ABSTRACT

MULKEY, STEVEN EDWARD. SSR Development and Mapping of Gray Leaf Spot Resistance Genes in St. Augustinegrass. (Under the direction of Dr. Susana Milla-Lewis).

St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] is a widely used turfgrass in the southern United States. Development and application of molecular tools in the species have lagged behind other warm season grasses. The present investigation was conducted in order to increase our understading of levels of genetic diversity and to develop molecular tools that could facilitate selective breeding.

To assess the genetic diversity and ploidy variation present within the the University of Florida (UF) St. Augustinegrass germplasm collection, a large pool of variation for the species, a combination of amplified fragment length primers (AFLPs) and flow cytometry were used. Four ploidy levels were designated based on flow cytometry results with most accessions falling into the diploid and proximal tetraploid categories. Unweighted pair group method with arithmetic averaging (UPGMA) cluster and principal coordinate (PCO) analyses grouped accessions largely by ploidy level. Among diploid accessions one cluster was identified that was unrepresented by any cultivars or plant introductions. These findings indicate that the UF St. Augustinegrass germplasm collection contains significant, untapped pools of diversity at diploid and polyploid levels and could be a great asset for cultivar development.

Illumina genomic sequencing data was used to develop simple sequence repeat (SSR) markers for St. Augustinegrass. Polymorphism was observed in 190 of 215 designed primer pairs among a panel of eight diverse germplasm accessions. Of these, 94 were subsequently used to assess levels of diversity within a diverse collection of *Stenotaphrum*

germplasm. Levels of polymorphism were high, with number of alleles amplified per loci ranging from 5 to 23 (μ=10) and with polymorphic information content (PIC) values ranging from 0.09 to 0.83 (μ =0.54). Analysis of population structure identified six subpopulations with some admixture present among them. Additionally, the ability of these newly developed SSRs to uniquely identify specific cultivars was assessed. A set of five primers were capable of producing unique banding patterns for all but two cultivars. These markers, the first developed for the species, should be useful for linkage mapping, diversity and pedigree analysis, accurate varietal identification, and marker assisted selection in St. Augustinegrass.

Genetic linkage maps of St. Augustinegrass were constructed for quantitative trait loci (QTL) analysis of resistance to gray leaf spot using a total of 107 AFLP fragments and 36 SSRs on a pseudo F_2 population of 77 progeny. Twenty two linkage groups were identified, covering 1216.8 cM. Ten linkage groups were ascribed to the susceptible maternal cultivar, ‘Raleigh’, nine linkage groups to the resistant paternal accession, PI410353, and three groups were found to be homologous. The Raleigh (67 markers) and PI410353 (39 markers) maps covered 525.3 cM and 394.8 cM, respectively, while the homologous linkage groups consisted of 37 markers covering 296.5 cM. The average marker density across all groups was 8.5 cM/marker. Four putative QTL were identified through interval and composite interval mapping. These results represent the first linkage map produced for St. Augustinegrass, providing a template for further genetic mapping. Additionally, markers linked to the QTL identified in this study can be used by breeders to transfer resistance to gray leaf spot from unadapted germplasm to elite breeding lines and cultivars

SSR Development and Mapping of Gray Leaf Spot Resistance Genes in St. Augustinegrass

by
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DEDICATION

To my mom, for being the pillar of strength and steady rock I could build my life upon. To my family and friends for all the understanding, excitement, and joy you bring to my life. Thank you all.

BIOGRAPHY

Steve was born in 1985 and grew up in Sebastian, Florida. As a child he spent much of his time running through the woods, building forts, avoiding alligators, and getting poison ivy. For most of his youth Steve went to school at the Kashi Ashram, where he developed a deep love of nature and respect for all forms of life. He also learned to accept people from all walks of life and to help those in need.

Steve went to Cornell University for his undergraduate education, for which he is eternally grateful. There he learned about love, life, and… Science! The knowledge and work ethic battered into him there have proven invaluable. The friendships he acquired there, forged through mutual struggle and shared optimism for the future, transformed his life and continue to brighten it to this day. Steve also had the opportunity to get first hand exposure to the wide world of plant research in the labs of Dr. Wolfe, Dr. Mohler, and Dr. Earle. Steve spent a year following graduation learning about molecular markers and playing with toxic chemicals in Dr. Mark Sorrells' lab.

When he isn't sitting in front of a computer counting ones and zeros, or pipetting reagents into tiny tubes, Steve likes to spend his time enjoying good food, good beer, and good friends. He enjoys home brewing, and has his sight set on some excellent hop varieties for his garden.

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Literature Review

General Overview of Turfgrass Production and Economic Importance in North Carolina

Throughout history humans have sought to change the world around them, adapting the environment to meet their needs while selectively breeding plants and animals to increase their utility and productivity. Beyond this we have purposely molded nature, making trusting companions and sculpting plants and landscapes to fulfill emotional and spiritual needs. To this end turfgrasses have been developed and cultivated across the globe, with just a handful of species chosen for their ability to thrive despite the extreme stresses (abiotic and biotic) found in recreational areas.

In this process, two primary groups of turfgrasses have arisen based on the climates in which they were domesticated. Cool-season turfgrasses are long-day plants belonging to the *Pooideae* subfamily of grasses (*Poaceae*). They typically use C_3 carbon fixation for photosynthesis and grow most vigorously during spring and fall. Examples include bentgrasses (*Agrostis* spp.), bluegrasses (*Poa* spp.), ryegrasses (*Lollium* spp.), and fescues (*Festuca* spp.). Warm-season turfgrasses are generally short-day or day-neutral and grow aggressively during late-spring and summer. They are found largely in the *Chloridoideae* and *Panicoideae* subfamilies and utilize C_4 photosynthesis. Examples include bermudagrass [(*Cynodon dactylon* (L.) Pers.], centipedegrass [*Eremochloa ophiuroides* (Munro) Hack.], zoysiagrass (*Zoysia* spp.), and St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze].

In the United States, cool-season turfgrasses are widely used in the cool northern latitudes to which they are adapted, ranging from the Northeast to the Pacific Northwest. Conversely, warm-season grasses do well in the hotter climates of the South, spanning from Florida to California. In between these two extremes lies the transition zone, an area with highly variable seasons ranging from cold bitter winters to hot, dry summers. This region, spanning from North Carolina and Virginia into the Midwest, poses a unique challenge for turf. While both cool- and warm-season turf types can be grown in the transition zone, neither is ideally adapted.

Warm-season lawns are generally established through sprigging and sod. These grasses are readily propagated through rhizomes and stolons, allowing them to more effectively compete against weeds and recover from damage in high traffic areas. Unfortunately, warm-season grasses have little cold or freezing tolerance and go dormant in the winter, a feature that many growers find undesirable. Most cool-season grasses are propagated through seed, providing a more economical method for lawn establishment than sod. These grasses can produce lush stands of high quality, fine textured turf, but struggle with heat and drought stress in the summer. This can lead to thin, patchy turf without a high level of maintenance. A common method to overcome these difficulties is to overseed warm-season turf with cool-season grasses annually in the fall, thus taking advantage of the strength of both seasonal types for a lush turf year-round.

The production and maintenance of turf is an important industry for North Carolina. In 1999, an estimated $1.2 billion was spent on the maintenance of approximately 2.14

million acres of turf (North Carolina Dept. of Agriculture and Consumer Services, 1999). An additional $700 million was spent on renting or procurement of the equipment used for turf maintenance. With a population growth of over 20% in the last decade (based on 2001-2011 population census data, North Carolina Office of State Budget and Management) these numbers can be expected to have increased significantly. Single family homes accounted for a vast majority of North Carolina land devoted to turf at 1.47 million acres, followed by roadsides and commercial properties at 0.35 and 0.12 million acres, respectively (North Carolina Dept. of Agriculture and Consumer Services, 1999). Golf courses, schools, churches, airports, parks, cemeteries, and other institutions composed the remainder. Similarly, single family homes, commercial properties, and golf courses spent the most on turf maintenance.

The most commonly used species overall were Kentucky 31 tall fescue (36.1%), centipedegrass (16.3%), bermudagrass (10.8%), bluegrass/fescue mix (7%), tall fescue (4.7), and bahiagrass (4.3). Ryegrass, zoysiagrass, and St. Augustinegrass together made up an additional four percent. Despite these overall usage rates, the actual topography of turfgrass usage varied depending on the sector analyzed, reflecting the ability of different turfgrasses to meet a variety of needs. For example, Kentucky 31 is among the most drought tolerant cool-season grasses (Huang and Gao, 1999), providing homeowners and commercial sites with a balance between turf quality and ease of maintenance. The ability of bermudagrass to maintain quality under high traffic made it the preferred turfgrass for golf courses and athletic fields. Bahiagrass and centipedegrass showed greater usage rates along roadsides and in parks and cemeteries, reflecting their hardiness and low maintenance requirements. Other grasses are used in specific contexts, such seashore paspalum (*Paspalum vaginatum* Swartz) or St. Augustinegrass, which are known for their high salinity and shade tolerances, respectively.

Genetics, Morphology, and Pathology of St. Augustinegrass

St. Augustinegrass is a widely used warm season turfgrass known for its tolerance to weed infestation (Busey, 2003; Long and Bashaw, 1961) and shade (Busey, 1995; Peacock and Dudeck, 1993). The species is characterized by broad leaves and rapid stoloniferous growth, producing lush stands on a wide range of soil types (Busey et al., 1982). Its adaptation to tropical and subtropical climates makes it a preferred turfgrass for home lawns and commercial landscapes throughout the Southern U.S. In Florida, St. Augustinegrass sod production expanded from 3,100 hectares in 1974 (Florida Department of Agriculture and Consumer Services, 1976) to over 52,000 acres in 2007 (Hodges and Stevens, 2010), accounting for the majority of sod produced in the state. While its usage is more limited in North Carolina, the versatility and potential for breeder-driven improvement of St. Augustinegrass ensures its continued importance in the turf profile of the state.

The genus consists of seven species indigenous to coastal areas along East Africa, India, and Southern China where they are commonly used for forage, though only *S. secundatum* has been widely cultivated for turf (Sauer, 1972). St. Augustinegrass has been found on all continents excluding Antarctica, though its exact origin is not known (Busey, 2003). The most plausible explanation may be a pre-colonial era transoceanic dispersal originating from Indian Ocean coastal areas, as multiple naturalized and divergent populations of St. Augustinegrass have been found in the Caribbean, the Southern U.S., and elsewhere. St. Augustinegrass does show some level of interspecific hybridization, and it is believed that the cultivar FX-10 arose from a cross between *S. secundatum* and *S. dimidiatum* (Busey, 1993).

Morphologically, accessions of St. Augustinegrass have been clustered into 'demes' (Sauer, 1972) or 'groups' and 'races' based on floral features such as spikelet length and number or stigma color (Busey et al., 1982, Busey, 1986). These clusters are largely parsimonious with geographic distribution, and have also been shown to correspond well with ploidy (Busey, 1995; Green et al., 1981). Polyploidy is common within *Stenotaphrum* germplasm. Triploids, tetraploids, and aneuploids have all been described (Busey, 1993; Milla-Lewis et al., 2013; Sauer, 1972), and it has been proposed that multiple instances of auto- and/or allo-polyploidy have led to the genetic diversity in the genus (Busey, 2003).

St. Augustinegrass is susceptible to a number of pests and diseases. One of the most important insect pathogens of St. Augustinegrass is the southern chinch bug [*Blissus insularis* Barbar]. Excessive feeding by nymphs can weaken and damage plants (Baker, 1982). During peak summer months this can be exacerbated due to drought stress, resulting in the death of large patches of turf. Fungal diseases of St. Augustinegrass include dollar spot [*Sclerotinia homoeocarpa*], large patch [*Rhizoctonia solani*], and gray leaf spot

[*Magnaporthe grisea* (T.T. Hebert) Yaegashi & Ugadawa]. Under ideal conditions these pathogens can potentially devastate large swaths of turf.

Magnaporthe is a common pathogen throughout the grass family, causing blast disease in rice, wheat, and barley, as well gray leaf spot in tall fescue and ryegrass. Because of its widespread prevalence in important food crops, the disease has been widely researched as a model for host-pathogen interactions in rice (Liu et al., 2010). Consequently many resistance genes have been identified in this species (Yang et al., 2009). In addition to these single genes which typically confer complete but race specific resistance, QTL conferring partial resistance have been identified in rice (Fukuoka and Okuno, 2001; Xu et al., 2008), ryegrass (Curley et al., 2005), and barley (Sato et al., 2001).

Control of GLS in most crops relies on the use of fungicides, making management of this disease costly and difficult for St. Augustinegrass in most home lawn and public use settings. Active breeding efforts in rice have managed to produce varieties with durable resistance (Skamniotie and Gurr, 2009; Wang et al., 1994). Incorporation of host resistance into planted cultivars would present an ideal alternative for the management of GLS is St. Augustinegrass. Specifically, the incorporation of quantitative trait loci, which are commonly associated with durable modes of resistance (Boyd, 2006; Kou and Wang, 2010), would present an ideal alternative to reliance upon fungicide for control of the disease.

A significant amount of variation has been observed for a number of important traits within *Stenotaphrum* germplasm, including growth habit (Busey, 1995), cold and shade tolerance (Peacock and Dudeck, 1993; Philley et al., 1995), and resistance to chinch bugs, gray leaf spot, and other diseases (Atilano and Busey, 1983; Flor et al., 2010; Reinert and Dudeck, 1974). ‘Raleigh’ is considered the most cold tolerant of St. Augustinegrass accessions, and ‘Seville’ displays a lower growth habit and good tolerance to shade. Unfortunately, much of the disease resistance that has been observed is located within polyploid germplasm. This presents a problem for breeders as many commonly used varieties are diploid and cannot be readily crossed with polyploid material. Despite these obstacles, a number of cultivars have been released. Polyploids such as ‘Bitterblue’ and ‘Floratam’ were released for their finer leaf texture and resistance to chinch bugs, respectively.

