theory this should be effective still at

separating accessions of this species. The lack of difference here suggests these accessions despite their different names are likely derived from the same clone. The other interpretation is that they are all derived from the same *M. sinensis.* However, since *M. sinensis* is self-infertile, this is highly unlikely. This contrasts to HRM which did separate the *M. x giganteus* accessions, but as noted earlier DNA methylation resulting from epigenetic effects could cause apparent differences even when sequences are identical. The idea of a bias due to species differences should not be ruled out however. The GoldenGate methods also identified the *M. x giganteus* "Kurt Blumel" as a *M. sacchariflorus* in agreement with morphological typing and the HRM analysis. Further it provided very clear evidence that all three accessions of *M. transmorisonensis* should be classified as a variant firmly within *M. sinensis.* GoldenGate appears the most robust of the methods examined for genotyping *Miscanthus* and most likely for any other plant to date, aided by the exceptional number of loci and polymorphisms that can be examined at one time(Hyten et al. 2008; Akhunov et al. 2009; Yan et al. 2009). Its ability to handle various ploidy levels as well as produce information about allele frequencies and paralogs is unrivaled; especially for species where the whole genome sequence is not available, as in the case of *Miscanthus*.

With the advancement of technology and resources, the potential genotyping platforms for *Miscanthus* evolved over the course of the research conducted in this dissertation. There are methods now in place to assess the relationships of *Miscanthus,* whether at the interspecific or intraspecific level. This research has allowed us to understand the level of genetic diversity available in *Miscanthus* accessions available within the United States. The use of any one of these three technologies can be used identify mislabeled collections as in the case of *M. x giganteus* "Kurt Blumel". This would avoid unsuspecting purchaser from planting a fertile and possibly invasive plant, when the expectation was that a sterile plant had been purchased. This research has shown methods that can aid breeding for seed producing and the synthesis of new sterile high yielding hybrids for clonal propagations. In addition

to allowing identification of genotypes from asexual plant parts, including rhizome propagules, these techniques provide a practical means to protect intellectual property in the form of newly developed clones, an important incentive and necessary precursor to commercial investment in improving this emerging crop.

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Appendix

Table CTAB Extraction Buffer Formulation

CTAB (2%) Extraction Buffer

Reagent	**Quantity (for 1L)**	**Final concentration**
Tris–HCl (1M, pH 9)	100mL	100 mM
NaCl	81.8g	1.4 M
Hexadecyltrimethylammonium bromide (CTAB)	20g	2% (w/v)
EDTA (0.5M, pH 7)	40mL	20mM
Polyvinyl Pyrrolidone (PVP)	20g	2%
PEG-600	10g	1%

Cost Breakdown for HRM Analysis

Cost of HRM Dye	**# of Reactions**	**Cost per Reaction**	**Cost per 96 Well Plate**	**Cost per 384 well plate**
$420	1000	$0.42	40.32	161.28