Germplasm Characterization

Though progress has been made in identifying morphological variation within subsets of St. Augustinegrass germplasm, our understanding of levels of genetic variation in the overall genus remains limited. Furthermore, only a handful of accessions are publicly available in the USDA’s National Plant Germplasm System. While The University of Florida maintains the largest St. Augustinegrass germplasm collection in the U.S., many of the accessions remain uncharacterized. This germplasm represents an enormous asset for breeders as a reservoir for novel traits and disease resistance genes. In order to make use of such a resource, breeders must first understand the extent of variation available and where accessions lie within this continuum. This knowledge informs which crosses are most likely to generate worthy genetic combinations for improvement of traits of interest such as disease resistance and abiotic stresses alongside turf quality traits.

Germplasm characterization within turfgrasses has generally focused on inflorescence characteristics (Hanna and Burton, 1978; Liu et al., 2003) and traits corresponding to turf quality, such as leaf width and density, internode length, color, spring green-up, etc. (Anderson, 2000; Murphy et al., 1997; Taliaferro, 1995). Susceptibility to disease and abiotic stresses (Bertrand et al., 2009; Huang and Gao, 1999) can also be included in this regard as sick and damaged turf is usually rated poorly for quality. However, morphological analysis provides only a broad picture of the diversity within a germplasm, and cannot always provide an accurate understanding of the underlying genetic variability (Mba and Tohme, 2005). Some morphological traits can be difficult or time consuming to accurately measure and can vary over time and with different environmental conditions. Additionally, closely related accessions often cannot be distinguished from each other using morphological observations alone. Such problems have been observed in zoysiagrass, where continuous variation in a number of traits made separation of accessions into distinct species difficult (Kimball et al., 2012; Patton and Reicher, 2007).

These problems can be overcome with the use of DNA-based molecular markers, which are insensitive to environmental influences. Additionally, molecular markers can generally identify polymorphism between even closely related accessions. Molecular markers have gained widespread popularity in all facets of biology due to their versatility, reproducibility, ease of assay, and ability to reliably detect high resolution genotypic variability (Arif et al., 2010; Kalia et al., 2011).

Molecular Marker Development and Applications in Plant Breeding

A number of DNA-based molecular marker systems have been applied towards the plant sciences, including Restriction Fragment Length Polymorphisms (RFPLs) (Saiki et al., 1985), Randomly Amplified Polymorphic DNA (RAPDs) (Williams et al., 1990), Amplified Fragment Length Polymorphisms (AFLPs) (Vos et al., 1995), Simple Sequence Repeats (SSRs) (Jacob et al., 1991), and Single Nucleotide Polymorphisms (SNPs). Molecular markers can be used to uniquely identify cultivars, assess levels of relatedness between various individuals, and analyze diversity within a germplasm pool. Additionally, analysis of segregation patterns of markers between related progeny can facilitate the construction of linkage maps, which, when combined with phenotypic data, can allow genes controlling important traits to be mapped. Markers closely linked to these traits can be utilized by breeders in marker assisted selection (MAS) to select progeny carrying genes of interest. Each marker system has its own advantages and weakness, making some better suited for certain tasks than others.

The earliest of these marker systems to receive widespread attention was RFLPs, which were important in developing the first DNA-based linkage maps for many crops, including rice (McCouch et al, 1988) and maize (Helentjaris, 1987), and elucidating the location of genes for numerous traits of interest (Louie et al., 1991; Ottaviano et al., 1991; Wang et al., 1994). Among turfgrasses, RFLPs have been used to analyze diversity in perennial ryegrass (Charmet et al., 1997), tall fescue (Busti et al., 2004), and zoysiagrass (Yaneshita et al, 1997). Early protocols for RFLPs required digestion of DNA fragments with restriction endonucleases, separation of the bands by size, and eventually visualization using X-rays. Because of the large amount of DNA required, relative difficulty of the process, and issues with reproducibility, RFLPs were rapidly replaced when polymerase chain reaction (PCR)-based marker systems were developed. In addition to requiring less DNA, PCR-based systems can incorporate fluorescently labeled primers for automation. Some techniques have been developed to combine the advantages of PCR with RFLPs (CAPS: Konieczny and Ausubel, 1993; TRFLP: Liu et al., 1997), but application of these systems has largely been limited to certain contexts.

RAPDs make use of short, non-specific primers to amplify random fragments. Similar to RFLPs, variations at or between primer sites lead to different banding profiles between individuals when using the same 'random' primers (Powell et al., 1996). The key advantage of this system is that no prior sequence information of the species being assayed is needed, making it especially useful for species with limited genetic resources. RAPDs have been used to some extent in turfgrasses for cultivar identification (Wu and Lin, 1994) and diversity studies (Al-Humaid and Motawei, 2004; Curley and Jung, 2004). Because of issues with reproducibility this system was later replaced by AFLPs (Mba and Tohme, 2005).

AFLPs make use of a combination of digestion by restriction enzymes with rare and common recognition sites, followed by ligation with adapters to allow for primer binding and PCR amplification. As with RAPDs, AFLPs do not require *a priori* sequence information. Additionally, AFLPs commonly show a high degree of polymorphism and are highly reproducible, making them very useful for germplasm characterization (Meudt and Clark, 2007; Mueller and Wolfebarger 1999). Unfortunately both RAPDs and AFLPs are dominant marker systems and cannot readily distinguish between homozygosity and heterozygosity at a locus, resulting in some limitations for phylogenic and linkage analysis. Additionally, the occurrence of homoplasy, in which two or more loci produce fragments of the same size, can increase error rates and confound results. Despite this, AFLPs have been used extensively among turfgrasses for germplasm characterization and linkage mapping. Even among warm-season grasses, which lag behind in DNA marker development and application, examples can be seen in bermudagrass (Anderson et al., 2009; Wu et al., 2006), carpetgrass (Wang et al., 2010), St. Augustinegrass (Milla-Lewis et al., 2013), and zoysiagrass (Cai et al., 2004; Kimball et al., 2012),

Simple sequence repeats (also known as microsatellites) are composed of tandemly recurring nucleotide patterns of one to six base pairs long which are repeated as many as 100 times. Primers roughly 20-25 base pairs long are designed to flank these regions and polymorphism within and between accessions arise as a result of differences in the number of repeats. Compared to other molecular markers, SSRs show high rates of mutation per generation, resulting in a high degree of polymorphism among genotypes (Buschiazzo and

Gemmell, 2006). This mutation rate appears to be controlled by the complex interaction of a number of factors including slippage during replication, mismatch repair mechanisms, recombination during meiosis, point mutations, and retrotransposition (Ellegren, 2002; Kalia et al, 2011; Li et al, 2002; Temnykh, 2001; Wang et al, 2009;). Generally it has been observed that higher mutation rates are associated with longer microsatellites (Kruglyak et al, 1998) resulting in greater levels of polymorphism.

Traditionally, flanking sequence information needed to design primers for new SSRs has been obtained by isolating genomic DNA and subsequently screening thousands of clones through hybridization with SSR-containing probes (Kalia et al, 2011). This process can be extremely inefficient and labor intensive, leading some to develop new techniques for microsatellite discovery and characterization. Next generation sequencing (NGS; alternatively, 'second generation sequencing') platforms such as the Roche 454 or Illumina's Genome Analyzer IIx allow for whole genomes to be cheaply sequenced directly from isolated genomic DNA, eliminating the need to transform and sequence numerous libraries. These platforms are capable of producing up to 1.2 and 100 million reads, respectively, in a matter of days (Zalapa et al., 2012) through a process known as 'sequencing by synthesis' (reviewed in: Mardis, 2008; Varshney et al., 2009). Next generation sequencing technology is increasingly being applied towards *de novo* sequencing (Garg et al., 2011; Hyten et al., 2008; Jun et al., 2011) and microsatellite isolation (Lu et al., 2011; Parchman et al., 2010; Wang et al., 2010a) of less researched organisms.

As a marker system, SSRs are highly polymorphic, reproducible, easy to use, codominant, and evenly distributed in most genomes (Kalia et al., 2011) making them ideal for cultivar identification, analysis of germplasm diversity, linkage mapping, and quantitative trait loci (QTL) discovery (Powell et al., 1996). In turfgrasses, SSRs have been recently used in the construction of linkage maps and QTL analysis for bermudagrass (Harris-Schultz et al., 2010), creeping bentgrass (Rotter et al., 2009) perennial ryegrass (Jones et al., 2002; Yamada et al., 2004), and zoysiagrass (Cai et al., 2005; Li et al., 2009). Microsatellites have also been used in turfgrass for germplasm characterization (Liu et al., 1995; Tsuruta et al., 2005; Wang et al., 2010b), comparative genomics (Saha et al., 2006; Sim et al., 2009; Wang et al., 2006), and cultivar identification (Jones et al., 2001; Wang et al., 2010c).

Single nucleotide polymorphisms (SNPs) have gained significant attention recently in light of advances in NGS technology. Based on single nucleotide changes (insertions, deletions, substitutions) in DNA sequence between two individuals, these markers are codominant, highly amenable to automation, and extremely abundant throughout all genomes (Vignal et al., 2002). Taking advantage of their abundance and automation, however, requires significant genetic resources (i.e. genome sequence) for the species being assayed, and consequently applications of this technology have largely been limited to model species and major crops (Varshney et al., 2009).

Linkage Mapping and Analysis of Quantitative Trait Loci

Linkage mapping is a tool that has been used by geneticists for roughly a century to understand gene organization within different organisms and identify associations between various traits. The basic concept of linkage mapping is that segregation patterns for various traits among related individuals with known pedigree can be analyzed to determine where the genes controlling them are located in relation to each other within an organism's genome (Laird and Lange, 2011). In addition to purely academic pursuits, linkage mapping can be applied towards the identification of markers closely linked to important traits such as disease resistance or yield (Collard et al., 2005). These, in turn, can be used in MAS by breeders to expedite breeding programs.

Early in the process of linkage mapping researchers relied upon morphological markers such as flower color, wing shape, etc. The advent of molecular genetic markers greatly improved this process, allowing researchers to readily assay numerous individuals and with greater numbers of markers (Young, 2000). Genetic markers have the added benefits of being free from environmental interactions and widely distributed throughout most genomes. Because of advances in marker technology, linkage mapping has become one of the most ubiquitous aspects of genetic research and a key part of most crop improvement programs. A similar approach, association mapping, has become popular recently due to its ability to locate target genes and QTL with greater accuracy and sensitivity (Laird and Lange, 2011). However, association mapping requires much larger population sizes and higher marker densities to be effective. While NGS technologies and

SNPs have made this possible for some crops, linkage mapping will remain the focus for most.

The first consideration for developing a linkage map is the mapping population. A number of schemes have been developed in plants, including F_2, backcross, and recombinant inbred lines (RILs) (Young, 2000). These populations are created by crossing two inbred parents and then selfing the progeny for one or more generations. Though they take more time and effort to produce, for most crops RILs make ideal populations as F_2s and early backcrosses cannot be maintained over time (Zhang, 2012). Many turfgrasses can be maintained perennially through clonal propagation, providing a unique advantage for F_2 populations. Unfortunately, many forage and turfgrass species are naturally outcrossing, making the creation of inbred parental lines difficult. The creation of a 'pseudo testcross' or 'pseudo F_2' population to overcome this problem has been discussed (Alves et al., 2010; Jansen, 2005; Lu et al., 2004; Maleipaard et al., 1997; Wu et al., 1992). These populations take advantage of the highly heterozygous nature of outcrossing species by examining segregation patterns within the F_1 generation. To accomplish this, markers that are heterozygous in at least one parent and share a maximum of one allele with the other can be selected for analysis among the progeny. This method has been used in a wide range of crops, including oil palm (Billotte et al., 2010), apple (Maleipaard et al., 1998), cassava (Okagbenin et al., 2008), and perennial ryegrass (Jones et al., 2002).

After a suitable mapping population has been developed and characterized, a linkage map must be constructed. The choice of method for deciding proper marker order is somewhat dependent on the number of markers used, population size, and computing power available (Young, 2000). Because earlier mapping populations were smaller and fewer markers were used, a two-point analysis was commonly used. In this method the recombination frequencies for each pair of markers are tested directly with a chi-square test. This process can become cumbersome as more markers and progeny are analyzed, leading to the development of multi-point analysis. Most linkage mapping programs utilize this method, which uses a logarithm of odds (LOD) score to test marker order (Laird and Lange, 2011). Additionally, programs such as Mapmaker (Lander et al., 1987) and JoinMap (van Ooijen and Voorrips, 2001) have developed algorithms and procedures to create primary marker groupings and accelerate the mapping process. Following this, the map distance is calculated. Several mapping functions have been developed to convert recombination frequencies into centiMorgans (cM) (Mather, 1938; Morgan, 1928; Haldane, 1919; Kosambi, 1994), all of which yield similar results when markers are reasonably close to each other and recombination frequencies are low.

Once a linkage map has been assembled, it can be compared against phenotypic data for a trait of interest. Similarly to linkage mapping, earlier research used single marker analysis where t-tests, analysis of variance (ANOVA), or linear regression were used to test for linkage between a marker and the trait (Collard et al., 2005). Newer methods, such as composite interval mapping (CIM) use hypothetical locations (‘intervals’) between markers and regression analysis to generate LOD scores which can be used to identify putative genes or QTL. These functions are commonly employed in QTL analysis software such as QTL Cartographer (Basten et al., 2001), MapManager QTX (Manly et al., 2001), and others.

Discovery of QTL can be confounded by a number of factors (Asins, 2002; Collard et al., 2005). A small mapping population means fewer recombination events to compare against, creating error in linkage mapping and possibly producing artificial associations between markers unlinked to putative QTL. A lack of marker density can lead to underestimation of the number of QTL involved and an overestimation of their effect. Genotyping errors and missing data can lead to false marker assignment (Hackett, 2002). Finally, estimation of QTL can be susceptible to environmental influences, either inflating the signal or dampening it (Lindhout, 2002; Paterson et al., 1991)

The application of molecular markers towards linkage mapping and QTL analysis for turfgrasses has been gaining ground in recent years. The majority of studies have focused on cool season grasses such as colonial bentgrass (Rotter et al., 2009), perennial ryegrass (Jones et al., 2002), and tall fescue (Saha et al., 2005), but warm season grasses are beginning to receive attention as well (Karaca et al., 2002; Cai et al., 2005). To date few turfgrass breeding programs have made use of such work for marker assisted selection (Roldan-Ruiz and Kolliker, 2010). Continued development of NGS technologies, dropping sequencing costs, and sustained marker development are likely to turn this around in the near future.

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-CHAPTER II-

Genetic Analysis of a St. Augustinegrass Germplasm Collection Using AFLP Markers

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ABSTRACT

St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] is a widely used turfgrass in the southern United States, favored for its shade tolerance and lush ground cover. The University of Florida (UF) St. Augustinegrass germplasm collection represents the largest pool of variation for the species, yet little is known about its diversity at the molecular or cytological levels. This study was conducted to assess levels of molecular and cytological diversity in this collection, compare them to those present among publicly available germplasm, and evaluate possible associations between molecular diversity and ploidy level. Four ploidy levels were designated based on flow cytometry results with most accessions falling into the diploid and proximal tetraploid categories. Twelve AFLP primer pairs produced a total of 747 scorable bands. Unweighted pair group method with arithmetic averaging (UPGMA) cluster and principal coordinate (PCO) analyses produced comparable groups of accessions that were based largely on ploidy level. Analysis of molecular variance indicated that both among and within ploidy levels were significant sources of variation. The number of observed bands largely increased with ploidy level, indicating greater levels of diversity among higher ploidy accessions. Among diploid accessions one cluster was identified that was unrepresented by any cultivars or plant introductions. The UF St. Augustinegrass germplasm collection contains significant, untapped pools of diversity at diploid and polyploid levels and could be a great asset for cultivar development.

Abbreviations: AFLP, amplified fragment length polymorphism; AMOVA, analysis of molecular variance; CTAB, cetyltrimethylammonium bromide; GS, genetic similarity; MI, marker index; NPGS, National Plant Germplasm System; PCO, principal coordinate analysis; PCR, polymerase chain reaction; PIC, polymorphic information content; PP, primer pairs; UF, University of Florida; UPGMA, unweighted pair group means analysis.

INTRODUCTION

St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] is a warm season turfgrass characterized by aggressive stoloniferous growth, high shoot density and broad leaf texture. The genus consists of seven species indigenous to coastal areas of the Old World tropics, ranging from East Africa to islands in the south Pacific (Busey, 1995; Sauer, 1972). Of these, only *S. secundatum* has been widely cultivated globally. The ability of St. Augustinegrass to tolerate shade (Busey, 1995; Peacock and Dudeck, 1993) and effectively out-compete weeds (Long and Bashaw, 1961) has promoted its widespread use for lawns and forage in tropical and sub-tropical climates throughout the world. While the University of Florida's (UF) St. Augustinegrass germplasm collection is a very extensive germplasm source for this species in the US, little is known about the genetic variability present at the genome level. Characterization of this germplasm would increase the efficacy of breeders utilizing the variation within the collection.

Previous analysis of the diversity in St. Augustinegrass has focused primarily on morphological characteristics (Busey et al., 1982; Busey, 1986) and adaptation for economic traits such as drought tolerance (Busey, 1996; Steinke et al., 2010), cold and freezing tolerance (Busey, 2003; Maier et al., 1994; Philley et al., 1995), and disease resistance (Atilano and Busey, 1983; Busey et al., 1993; Flor et al., 2010; Reinert and Dudeck, 1974). Organization of St. Augustinegrass accessions into races and groups via flower and leaf morphology has been successful at predicting genetic and phenotypic diversity (Busey et al., 1982). Much of the observed variation can be ascribed to differences in ploidy level. Early work by Long and Bradshaw (1961) identified diploids ($2n = 2x = 18$), triploids ($2n = 3x = 27$), and tetraploids ($2n = 4x = 36$). Aneuploids and African introductions with higher ploidy levels were also reported (Busey, 1979; Busey et al., 1993). More recently, flow cytometry has been paired with cytological and molecular analysis to assess intra- and inter-specific variation within publicly available sources of *Stenotaphrum* germplasm (Milla-Lewis et al., 2013). Accessions were found to form ploidy level-based clusters that compared favorably with the taxonomic groups identified by Busey (1986). Another major finding of this research was the identification of discrepancies between flow-cytometry inferred ploidy levels and cytological chromosome counts for higher ploidy levels. This was interpreted as an indication that polyploids were developed through hybridization of diploids of different genome size, or that this group has undergone significant genome reorganization after polyploidization.

Knowledge of genetic diversity and levels of speciation within a germplasm collection can aid in the selection of parental material in a breeding program. Molecular markers are commonly employed as an efficient means to evaluate numerous accessions at the whole genome level. The amplified fragment length polymorphism (AFLP) method (Vos et al., 1995) is a robust technique that allows for the simultaneous evaluation of numerous loci without prior sequence knowledge of the genome being analyzed. These markers commonly show a high degree of polymorphism and are highly reproducible (Meudt and Clark, 2007). A number of warm-season turfgrasses have been characterized using AFLPs, including bermudagrass [*Cynodon dactylon* (L.) Pers.] (Anderson et al., 2009; Wu et al., 2006), carpetgrass [*Axonopus fissifolius* (Raddi) Kuhlm.] (Wang et al., 2010), St. Augustinegrass (Milla-Lewis et al., 2013), and *Zoysia* spp. (Kimball et al., 2012).

Within angiosperms polyploidy occurs frequently, and it is thought to play a role in facilitating speciation (Leitch and Bennett, 1997; Wendel, 2000; Wood et al., 2009). It has been shown that the incidence of polyploidy can result in a change in gene regulation at the cellular level (Allen and Eccleston, 1998; Gastony, 1991). Additionally, the duplication of genes can increase plasticity within the genome, allowing novel gene mutations and chromosomal rearrangements (Volkov et al., 1999; Wendel, 2000). Through these mechanisms polyploidy can drive diversification within a species. Though differences in ploidy may not always act as a barrier to gene flow (Slotte et al., 2008), it has at least been shown to increase reproductive isolation in some instances (Husband and Sabara, 2003; Sandhu et al., 2010). This would indicate that polyploidy can also act as a wedge between pools of individuals in a population. Within *Stenotaphrum* germplasm clear phenotypic differences between diploid and polyploid accessions can be seen (Busey, 1986), with increased variation observable in the latter for a number of traits (Busey, 1979; Reinert et al., 1986; Busey; 1995). Though the relationship between ploidy level and genetic diversity at the molecular level has been investigated previously (Milla-Lewis et al., 2013), analysis was limited only to publicly available germplasm.

The objectives of this study were to (i) assess levels of molecular diversity among 235 accessions from the University of Florida's St. Augustinegrass germplasm collection,

(ii) compare them to those present among publicly available germplasm represented by eight plant introductions and 15 commercial cultivars, (iii) use flow cytometry to determine the ploidy of these accessions, and (iv) examine possible associations between ploidy level and molecular variability.

MATERIALS AND METHODS

Plant Materials. Two hundred and fifty eight *Stenotaphrum* genotypes were analyzed in this study. Of these, fourteen were cultivars obtained from the University of Florida (UF) (Gainesville, FL), and nine were plant introductions from the National Plant Germplasm System (NPGS) (S9 Germplasm Repository, Griffin, GA). One plant introduction, PI 289789, belongs to the *S. dimidatum* species, and was included for comparison to a closely related species. The remaining 235 genotypes belonged to the UF's St. Augustinegrass germplasm collection which consists of breeding lines and a large collection of accessions collected from the US Gulf Coast (Texas, Louisiana, Mississippi, Alabama and Florida) and the southern portion of the US Atlantic coastline (North and South Carolina, Georgia and Florida). All samples were received as vegetative plugs and were transplanted into plastic pots containing Fafard 4P potting mix (Conrad Fafard Inc., Agawam, MA) in the greenhouse. Plants were mowed weekly to 7.62cm (3inch) and fertilized every two weeks with Scotts® Starter® Fertilizer (The Scotts Company LLC, Marysville, OH).

Flow Cytometry and Ploidy Estimation. In order to estimate ploidy levels, flow cytometry was performed on all 235 UF accessions using the methods described by Doležel et al. (2007). Nuclear DNA content was estimated using fresh leaf tissue taken from greenhouse-grown plants. Soybean (*Glycine max* Merr. cultivar 'Roy') and corn (*Zea mays* L. cultivar 'Bicolor Sweet Frisky F_1') were used as internal standards. Analysis was carried out at the North Carolina School of Veterinary Medicine with a FACSCalibur flow cytometer (Becton-Dickinson Biosciences San Jose, CA) using a 15 mW argon laser. DNA content was calculated as described in Milla-Lewis et al. (2013). Ploidy levels were estimated based on the proportion of measured nuclear DNA of an accession relative to the known diploid cultivar, 'Raleigh'. Nuclear DNA contents for all cultivars and plant introductions were obtained from Milla-Lewis et al (2013).

DNA Extraction and Quantification. Four to five young leaves were collected from each plant for total genomic DNA extraction. DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method, as described in Stein et al. (2001), with the exception that a Fast Prep FP120 (Qbiogene, Carlsbad, CA) was used to grind sample tissue. Samples were quantified using a Hoefer fluorometer (Hoefer Scientific Instruments, San Francisco, CA) and then diluted to 25 ng μL^{-1}. Sample DNA was stored at 4°C.

Amplified Fragment Length Polymorphism (AFLP) Genotyping. All AFLP reactions were carried out as described by Milla et al. (2005). Labeled *Eco*RI + 3 primers were obtained from LICOR Inc. (Lincoln, NE). All other primers and adapters were purchased from Sigma Genosys (The Woodlands, TX). Following PCR amplification, AFLP fragments were separated by polyacrylamide gel electrophoresis. Samples were first denatured at 94°C and then run on a LICOR 4300 DNA Analyzer Sequencer using 8% *v/v* denaturing polyacrylamide gels. Twelve AFLP primer pairs (PP) with known quality of amplification and polymorphism rates (Milla-Lewis et al., 2013) were selected to genotype all 258 accessions. The reproducibility of the banding patterns was confirmed by generating two completely independent banding patterns for six samples. Mean reproducibility values were calculated as the percentage of bands that were identical in both replications.

Genetic Data Analysis. Amplified fragment length polymorphism bands were scored as either present (1) or absent (0) using AFLP-Quantar 1.0 software (Keygene Products, 2000). Summary statistics were calculated for each primer pair, including: total number of loci, number of polymorphic loci, number of scored loci, and percentage of scored polymorphic loci. Polymorphic information content (PIC) and marker index (MI) values were calculated according to Geuna et al. (2003) and Varshney et al. (2007), respectively to determine the discriminatory power of each PP.

To identify which factors contributed the most to diversity within the germplasm, genotypes were grouped according to both germplasm type (cultivar, plant introduction, UF accession) and ploidy level. However, because of the inaccuracies in differentiating between higher ploidy levels in St. Augustinegrass with flow cytometry (Milla-Lewis et al., 2013), only four ploidy levels were defined: diploid, triploid, proximal tetraploid (includes individuals with DNA content levels between that of triploids and tetraploids) and higher ploidy. Dice genetic similarity (GS) coefficients (Dice, 1945) were calculated for all pairwise genotype combinations using NTSYSpc v2.2 (Rohlf, 2000), and a dendrogram was constructed via the unweighted pair group method with arithmetic averaging (UPGMA) (Sokal and Michener, 1958). Principal coordinate analysis (PCO) was also conducted to assess genetic relationships among accessions. A distance matrix using the Eigen function of NTSYS was calculated in order to perform PCO, and the first three resulting scores were plotted for visualization. Additionally, an analysis of molecular variance was performed using Arlequin v.3.0 (Excoffier et al., 2005) to evaluate the significance of variation among and within germplasm types and ploidy levels.

The random permutation method of Fu (2010) was used to test for significant differences in allele numbers among ploidy levels and germplasm types. This method reduces the potential bias associated with comparing groups of different sizes. With this method, a PROC IML program (SAS Institute, 2008) calculated the observed frequency of an allele among the entire group of 258 accessions. This frequency was used to randomly allocate the allele without replacement to all 258 accessions regardless of ploidy level. These steps were repeated for all 747 alleles to produce a total allelic count for each simulated accession, which could then be used to calculate an expected allele count for each ploidy level. This process was repeated 5000 times, with the expected difference between simulated ploidy levels compared to the observed differences after each permutation. The averages of these 5000 permutations were used to determine the overall probability of observing the difference between actual and expected values due to chance.

A regression analysis using PROC REG (SAS Institute, 2008) was performed according to Gulsen et al. (2009) in order to test for associations between ploidy level and band frequency. The frequency of scored bands was plotted against ploidy level.

RESULTS

Ploidy Levels. Nuclear DNA contents for all genotypes analyzed are listed in Table 2.1. Measurements for 2C content ranged from 1.68 (NUF 25) to 7.94 (NUF 124). According to these values accessions could be classified into six ploidy levels: diploid, triploid, tetraploid, aneutetraploid (2C = 4.0 ± > 0.2), hexaploid, and octoploid. However, based on the discrepancy between flow cytometry and chromosome counts at higher ploidy levels observed by Milla-Lewis et al. (2013), four categories were used here to describe results. In addition to diploids and triploids, the term 'proximal tetraploids' is applied to all accessions found to contain a 2C value greater than 3 but less than 5. The term 'higher ploidy' is used to refer to samples containing a 2C value greater than 5. Results of flow cytometry analysis were used to categorize 127 accessions as diploids, 17 as triploids, 88 as proximal tetraploids, and three accessions as having higher ploidy within the NUF collection.

AFLP Marker Diversity. Standard statistics for the twelve AFLP PPs are summarized in Table 2.2. A total of 1229 bands were amplified, with fragment sizes ranging from 66 to 732 bp. For individual PPs, the total number of fragments produced ranged from 53 (E-CCT/M-ACC) to 176 (E-ACT/M-CGT), and the number of scored polymorphic fragments ranged from 35 (E-CTA/M-ACC) to 127 (E-ACT/M-CGT). Of 973 polymorphic bands identified, only bands with reproducibility values higher than 90% between the six individuals across two amplifications were scored. Thus, 747 distinct and readily scorable bands were used for analysis. Polymorphic information content values ranged from 0.21 to 0.29, with an average of 0.26. Marker index values ranged from 3.19 to 26.95, with an average of 10.22. For both estimates of marker utility, PP E-ACT/M-CGT showed the highest values. Primer pairs E-CCC/M-ACA and E-CTA/M-ACC showed the lowest values for PIC and MI, respectively.

Genetic Diversity and Population Structure. Average genetic similarity values (GS) for accessions grouped by ploidy or germplasm type are shown in Table 2.3. Higher similarity values were obtained within ploidy levels than between ploidy levels. Diploids (GS=0.68), triploids (GS=0.69), and proximal tetraploids (GS=0.66) showed comparable levels of within ploidy similarity, while higher ploidy accessions were somewhat more diverse (GS=0.59). Similarities between ploidies decreased as ploidy increased. For example, comparison of diploid accessions with triploids, proximal tetraploids, and higher ploidies yielded similarity values of 0.57, 0.55, and 0.37, respectively. Similarly, comparisons of triploids with proximal tetraploids and higher ploidies gave similarity values of 0.64 and 0.42, respectively. When comparing accessions by germplasm type, cultivars (GS=0.70) and plant introductions (GS=0.51) showed the highest and lowest levels, respectively of within-group similarity.

A UPGMA dendrogram was created using Dice similarity coefficients (Figure 2.1). The 258 accessions formed two major groups based largely on ploidy level. Group A was composed of 140 accessions, including all diploid cultivars and plant introductions, as well as one proximal tetraploid (NUF 229). Within Group A most accessions fell into six major clusters. Most diploid cultivars grouped together in Cluster III, with the exception of Raleigh and 'Deltashade' (Cluster II), 'Mercedes' (Cluster IV), and 'Majestic' (Cluster V). Cluster V also contained two plant introductions. The two remaining diploid plant introductions grouped together in an indistinct cluster that was markedly different from most other diploid accessions.

Group B consisted almost entirely of polyploids, which showed some degree of clustering according to ploidy level. For example, Cluster VII consisted almost entirely of triploid accessions, including cultivars 'Bitterblue' and 'Floratine'. Similarly, Cluster VIII was composed entirely of proximal tetraploids from the UF Collection. Cluster IX contained a heterogeneous mixture of triploids and proximal tetraploids, including 'FX-10'. Three triploid plant introductions grouped with several proximal tetraploids in Cluster X. Two diploids and one proximal tetraploid formed a small cluster that grouped distantly from all other accessions. The three higher ploidy accessions identified from the UF Collection also clustered with PI 289729 (*S. dimidiatum*).

Principal coordinate analysis (Figures 2.2A-C) revealed genetic relationships that corresponded favorably with the UPGMA dendrogram. The first three eigenvectors accounted for 70.25% of the total variation. In two dimensions, diploid accessions separated clearly from higher ploidies (Figure 2.2A and C), and formed two very distinct subgroups with all diploid cultivars and accessions clustering in just one of these subgroups (Figures 2.2A and B). As in the dendrogram, NUF 6, NUF 55, and NUF 72 separated from the other diploids in the third dimension with NUF 34, a proximal tetraploid (Figure 2.2C). Similarly, NUF 10, NUF 114, and NUF 124 clearly clustered with the *S. dimidatum* accession PI 289729 and distantly from all other accessions (Figures 2.2B and C). A large degree of genetic variation was observed among the triploid and proximal tetraploid accessions (Figures 2.2A-C). These also formed two subgroups, though they were not as clearly separated as in the diploid accessions (Figures 2.2A and B). Groups of triploid accessions corresponding to Cluster VII and IX from the dendrogram were readily identifiable. Interestingly, Floratine and Bitterblue failed to group closely using this method. None of the cultivars or plant introductions included in this study fell within Cluster I, IV, VI, or VIII.

Analysis of Molecular Variance. To determine the optimal classification system to compare variation among the samples through AMOVA, accessions were partitioned both according to ploidy level (diploid, triploid, proximal tetraploid, and higher ploidy) and germplasm type (cultivars, UF collection, plant introductions) (Table 2.4). Results for AMOVA by ploidy revealed significant ($p=0.01$) variation among ploidy levels accounting for 23.85% of the total variation. Significant levels of variation ($p=0.001$) were also seen among germplasm types within ploidy levels, and within germplasm types, accounting for 10.87% and 65.28% of the total variation, respectively. By comparison, results for AMOVA by germplasm type did not reveal significant variation among germplasm types. A significant (p=0.001) amount of variation was observed both among ploidy levels within germplasm type, and within ploidy levels. Though both systems revealed significant levels of variation among populations within groups (V_b) and within populations (V_c), grouping accessions by ploidy level appears to provide better separation among groups.

Comparison of Levels of Variation among Ploidies. Calculations for band frequencies and their regression with ploidy level are highlighted in Figure 2.3. Frequencies ranged from 0.2 to 0.49, with an average of 0.33. Average frequencies were 0.29, 0.37, 0.37, and 0.36 for diploid, triploid, proximal tetraploid, and higher ploidy accessions, respectively. Regression analysis identified a cubic relationship between ploidy level and band frequency as the best fit ($p = 0.01$), with an adjusted R^2 of 0.44. Three samples were found to be significant outliers, corresponding with missing data, and were not included in the regression.

Significant differences between ploidy levels (Table 2.5) were also identified using the random permutation method of Fu (2010), though no significant differences were observed when comparing accessions by germplasm type alone (data not shown). Proximal tetraploids were found to have the highest average allelic count (718), followed by diploids (700), triploids (619), and higher ploidy levels (416). Statistical comparisons of these groups showed that the proximal tetraploids had significantly ($p<0.001$) more alleles than both diploids and triploids. Additionally, diploids were found to have significantly ($p<0.001$) more alleles than triploids. Though higher ploidy accessions had lower allelic counts than all other groups, these differences were not found to be significant.

Fu's random permutation test (Fu, 2010) was also used to compare differences between germplasm types for each ploidy level, and conversely, differences between ploidy levels for each germplasm type (Table 2.6). Diploids within the UF collection were found to have significantly ($p<0.001$) more alleles than diploid cultivars or PIs. When comparing triploid accessions, cultivars were found to have significantly ($p<0.05$) more alleles than PIs. No significant differences were observed between germplasm types within proximal tetraploid accessions. When comparing different ploidy levels within each germplasm type, diploids within the UF collection were found to have significantly ($p<0.001$) more alleles than either the triploids or proximal tetraploids. Conversely, proximal tetraploids within the PIs were found to have significantly ($p<0.001$) more alleles than either diploid or triploid PIs.

DISCUSSION

The University of Florida St. Augustinegrass germplasm collection is an extensive germplasm source for this species in the US, being several-fold larger than the national collection. Therefore, it potentially represents an important portion of the total variability present within *S. secundatum*. Historically, efforts to characterize the diversity within St. Augustinegrass have focused on cytological, morphological, and disease/pest resistance traits, with only one study using molecular markers (Green et al., 1981). More recently flow cytometry and AFLP markers were utilized to assess differences in ploidy level and molecular diversity within publicly available germplasm (Milla-Lewis et al., 2013). By applying these techniques towards the University of Florida collection, this study significantly expands our knowledge of the total variability within *Stenotaphrum* germplasm.

The value of flow cytometry for the determination of ploidy level has been shown in other turfgrasses, including *Agrostis spp.*(Bonos et al., 2002), buffalograss [*Buchloe dactyloides* (Nutt.) Engelm.] (Budak et al., 2005), and *Zoysia spp.* (Schwartz et al., 2010). However, small differences in chromosome number can be hard to distinguish using flow cytometry (Doležel et al., 2007; Emshwiller, 2002). Discrepancies between 2C content and cytological counts have been identified for higher ploidy levels in St. Augustinegrass (Milla-Lewis et al., 2013). Consequently, despite the tremendous degree of 2C content variation identified by flow cytometry, only four ploidy levels were used to describe accessions in this study. Slightly more than half of the accessions fell within the diploid category. As most widely used cultivars are diploid, this represents a tremendous increase in readily available germplasm for breeding purposes. Additionally, a large group of triploid and proximal tetraploid accessions were identified. Previous work has shown the potential for resistance to a number of serious St. Augustinegrass diseases and pests within these ploidy levels (Busey, 1979; Busey, 1990; Busey, 1995). Though differences in ploidy pose a barrier to gene transfer between these groups of accessions, methods for embryo rescue and tissue culture have been developed to facilitate interploid crosses (Genovesi et al., 2009).

The frequent use of AFLPs to assess molecular diversity in other turfgrasses (Anderson et al., 2009; Kimball et al., 2012; Wang et al., 2010; Wu et al., 2006) attests to their utility as an easy to use, informative, and highly reproducible marker system. Here, 747 clear, polymorphic bands were amplified using twelve primer pairs. Grouping of accessions according to the marker data revealed considerable ordering of accessions by ploidy, which is in agreement with the molecular analysis of Milla-Lewis et al. (2013). Both UPGMA and PCO analyses separated diploids distinctly from polyploids, though segregation of triploids and proximal tetraploids was less pronounced. The three higher ploidy samples that were identified through flow cytometry formed a separate group with PI 289729, providing possible evidence for the presence of *S. dimidatum* accessions within the UF collection.

Though PCO analysis was capable of identifying two large subgroups within each major group of accessions, the dendrogram provided a more detailed picture. In many cases the subgroups identified through UPGMA analysis reflected the 'races' and 'groups' identified through morphological variation (Busey et al., 1982; Busey, 1986). For example, the presence of Raleigh in Cluster II, and 'Jade' within Cluster III corresponds well with the Gulf Coast and Dwarf groups within the Breviflorus race. The tight clustering of Bitterblue and Floratine strongly implicates Cluster VII as the Bitterblue group within the Longicaudatus race. Significant homology was also seen in the ordering of cultivars and PIs in previous analysis of publicly available germplasm (Milla-Lewis et al., 2013). The tight clustering between Raleigh and Deltashade, 'Classic' and 'Sapphire', Bitterblue and

Floratine, PI 647924 and PI 647925, and PIs 291594, 300129, and 300130 was preserved, highlighting both the high degree of relatedness between these samples, and the reproducibility of AFLP markers. Additionally, UPGMA analysis identified several clusters composed solely of NUF accessions. When the similarity between samples was visualized via PCO, most PIs and cultivars clearly clustered together while the NUF accessions formed much broader pools of diversity. Among all diploid accessions, those from the UF collection were found to have significantly ($p<0.001$) more alleles than either PIs or cultivars (Table 6). As most cultivars in use today are diploid, these results are especially significant as they identify a readily accessible source of genetic diversity for turfgrass improvement.

Within angiosperms, the relatively frequent occurrence of polyploidy has helped facilitate speciation (Leitch and Bennett, 1997; Wendel, 2000; Wood et al., 2009). While duplication of genes can provide more flexibility for the development of novel mutations, it can also act as a reproductive barrier, preventing gene flow between members of the same or closely related species (Comai, 2005; Husband and Sabara, 2003; Leitch and Leitch, 2008; Sandhu et al., 2010; Widmer et al., 2009). In St. Augustinegrass, diploids have generally been found to be fertile, while higher ploidies often show reduced fertility (Busey, 1995). Though crosses between ploidy levels can occasionally produce viable offspring, difficulty in manually creating these crosses (Busey, 1995) indicates that this is presumably a rare occurrence and gene flow between ploidy levels is probably low. Consequently one would expect distinct levels of genetic diversity for the different ploidies. The average genetic similarity values calculated here were highest within ploidy levels and rapidly decreased as the difference between ploidy levels increased. Similarly, analysis of the molecular variance found a significant portion of the diversity explained by variation both among ($p<0.01$) and within ($p<0.001$) ploidies. Concurrent with the UPGMA and PCO analysis, these results indicate that polyploidy has served to create partially isolated pools of diversity within *Stenotaphrum* germplasm.

Previous studies have examined variation in ploidy and genetic diversity in other turf species (Budak et al., 2005; Gulsen et al., 2009). Within *Cynodon* accessions Gulsen et al. (2009) found that diversity increased in a quadratic fashion with ploidy. Investigation of the relationship between diversity and ploidy in this study revealed a cubic relationship, with total allelic counts peaking among tetraploids and then decreasing among higher ploidies. While unexpected, this trend may be an artifact of sampling, as the higher ploidies are represented by only a few accessions. To account for this we used a random permutation method that was developed to reduce bias associated with making comparisons between populations of varying size (Fu, 2010). While the higher ploidy samples had a lower total allelic count, this was not found to be significant in any of the comparisons. Congruent with expectations, the proximal tetraploid group was found to have significantly more alleles than both diploids and triploids. Though a small sample size from each ploidy class may partially obscure the results, our investigation of the relationship between ploidy and marker variability appears to agree with the hypothesis that polyploidy acts to increase genetic diversity.

Characterization of the diversity within a germplasm collection is essential for breeders to identify ideal pedigrees to make crosses in a breeding program. Molecular analysis of the UF St. Augustinegrass germplasm collection has revealed significant untapped sources of variation among diploid accessions that can be readily utilized by breeders. The collection also contains a large pool of polyploid accessions which can be expected to contain a wealth of novel traits. With the recent application of embryo rescue and tissue culture techniques it has been demonstrated that ploidy barriers can be overcome in St. Augustinegrass, thus opening the diversity within polyploid accessions for introgression into elite diploid germplasm.

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Table 2.1. List of *Stenotaphrum* accessions used in assessment of molecular diversity with AFLP markers organized by nuclear DNA content and inferred ploidy level based on flow cytometry.

Identity[†]	2C Value	Inf. Ploidy[‡]	Identity	2C Value	Inf. Ploidy	Identity	2C Value	Inf. Ploidy
NUF-25	1.68	Diploid	Mercedes	2.03[§]	Diploid	Deltashade	2.07[§]	Diploid
NUF-231	1.90	Diploid	NUF-172	2.03	Diploid	NUF-13	2.07	Diploid
NUF-234	1.91	Diploid	NUF-135	2.03	Diploid	NUF-184	2.07	Diploid
NUF-112	1.92	Diploid	NUF-6	2.03	Diploid	NUF-132	2.07	Diploid
NUF-228	1.92	Diploid	NUF-183	2.03	Diploid	NUF-128	2.07	Diploid
PI 647925	1.93[§]	Diploid	NUF-146	2.03	Diploid	NUF-249	2.07	Diploid
NUF-66	1.94	Diploid	NUF-201	2.03	Diploid	NUF-155	2.07	Diploid
NUF-110	1.94	Diploid	NUF-165	2.03	Diploid	NUF-213	2.07	Diploid
NUF-226	1.95	Diploid	NUF-148	2.03	Diploid	NUF-164	2.07	Diploid
NUF-39	1.95	Diploid	NUF-200	2.03	Diploid	NUF-89	2.07	Diploid
NUF-235	1.95	Diploid	NUF-255	2.03	Diploid	NUF-251	2.07	Diploid
NUF-73	1.95	Diploid	NUF-211	2.03	Diploid	Sapphire	2.07[§]	Diploid
PI 647924	1.95[§]	Diploid	NUF-87	2.03	Diploid	NUF-163	2.08	Diploid
NUF-118	1.96	Diploid	NUF-129	2.03	Diploid	NUF-166	2.08	Diploid
NUF-236	1.96	Diploid	NUF-28	2.03	Diploid	NUF-122	2.08	Diploid
NUF-22	1.96	Diploid	NUF-173	2.03	Diploid	NUF-85	2.08	Diploid
NUF-180	1.96	Diploid	NUF-248	2.04	Diploid	NUF-143	2.08	Diploid
NUF-147	1.96	Diploid	NUF-61	2.04	Diploid	NUF-41	2.08	Diploid
NUF-57	1.97	Diploid	NUF-238	2.04	Diploid	NUF-63	2.08	Diploid
NUF-77	1.97	Diploid	NUF-203	2.04	Diploid	NUF-153	2.08	Diploid
NUF-230	1.97	Diploid	NUF-16	2.04	Diploid	NUF-170	2.08	Diploid
NUF-9	1.97	Diploid	NUF-116	2.04	Diploid	NUF-212	2.08	Diploid
NUF-12	1.98	Diploid	NUF-152	2.04	Diploid	NUF-181	2.08	Diploid
NUF-78	1.98	Diploid	NUF-76	2.04	Diploid	Captiva	2.09[§]	Diploid
NUF-177	1.98	Diploid	NUF-174	2.04	Diploid	Delmar	2.10[§]	Diploid
NUF-171	1.98	Diploid	NUF-88	2.04	Diploid	Jade	2.10[§]	Diploid
NUF-179	1.98	Diploid	Amerishade	2.05[§]	Diploid	NUF-134	2.10	Diploid
NUF-17	1.99	Diploid	NUF-14	2.05	Diploid	NUF-79	2.10	Diploid
NUF-43	1.99	Diploid	NUF-8	2.05	Diploid	NUF-142	2.10	Diploid
NUF-38	1.99	Diploid	NUF-91	2.05	Diploid	NUF-80	2.11	Diploid
NUF-233	1.99	Diploid	NUF-84	2.05	Diploid	NUF-20	2.12	Diploid
NUF-109	1.99	Diploid	NUF-93	2.05	Diploid	PI 410361	2.12[§]	Diploid
Raleigh	2.00[§]	Diploid	NUF-121	2.05	Diploid	Sunclipse	2.12[§]	Diploid
NUF-162	2.00	Diploid	NUF-46	2.05	Diploid	NUF-149	2.13	Diploid
NUF-111	2.00	Diploid	NUF-55	2.05	Diploid	NUF-253	2.13	Diploid
NUF-24	2.00	Diploid	NUF-150	2.05	Diploid	NUF-137	2.13	Diploid
NUF-37	2.00	Diploid	NUF-175	2.05	Diploid	NUF-168	2.15	Diploid
NUF-208	2.00	Diploid	NUF-45	2.05	Diploid	PI 410363	2.16[§]	Diploid
NUF-256	2.01	Diploid	NUF-11	2.05	Diploid	NUF-240	2.18	Diploid
NUF-4	2.01	Diploid	NUF-210	2.05	Diploid	NUF-144	2.89	Triploid
NUF-232	2.01	Diploid	NUF-75	2.05	Diploid	NUF-161	2.96	Triploid
NUF-69	2.01	Diploid	NUF-117	2.05	Diploid	NUF-67	2.96	Triploid
NUF-176	2.01	Diploid	NUF-15	2.06	Diploid	NUF-127	2.99	Triploid
Majestic	2.01	Diploid	NUF-47	2.06	Diploid	NUF-35	3.00	Triploid
NUF-178	2.02	Diploid	NUF-90	2.06	Diploid	NUF-21	3.01	Triploid
NUF-154	2.02	Diploid	NUF-26	2.06	Diploid	NUF-7	3.04	Triploid
NUF-5	2.02	Diploid	NUF-250	2.06	Diploid	Floratine	3.06[§]	Triploid
NUF-72	2.02	Diploid	NUF-96	2.06	Diploid	NUF-102	3.08	Triploid
NUF-97	2.02	Diploid	NUF-136	2.06	Diploid	NUF-50	3.10	Triploid
NUF-23	2.02	Diploid	NUF-252	2.06	Diploid	NUF-48	3.10	Triploid
Classic	2.03[§]	Diploid	NUF-44	2.06	Diploid	NUF-98	3.11	Triploid
Floraverde	2.03[§]	Diploid	NUF-254	2.06	Diploid	NUF-70	3.12	Triploid

Table 2.1 (cont.). List of *Stenotaphrum* accessions used in assessment of molecular diversity with AFLP markers and their nuclear DNA content and inferred ploidy level based on flow cytometry.

Identity	2C Value	Inf.	Identity	2C Value	Inf. Ploidy	Identity	2C Value	Inf. Ploidy
NUF-19	3.13	Triploid	NUF-29	3.93	Prox Tetra	NUF-140	4.00	Prox Tetra
NUF-241	3.14	Triploid	NUF-51	3.93	Prox Tetra	NUF-141	4.00	Prox Tetra
Bitterblue	3.15[§]	Triploid	NUF-83	3.93	Prox Tetra	NUF-160	4.00	Prox Tetra
NUF-120	3.15	Triploid	NUF-60	3.93	Prox Tetra	PI 300130	4.07[§]	Prox Tetra
NUF-239	3.17	Triploid	NUF-220	3.93	Prox Tetra	FX-10	4.19[§]	Prox Tetra
PI 291594	4.03[§]	Triploid	NUF-33	3.94	Prox Tetra	NUF-18	4.20	Prox Tetra
PI 300129	4.07[§]	Triploid	NUF-259	3.94	Prox Tetra	NUF-27	4.20	Prox Tetra
NUF-65	3.26	Prox Tetra	NUF-187	3.95	Prox Tetra	NUF-30	4.20	Prox Tetra
NUF-139	3.71	Prox Tetra	NUF-224	3.95	Prox Tetra	NUF-32	4.20	Prox Tetra
NUF-223	3.71	Prox Tetra	NUF-52	3.96	Prox Tetra	NUF-185	4.20	Prox Tetra
NUF-3	3.78	Prox Tetra	NUF-258	3.96	Prox Tetra	NUF-204	4.20	Prox Tetra
NUF-158	3.78	Prox Tetra	NUF-54	3.96	Prox Tetra	NUF-206	4.20	Prox Tetra
NUF-131	3.78	Prox Tetra	NUF-221	3.96	Prox Tetra	NUF-207	4.20	Prox Tetra
NUF-2	3.79	Prox Tetra	NUF-86	3.96	Prox Tetra	NUF-81	4.20	Prox Tetra
NUF-159	3.79	Prox Tetra	NUF-40	3.97	Prox Tetra	NUF-94	4.20	Prox Tetra
NUF-108	3.81	Prox Tetra	NUF-34	3.97	Prox Tetra	NUF-99	4.20	Prox Tetra
NUF-107	3.84	Prox Tetra	NUF-62	3.97	Prox Tetra	NUF-100	4.20	Prox Tetra
NUF-219	3.84	Prox Tetra	NUF-53	3.97	Prox Tetra	NUF-209	4.20	Prox Tetra
NUF-31	3.85	Prox Tetra	NUF-64	3.98	Prox Tetra	NUF-214	4.20	Prox Tetra
NUF-101	3.85	Prox Tetra	NUF-68	3.98	Prox Tetra	NUF-113	4.20	Prox Tetra
NUF-103	3.85	Prox Tetra	NUF-218	3.98	Prox Tetra	NUF-130	4.20	Prox Tetra
NUF-126	3.85	Prox Tetra	NUF-92	4.00	Prox Tetra	NUF-138	4.20	Prox Tetra
NUF-105	3.87	Prox Tetra	NUF-42	4.00	Prox Tetra	NUF-1	4.40	Prox Tetra
NUF-227	3.87	Prox Tetra	NUF-167	4.00	Prox Tetra	NUF-188	4.40	Prox Tetra
NUF-186	3.89	Prox Tetra	NUF-169	4.00	Prox Tetra	NUF-59	4.40	Prox Tetra
NUF-151	3.89	Prox Tetra	NUF-189	4.00	Prox Tetra	NUF-74	4.40	Prox Tetra
NUF-133	3.90	Prox Tetra	NUF-205	4.00	Prox Tetra	NUF-82	4.40	Prox Tetra
NUF-225	3.90	Prox Tetra	NUF-71	4.00	Prox Tetra	NUF-216	4.40	Prox Tetra
NUF-261	3.91	Prox Tetra	NUF-95	4.00	Prox Tetra	NUF-119	4.40	Prox Tetra
NUF-49	3.91	Prox Tetra	NUF-104	4.00	Prox Tetra	NUF-145	4.40	Prox Tetra
NUF-36	3.93	Prox Tetra	NUF-215	4.00	Prox Tetra	NUF-10	5.73	H Ploidy
NUF-222	3.93	Prox Tetra	NUF-217	4.00	Prox Tetra	NUF-114	6.00	H Ploidy
NUF-123	3.93	Prox Tetra	NUF-106	4.00	Prox Tetra	PI289729[¶]	6.02[§]	Prox Tetra
NUF-56	3.93	Prox Tetra	NUF-115	4.00	Prox Tetra	NUF-124	7.94	H Ploidy

[†] All cultivars and University of Florida Germplasm accessions ('NUF') were obtained from the University of Florida, Gainesville, FL; Plant Introductions (PIs) were obtained from the National Plant Germplasm System, Griffin, GA.

[‡] 'Prox Tetra' = proximal tetraploid, 'H Ploidy' = higher ploidies.

[§] Ploidies for all cultivars and PIs were obtained from Milla-Lewis et al. (2012)

[¶] PI 289729 is an accession of *Stenotaphrum dimidatum*.

Table 2.2. Standard statistics for AFLP primer combinations used to study molecular diversity within the University of Florida St Augustinegrass collection.

Primer Combination	Total No. bands	No. of scored bands	No. of polymorphic bands	% scored	% polymorphic	PIC†	MI‡
E-ACT/M-CGT	176	147	127	84	72	0.29	26.95
E-CAA/M-ACG	124	103	71	83	57	0.27	10.94
E-CAC/M-AAA	91	79	67	87	74	0.29	14.14
E-CAG/M-ACA	78	63	51	81	65	0.26	8.58
E-CAG/M-AGA	120	101	71	84	59	0.22	9.30
E-CCA/M-AAT	114	95	78	83	68	0.26	13.97
E-CCC/M-ACA	96	79	64	82	67	0.21	8.83
E-CCG/MACG	94	73	53	78	56	0.25	7.59
E-CCT/M-ACC	53	51	36	96	68	0.29	7.03
E-CGC/M-ACG	103	79	50	77	49	0.26	6.39
E-CGG/M-ATT	91	60	44	66	48	0.27	5.71
E-CTA/M-ACC	89	43	35	48	39	0.23	3.19
Total	1229	973	747	-	-	-	-
Average	102	81	62	72	51	0.26	10.22
Minimum	53	43	35	48	39	0.23	3.19
Maximum	103	79	50	96	68	0.29	26.95

†PIC, polymorphic information content.

‡MI, marker index.

Table 2.3. Average genetic similarity values (GS) within and between groups of St. Augustinegrass materials studied for amplified fragment length polymorphism (AFLP) diversity organized according to ploidy level or germplasm type .

By ploidy

	No. of individuals	Diploid	Triploid	Prox. Tetraploid	Higher Ploidy
Diploid	143	0.68			
Triploid	23	0.57	0.69		
Proximal Tetraploid	90	0.55	0.64	0.66	
Higher Ploidy	3	0.37	0.42	0.41	0.59

By germplasm type

	No. of individuals	UF Collection	Cultivars	Plant Introductions
UF Collection	235	0.61		
Cultivars	15	0.63	0.70	
Plant Introductions	9	0.52	0.55	0.51

Table 2.4. Results of the analysis of molecular variance for St. Augustinegrass accessions analyzed for amplified fragment length polymorphism (AFLP) diversity grouped by ploidy and germplasm type.

Source of variation	**df**	**Sums of squares**	**Variance components**†	**Variation accounted for (%)**
By ploidy level (diploid, triploid, proximal tetraploid, and higher ploidy)				
Among ploidy levels (V_a)	3	1908.02	9.14 (V_a)**	23.85
Among germplasm types within ploidy levels (Vb)	6	324.61	4.17 (V_b)***	10.87
Within germplasm types (V_c)	249	6232.08	25.03 (V_c)***	65.28
Total	258	8464.7	38.34	
By germplasm type (UF collection, cultivars, and plant introductions)				
Among germplasm types (V_a)	2	170.01	-4.31 (V_a)	-12.64
Among ploidy levels within germplasm types (V_b)	7	2062.62	13.35 (V_b)***	39.17
Within ploidies (V_c)	249	6232.08	25.03 (V_c)***	73.46
Total	258	8464.7	34.07	

**, ***Significant at the 0.01, and 0.001 probability levels, respectively.

† V_a, among variance component; V_b, among within variance component; V_c, within variance component.

Table 2.5. Pair-wise differences in allelic counts among St. Augustinegrass accessions belonging to four ploidy levels using the random permutation method of Fu (2010).

	No. of Entries	No. of Alleles	Diploid	Triploid	Proximal Tetraploid	Higher Ploidy
Diploid	143	700	-	-	-	-
Triploid	20	619	**-81*****	-	-	-
Proximal Tetraploid	93	718	**18*****	**99*****	-	-
Higher Ploidy	3	416	-284 (NS)	-203 (NS)	-302 (NS)	-

***Significant at the 0.001 probability level.

Table 2.6. Pair-wise differences in allelic counts among St. Augustinegrass accessions grouped by germplasm type within ploidy level and by ploidy level within germplasm type.

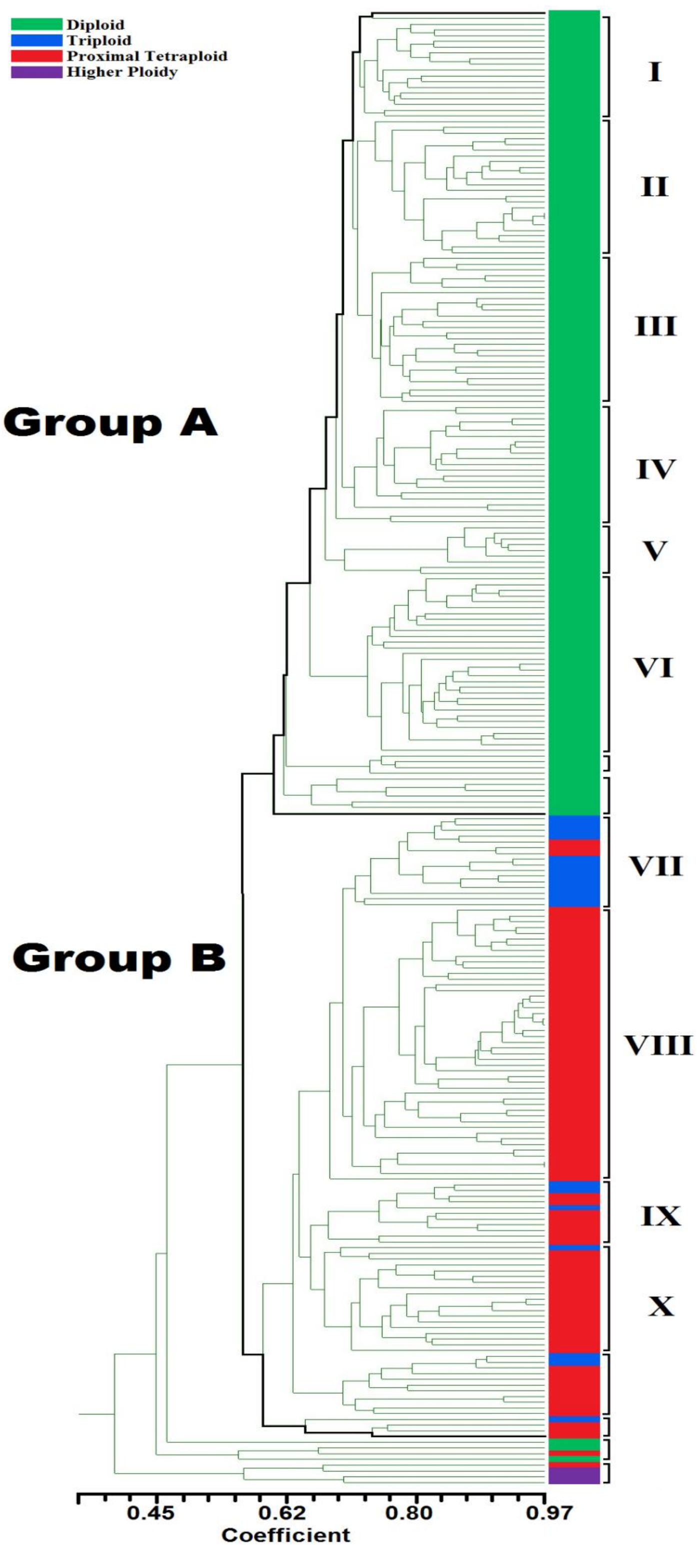
Germplasm within Ploidy †			
	UF vs Cultivar	UF vs PI	Cultivar vs PI
Diploid	**-288*****	**-369*****	-81(NS)
Triploid	-95(NS)	-119(NS)	**-24***
Proximal Tetraploid	-209(NS)	-141(NS)	68(NS)
Ploidy † ***within Germplasm***			
	2X vs 3X	2X vs ≈4X	3X vs ≈4X
UF Collection	**-291*****	**-211*****	80(NS)
Cultivar	-98(NS)	-132(NS)	-34(NS)
PI	-41(NS)	**17*****	**58*****

*, *** Significant at the 0.05 and 0.001 probability levels, respectively.

† Accessions belonging to higher ploidies were not included.

‡ 2X, 3X, ≈4X refers to diploids, triploids, proximal tetraploids, respectively.

Figure 2.1. Unweighted pair group method with arithmetic averaging (UPGMA) dendrogram of 258 *Stenotaphrum* genotypes constructed using 747 amplified fragment length polymorphism (AFLP) markers. Inferred ploidy levels based on flow cytometry results for each sample are indicated by color in the column to the right.


Diploid
Triploid
Proximal Tetraploid
Higher Ploidy
Group A
Group B
I
II
III
IV
V
VI
VII
VIII
IX
X
0.45
0.62
0.80
0.97
Coefficient

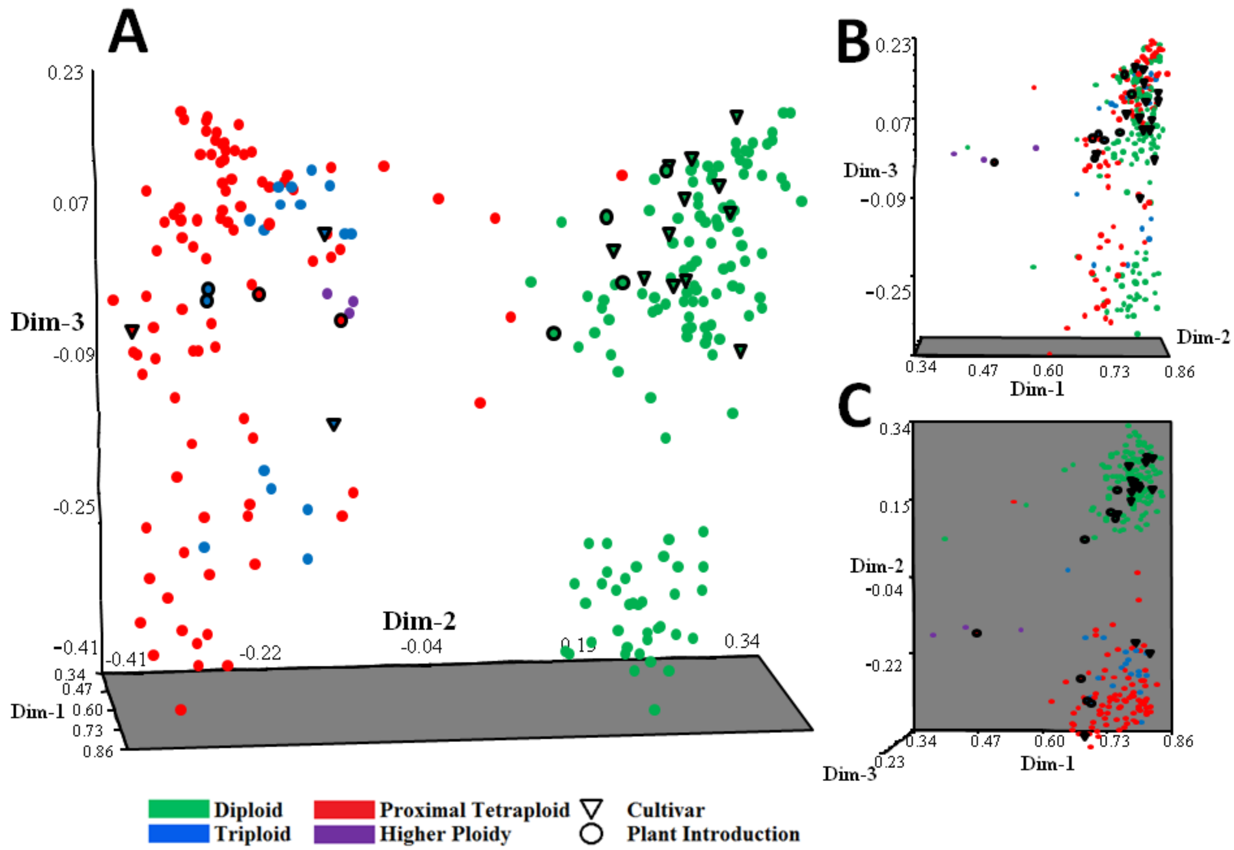


Figure 2.2. Three dimensional principal coordinate plot of 258 *Stenotaphrum* accessions estimated with 747 amplified fragment length polymorphism (AFLP) markers. a) separation of accessions across the 2nd and 3rd dimensions. b) plot rotated 90° c) plot from overhead.

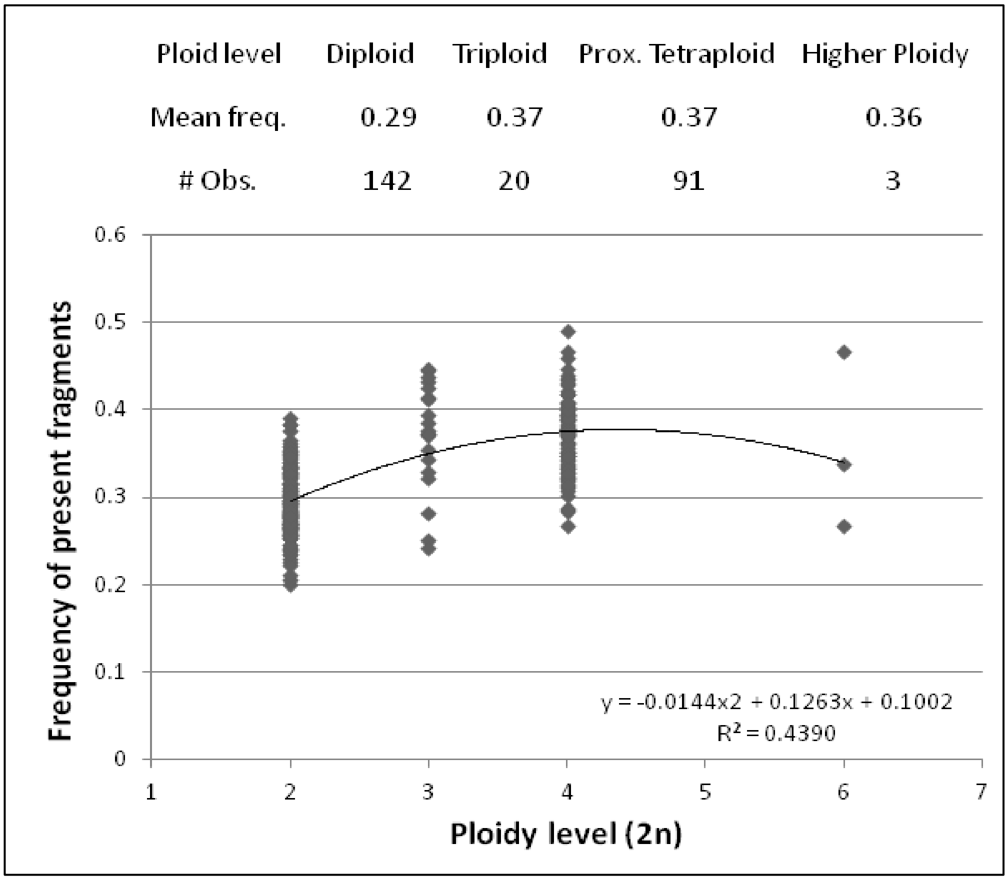


Figure 2.3. Distribution of AFLP fragment frequencies and ploidy levels in 258 *Stenotaphrum* accessions evaluated for genetic diversity.

-CHAPTER III-

Development and Characterization of Simple Sequence Repeat (SSR) Markers for St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze]

Submitted to Crop Science

ABSTRACT

Illumina sequencing data was used to develop simple sequence repeat (SSR) markers for St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze], an economically important warm-season turfgrass. Polymorphism was observed in 190 of 215 designed primer pairs among a panel of eight diverse germplasm accessions. Of these, 94 were subsequently used to assess levels of diversity within a diverse collection of *Stenotaphrum* germplasm. A total of 982 alleles ranging in size from 63 to 250 bp were amplified. Levels of polymorphism were high, with number of alleles amplified per loci ranging from 5 to 23 (μ=10) and polymorphic information content (PIC) values ranging from 0.09 to 0.83 (μ =0.54). Cluster and principal coordinate analyses of *Stenotaphrum* accessions showed groupings based primarily on ploidy level. Analysis of population structure identified six subpopulations with some admixture present among them. Additionally, the ability of these newly developed SSRs to uniquely identify specific cultivars was assessed. A set of five primers were capable of producing unique banding patterns for all but two cultivars. These markers, the first developed for the species, should be useful for linkage mapping, diversity and pedigree analysis, accurate varietal identification, and marker assisted selection in St. Augustinegrass.

Abbreviations: AFLP, amplified fragment length polymorphism; AMOVA, analysis of molecular variance; D, power of discrimination; DI, diversity index; MAS, marker assisted selection; NCSU, North Carolina State University; NGS, next generation sequencing; PCO, principal coordinate analysis; PCR, polymerase chain reaction; PIC, polymorphic information content; PP, primer pairs; QTL, qauntitative trait loci; SSR, simple sequence repeat; UPGMA, unweighted pair group means analysis

INTRODUCTION

St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] is a widely used warm season turfgrass known for its tolerance to weed infestation (Busey, 2003a; Long and Bashaw, 1961) and shade (Busey, 1995; Peacock and Dudeck, 1993). The genus consists of seven species indigenous to coastal areas of the Old World tropics, but only *S. secundatum* has been widely cultivated (Sauer, 1972). The species is characterized by broad leaves and rapid stoloniferous growth, producing lush stands on a wide range of soil types (Busey et al., 1982). Its adaptation to tropical and subtropical climates makes it a preferred turfgrass for home lawns and commercial landscapes throughout the Southern U.S. Its use, however, is limited by a lack of cold hardiness and susceptibility to a number of pests and diseases. Additionally, cultivar identification currently relies on analysis of morphological traits, which can be unreliable. Use and characterization of St. Augustinegrass germplasm would be enhanced by the availability of highly polymorphic DNA markers which could be used to identify cultivars, assess diversity among accessions, and determine linkage with important traits for marker-assisted selection (MAS).

Molecular markers have been used in other turfgrasses for these applications. For example, simple sequence repeats (SSR) (Jacob et al., 1991), have been used for cultivar identification in bermudagrass [*Cynodon dactylon* L.] (Wang et al., 2010c) and perennial ryegrass [*Lolium perenne* L.] (Jones et al., 2001). Amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995) and SSRs have been utilized to examine germplasm diversity in bahiagrass [*Paspalum notatum* Flügge] (Cidade et al., 2008), bermudagrass (Wu et al., 2006), carpetgrass [*Axonopus fissifolius* (Raddi) Kuhlm.] (Wang et al., 2010b), Kentucky bluegrass [*Poa pratensis* L.] (Curley and Jung, 2004; Honig et al., 2010), perennial ryegrass (Brazauskas et al., 2011; Hu et al, 2011), centipedegrass (Milla-Lewis et al., 2012), and zoysiagrass [*Zoysia spp.*] (Kimball et al., 2012a; 2013). Similarly, AFLPs and SSRs have been used to construct linkage maps for perennial ryegrass (Jones et al., 2002), bermudagrass (Harris-Shultz et al., 2010), zoysiagrass (Cai et al., 2004; Li et al., 2009), and colonial bentgrass [*Agrostis capillaris* L.] (Rotter et al., 2009).

Previous work on germplasm characterization in St. Augustinegrass primarily has focused on morphological characteristics (Busey, 1986; Busey et al., 1982) and adaptation for economic traits such as drought tolerance (Busey, 1996; Steinke et al., 2010), cold and freezing tolerance (Busey, 2003b; Maier et al., 1994; Philley et al., 1995), and disease resistance (Atilano and Busey, 1983; Busey et al., 1993; Flor et al., 2010; Reinert and Dudeck, 1974). More recently, flow cytometry and AFLP markers have been applied to assess intra- and inter-specific variation within public (Milla-Lewis et al., 2013) and private (Mulkey et al., 2013) sources of *Stenotaphrum* germplasm. Accessions were found to form ploidy level-based clusters that compared favorably with the taxonomic groups previously identified based on morphology (Busey, 1986). Though knowledge of diversity within a species can be informative when choosing which parents to use for a breeding program, selection of superior progeny segregating for important traits resulting from such a cross remains labor intensive and time consuming. Molecular markers displaying strong linkage with genes controlling important traits can be used within a MAS scheme to identify elite progeny and expedite the cultivar development process (Collard et al., 2005). Though previous studies have shown that AFLPs can produce an abundant supply of polymorphic markers in St. Augustinegrass (Milla-Lewis et al., 2013; Mulkey et al., 2013), their dominant nature limits their applicability for linkage analysis or MAS.

Compared to other molecular markers, SSRs are highly polymorphic. They are also highly repeatable, easy to use, codominant, and evenly distributed in most genomes (Kalia et al., 2011) making them ideal for cultivar identification, analysis of germplasm diversity, linkage mapping, and quantitative trait loci (QTL) discovery (Powell et al., 1996). Microsatellites have recently been developed for a number of turf and forage species for the construction of new linkage maps (Harris-Schultz et al., 2010; Jones et al., 2002; 2003; Li et al., 2009; Saha et al., 2005).

Traditionally, flanking sequence information needed to design primers for new SSRs has been obtained by isolating genomic DNA and subsequently screening thousands of clones through hybridization with SSR-containing probes (Kalia et al, 2011). This process can be extremely inefficient and labor intensive, leading some to develop new techniques for microsatellite discovery and characterization. Next generation sequencing (NGS) platforms such as the Roche 454 or Illumina's Genome Analyzer IIx allow for whole genomes to be cheaply sequenced directly from isolated genomic DNA, eliminating the need to transform and sequence numerous libraries. The techniques used by these two platforms and their differences have been reviewed previously (Mardis, 2008; Varshney et al., 2009). Though much of the initial research utilizing NGS has focused on model organisms and major crops for which reference genomes exist (Varshney et al., 2009), these platforms are increasingly being applied towards *de novo* sequencing of less researched organisms (Garg et al., 2011; Hyten et al., 2008; Jun et al., 2011) and microsatellite isolation (Lu et al., 2011; Parchman et al., 2010; Wang et al., 2010a). Use of NGS on St. Augustinegrass could provide a wealth of sequence information which could be used to isolate numerous SSRs at a fraction of the cost and time of conventional techniques. These molecular markers would be valuable tools for cultivar identification, assessment of genetic diversity within a germplasm or population, linkage mapping, and MAS. The objectives of this study were to (i) obtain Illumina sequencing data for the St. Augustinegrass cultivar 'Raleigh' and use this information to identify and develop primers for a set of SSR markers, (ii) assess levels of diversity within a diverse collection St. Augustinegrass accessions using these markers, (iii) assess the ability of these markers to uniquely identify specific cultivars, and (iv) assess the transferability of these markers to three economically important closely related grass species: centipedegrass [*Eremochloa ophiuroides* (Munro) Hack.], seashore paspalum [*Paspalum vaginatum* Swartz], and switchgrass [*Panicum virgatum* L.].

MATERIALS AND METHODS

Plant Materials. Twenty-two cultivars and 16 plant introductions (PIs) of St. Augustinegrass were used in this study (Table 3.1). Additionally, two accessions of *S.*

dimidatum, a closely related species, were also evaluated. In order to evaluate SSR transferability to other important grasses, seven centipedegrass genotypes, three seashore paspalum cultivars, and three switchgrass cultivars were also included in the analysis (Table 1). All St. Augustinegrass and centipedegrass cultivars were obtained from the North Carolina State University (NCSU, Raleigh, NC) turfgrass breeding program's germplasm collection. All PIs were obtained from the USDA Plant Germplasm System (Griffin, GA). Seashore paspalum cultivars were collected from a field trial at the Lake Wheeler Turfgrass Field Laboratory (Raleigh, NC). Switchgrass genotypes were obtained as calli from Dr. Ron Qu (NCSU, Raleigh, NC).

St. Augustinegrass and centipedegrass plants were maintained in the greenhouse in plastic containers containing Fafard potting mix (Conrad Fafard Inc., Agawam, MA). Plants were fertilized at a rate of approximately 1 lbs. of nitrogen per 1000 sq. ft. every month using Scotts® Starter® Fertilizer (The Scotts Company LLC, Marysville, OH) and mowed to a height of 5.5-7.5 cm. Centipedegrass entries were fertilized monthly at the same rate and mowed to a height of 2.5-5.5 cm. Switchgrass calli were grown on LP9 media at 25°C according to Burris et al. (2009).

DNA extractions for St. Augustinegrass and centipedegrass were performed following the protocols used in Kimball et al. (2012a). The same protocol was followed for switchgrass samples, with the exception that calli were first frozen at -80◦C and manually ground in sterile mortar and pestles. Following extraction, samples were quantified and stored according to the procedures used in Kimball et al. (2012a).

Genomic Sequencing and Primer Design. Total genomic DNA was extracted from fresh leaf tissue of St. Augustinegrass cultivar 'Raleigh' as described above. Library construction, sequencing, and assembly were performed at the NCSU Genomic Sciences Laboratory (Raleigh, NC). Genomic DNA was sheered using a Covaris Ultrasonicator (Covaris Inc., Woburn, MA) to create 200-400 bp fragments prior to the ligation of Illumina paired-end PCR primers (Illumina Inc., San Diego, CA). Fragment size and quantity were measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Libraries were prepared with an Illumina cBot following the recommended manufacturer protocols. An Illumina Genome Analyzer IIx was used to perform paired-end 72 bp sequencing. Illumina Genome Analyzer Pipeline software was used to provide base calls, assess quality, and align contiguous sequences. SciRoKo v3.4 (Kofler et al., 2007) was used to identify and compile a list of contigs containing SSRs with 50 bp flanking regions. To increase the likelihood of finding polymorphic SSRs, a minimum of 10 repeats was set as a requisite for selection. Primer3Plus (Untergasser et al., 2007) was used to design forward and reverse primers for each repeat. Primers were typically designed using the default program parameters with the following exceptions: primer size ranging from 17-27 bases; melting temperature ranging from 56-64°C; product sizes ranging from 80-150 bases.

SSR Screening. Each primer pair was prescreened for amplification and polymorphism on eight genetically diverse St. Augustinegrass accessions (Raleigh, 'Captiva', 'Floratam', and plant introductions 365031, 410357, 290888, 647925, and 289729). Polymerase chain reactions for SSR detection were performed using the fluorescent M13-tail labeling method described in Schuelke (2000). Microsatellites were amplified in 10µL reactions containing 1X PCR buffer, 0.25 mM dNTPs, 1.15 mM additional $MgCl_2$, 0.15 µM forward SSR primer, 0.3 µM reverse SSR primer, 0.1 µM fluorescently labeled M13 primer (LI-COR, Lincoln, NE), 1 M betaine, 0.6 U Taq polymerase, and 20 ng DNA. Reactions were carried out in a MyCycler (Bio-Rad Laboratories, Hercules, CA) using a profile of: one cycle at 94°C for 4 min, 30 cycles of 94°C for 30 sec, "annealing temp" for 30 sec, and 72°C for one min, and one final extension cycle of 72°C for 10 min. For prescreening, "annealing temp" was the average annealing temperature for the eight primer pairs being tested on a single 96-well plate. Following selective amplification, samples were denatured and run on polyacrylamide gels following the procedures of Kimball et al. (2012a). Primers that amplified clear, easily distinguishable polymorphic bands were selected for germplasm characterization.

Germplasm Characterization. A set of 108 primer pairs (PP) were selected to assess genetic diversity among 40 *Stenotaphrum* accessions and to test for transferability to 13 accessions representing closely related species. Polymerase chain reaction amplification and gel electrophoresis conditions were the same as described for primer prescreening with the exception that the average melting temperature of each forward/reverse PP was used as the annealing temp. Microsatellite bands were scored as binary data (present vs. absent) for each individual allele using the AFLP-Quantar 1.0 software (Key Gene Products, 2000). Three samples ('Deltashade', 'Floratine', and PI 365031) were repeated as controls to check for reproducibility and consistent scoring.

Data Analysis. Summary statistics were calculated for the sequencing data, including total number of contiguous sequences generated, number of sequences containing SSRs, and number of SSR containing sequences with both 5' and 3' flanking regions (Table 3.2). Summary statistics were also calculated for germplasm characterization, including the total number of markers screened, percentage of primers that successfully amplified, percentage showing polymorphism, and average number of alleles (Table 3.3). Additionally, to identify SSRs that are potentially useful for cultivar identification, the diversity index (DI) (Weir, 1990) and power of discrimination (D) (Tessier et al, 1999) were calculated for each marker.

Microsatellite data was used to estimate genetic similarity coefficients of all pairwise comparisons according to Dice (1945) using the SIMQUAL module of NTSYSpc v 2.2 (Rohlf, 2000). To generate a dendrogram, cluster analysis was conducted using the unweighted pair group method with arithmetic averaging (UPGMA; Sokal and Michener, 1958) within the SAHN module of NYTSYSpc. Principle coordinate analysis (PCO) was performed using the Dcenter and Eigen functions of NTSYS to construct a three dimensional eigenvector map to aid in visualization of relatedness among accessions. Population structure was also analyzed using a model-based approach using STRUCTURE v2.3 (Falush et al., 2003; 2007; Pritchard et al., 2000). While allowing for admixture, multiple settings for burning period and run length were tested to identify which provided the most consistent likelihood estimates for each K across runs. A setting of 100,000 iterations following a burning period of 50,000 was used for further analysis. The ΔK method (Evanno et al., 2005) was used to identify the number of populations that most accurately described the variation within our samples. Finally, an analysis of molecular variance (AMOVA) was performed in Arlequin v 3.0 (Excoffier et al., 2005) to calculate population pairwise comparisons and significance of variation among and within genotypes when grouped according to ploidy, and to the groups identified in STRUCTURE.

RESULTS

Sequencing Results and Microsatellite Discovery. Illumina sequencing produced approximately 28 million paired-end reads of cultivar Raleigh's genomic DNA. Twenty-four million of those had high enough quality scores to be used for further analysis. Sequence alignment of raw reads produced 21,877 contigs containing 30,895 SSRs of which 25,011 had at least 30 bp of both 5' and 3' flanking sequences, and 5,729 had at least 50 bp 5' and 3' flanking sequences (Table 3.2). The latter of those contained 1,418 SSRs with a three-base motif. To increase the likelihood of identifying polymorphism, microsatellites with the largest number of repeats were selected first for primer design. Out of the 215 PPs designed (Appendix 1) with Primer3Plus, twenty three failed to amplify and two appeared to be monomorphic (Table 3.2). The number of alleles amplified during screening ranged from two to 23 with an average of eight. Product sizes ranged from 60 to 250 bp.

Germplasm Characterization, Cultivar Identification, and Transferability. Primer pairs showing strong, clear amplification and a high degree of polymorphism were selected for further analysis on the germplasm collection. Of 108 PPs initially selected, 14 were later discarded due to poor amplification on a large number of samples or the appearance of multiple loci (i.e. more than two alleles for diploid accessions). The remaining 94 SSRs (Table 3.3) amplified a total of 982 alleles ranging in size from 63 to 250 bp. Minimum and maximum numbers of alleles for individual loci were 5 and 23, respectively, with an average of 10. Estimations of DI and PIC ranged from 0.12 and 0.09 to 0.85 and 0.83, with an average of 0.57 and 0.54, respectively. Estimations of the power of discrimination typically ran much higher, ranging from 0.64 to 0.97, with an average of 0.90.

Examination of the banding patterns for each marker on all cultivars revealed that each could be uniquely identified using just five markers (SAM01646, SAM01709, SAM23382, SAM27269, SAM28482) with the exception of cultivars ‘Palmetto’ and ‘Majestic’ for which no unique patterns were identified. Each marker produced, on average, four unique patterns among the cultivars, although some were able to uniquely identify as many as 10 cultivars (Figure 3.1).

Very low levels of transferability were observed, with only 18 markers amplifying a product for any of the seashore paspalum, centipedegrass, or switchgrass accessions. The majority of these only amplified single bands on just one or a few samples. Six markers (SAM06818, SAM09654, SAM12178, SAM12233, SAM27205, and SAM27269) showed amplification across multiple species, and only three of these (SAM12178, SAM12233, SAM27269) showed polymorphism among non-St. Augustinegrass species. Interestingly, just five of the 18 markers (SAM06818, SAM11437, SAM12178, SAM12233, and SAM27269) produced alleles not found among the *Stenotaphrum* accessions.

Genetic Similarity and Population Structure. Average genetic similarities (GS) were calculated for accessions grouped either by germplasm type (cultivar, plant introduction, and *S. dimidatum*) or ploidy level (diploid, triploid, aneuploid, higher ploidy) (Table 3.4). Within germplasm type, cultivars and PIs showed the highest (GS=0.45) and lowest (GS=0.26) similarities, respectively. Low levels of GS were observed between germplasm types, ranging 0.13 (PI x *S. dimidatum*) to 0.2 (cultivar x PI). Higher levels of within group GS were observed for accessions when grouped by ploidy, with values ranging from 0.29 (*S. dimidatum*) to 0.51 (triploids). Among ploidy levels, triploids and aneuploids showed the highest similarity (GS=0.2). Low levels of GS were observed between *S. dimidatum* and cultivars (GS=0.14), triploids (GS=0.17), and aneuploids (GS=0.15).

Dice similarity coefficients were also used to create an UPGMA dendrogram (Figure 3.2A). High bootstrap values (80-100%) were observed for most branches of the dendrogram, and at the 0.39 similarity level there were eight major clusters identified. The first cluster consisted of all diploid cultivars except for ‘Sapphire’, ‘Mercedes’, and ‘Floraverde’ which grouped with the diploid South African PI 365032 in cluster two. The third cluster consisted only of PI 509038 and PI 509039, both Argentinian introductions. Cluster IV was composed of the remaining diploid plant introductions (all of South African origin) with the exception of PI 414079, PI 647924, and PI 647925, which grouped distantly from all other accessions (cluster VIII). Cluster V was composed of the triploid and aneuploid accessions ‘Bitterblue’, ‘Floratine’, ‘Floratam’, and ‘Floralawn’. Cultivar ‘FX-10’ and PI 290888 formed cluster VI, while PI 291594, PI 300130, and PI 300129 formed a closely grouped seventh cluster. The two *S. dimidatum* accessions grouped together in cluster IX, although at a lower similarity than that used to describe all other clusters (0.28).

Analysis of the 40 *Stenotaphrum* accessions using a model-based approach (Pritchard et al., 2000) provided clear evidence of population structure. Using the ΔK method, it was determined that K=6 best described the data. Figure 3.2B shows the 40 *Stenotaphrum* accessions organized by STRUCTURE assigned populations, and the degree of admixture between them. Groups assigned by STRUCTURE matched well with the UPGMA analysis, with most diploid cultivars and PIs forming Groups 1 and 2, respectively. Of the 14 accessions within Group 1, half showed varying levels of admixture with Group 2. Similarly, the three cultivars within Group 2 (Floraverde, Sapphire, and Mercedes) showed admixture with Group 1. Interestingly, Raleigh (Group 1) and Sapphire showed a high degree (>25%) of admixture with Group 4 (PI 289729, PI 365031, PI 509038, and PI 509039), which consists of two polyploids and the two *S. dimidatum* accessions. As in the dendrogram, PI 414079, PI 647924, and PI 647925 clustered together (Group 3) showing only a small degree of admixture with Mercedes. Five accessions (Floratam, Floralawn, Floratine, Bitterblue, FX-10, and PI 290888) formed Group 5, which showed some admixture with Group 6 (PI 291594, PI 300129, and PI 300130).

Principal coordinate analysis (Figure 3.2C) was performed to better visualize genetic relationships among the accessions. The first three eigenvalues accounted for 51.5% of the total variation and produced clusters in agreement with UPGMA and STRUCTURE groupings. Polyploid accessions clearly separated from the diploids, but did not form distinct groups for the different ploidy levels. Furthermore, *S. dimidatum* accessions did not form a separate group. Most diploid accessions separated into two clusters with the exception of two groups. Plant introduction 414079, PI 647925, and PI 647925 formed a tight group clearly distinct from all other *Stenotaphrum* germplasm. Plant introductions 509038 and 509039, identified by STRUCTURE as having admixture with *S. dimidatum* accession PI 365031 fell in between the polyploid and diploid groups. Cultivars and PIs clustered at separate poles of the diploid group with a few cultivars identified as having admixture falling in between.

To compare the significance of variation explained by partitioning accessions according to the different models (by ploidy or by groups defined by STRUCTURE for K=4, 5, 6, and 7) an AMOVA was performed. F-tests between the STRUCTURE based models (K=4 vs. K=5, K=5 vs. K=6, K=6 vs. K=7; data not shown) found significant ($p=0.01$) increases in the amount of variation explained by each model as K increased to six. At K=7 the increase in explained variation was only significant at the $p=0.05$ level, supporting the earlier observations that K=6 best describes these samples. Among group differences explained 27% ($p<0.001$) of the total variation when partitioning samples by ploidy, and increased to 47% using the K=6 model identified with STRUCTURE (Table 5). Pairwise differences were significant ($p<0.05$) between every combination of K=6 group, while significant differences were only seen between diploids and all non-diploid groups ($p<0.05$) when grouping by ploidy (Table 3.6).

DISCUSSION

Next generation sequencing technologies are increasingly being applied towards sequencing, *de novo* assembly, and marker development in non-model species. Due to their high rate of polymorphism and codominant nature, SSRs remain important for crop species with less genomic resources. Using the Illumina platform, we identified over 25,000 testable genomic SSRs containing five to 21 repeats. Out of 215 SSRs for which primers were designed, 93% showed successful amplification. This can be compared to similar NGS studies in cucurbits (*Cucurbita pepo* L.; Blanca et al., 2011), peppers (*Capsicum annum* L.; Ashrafi et al., 2012), and sweet potato (*Ipomoea batatas* [L.] Lam.; Schafleitner et al., 2010) in which 1,882, 10,398, and 1,661 SSRs were identified, respectively. For these studies, cDNA libraries were used, however. It can be concluded from these results that the Illumina platform presents a rapid, powerful, and cost effective option for the development of SSR markers in non-model crop species.

The 94 PPs selected for germplasm evaluation were highly polymorphic and amplified nearly 1,000 alleles. While less similarity was observed among accessions here than in previous studies (Milla-Lewis et al, 2013; Mulkey et al, 2013), lower levels of similarity are commonly identified by SSRs as compared with other marker systems due to their high rates of polymorphism and codominant nature. The results of this study correlate well with other molecular diversity analyses using AFLPs (Milla-Lewis et al, 2013; Mulkey et al, 2013) as well as morphological studies (Busey, 1986) in that St. Augustinegrass accessions cluster mainly according to ploidy level. Higher genetic similarity values were observed between similar ploidy levels than between very different ploidy levels. Diploid accessions were clearly differentiated from other ploidy levels in the UPGMA cluster, STRUCTURE, and PCO analyses. In addition, all of these analyses clearly differentiated four unique pools of variation among diploid genotypes. These groups correlated well with previous work using AFLPs, but with a few key differences. Within the dendrogram, Deltashade, TX Common, Palmetto, Polaris, and Majestic could not be clearly distinguished in this study, whereas these accessions were more clearly delineated in the study of Milla-Lewis et al. (2013). Whereas Sapphire was previously seen to cluster within the main group of diploid cultivars (Milla-Lewis et al., 2013; Mulkey et al., 2013), our results had it clustering with Mercedes, Floraverde, and PI 365032 in a separate branch. Results from STRUCTURE indicated that the South African diploid plant introductions displayed admixture with these cultivars and could be in their pedigrees. In all three analyses PI 414079, PI 647924, and PI 647925 clearly grouped separately from all other accessions. Based on these findings and on the very different origins (U.S. and China) of these accessions, it can be inferred that these materials constitute a unique pool of genetic diversity. Perhaps most interesting was the finding that Raleigh and Sapphire display admixture with a group of polyploids containing the two *S. dimidatum* accessions. This could indicate that these cultivars contain introgression from this species. Cytogenetic analysis of these materials would help elucidate this issue.

The ability to identify unique subpopulations can greatly aid in understanding variation and diversity within a species. In this study, using groups identified in STRUCTURE to run an AMOVA identified more variation among STRUCTURE groups than among ploidy levels. Likewise, Fst pairwise comparisons for STRUCTURE groups were higher than comparisons between ploidy levels. The ability to further separate accessions into more clearly defined subpopulations helps to explain the genetic relationships among accessions of St. Augustinegrass. In zoysiagrass, where a continuous range of variation often skews analyses, subpopulations identified through UPGMA analysis and STRUCTURE revealed the ability to better differentiate between phenotypically overlapping species and their hybrids (Kimball et al., 2013). In this study we were able to identify the underlying architecture of variation within ploidy levels of St. Augustinegrass and identify instances of genetic overlap. The identification and clearer understanding of these subpopulation types may contribute to better parental selection for breeders.

One of the objectives of this study was to identify a set of SSRs that could be used to uniquely identify varieties. Varietal identification in St. Augustinegrass has relied upon assessment of node length, leaf architecture, and floral morphology. While morphological analysis can reliably attribute accessions to specific groups and races, these methods are less discriminating when applied towards closely related accessions or crossed material in a breeding program. Additionally, maintenance of varietal purity remains a problem in clonally propagated crops and turfgrasses. A recent study by Kimball et al. (2012b) found significant genetic variation among St. Augustinegrass cultivar Raleigh samples collected from sod farms throughout the Southeast. Because of their ease of use and high rates of polymorphism, SSRs can be used to quickly identify genetic differences between closely related materials even when morphological differences cannot be readily discerned. In this study, we identified 94 polymorphic SSRs, 34 of which had at least eight alleles among the cultivars tested. These markers were consistently able to identify multiple cultivars through unique allele combinations. A set of just five SSRs was able to identify 20 of the 22 cultivars included in this study. These markers will greatly increase the accuracy of cultivar identification and the ease with which it can be performed. This has immediate utility for both the sod industry in their attempts to maintain varietal purity and for breeders hoping to distinguish between breeding materials.

Another goal of this study was to assess the transferability of these markers to closely related members of the Poaceae family. Previous work has shown moderate levels of transferability from major cereal crops to seashore paspalum (Wang et al., 2006), and from bermudagrass to zoysiagrass (Harris-Shultz et al., 2012). Using EST-probes developed from rice cDNA libraries, Jo et al. (2007) were able to identify expression of homologues to defense-related genes in St. Augustinegrass. In this study we found very low levels of transferability, with only 19 markers amplifying outside of St. Augustinegrass and only five markers showing within species polymorphism. In most cases, amplification was limited to only a small number of samples or was monomorphic within a species. While it is unlikely that the markers described here will have applicability outside of St. Augustinegrass, it may be possible in future projects to increase the likelihood of transferability by using a cDNA library for sequencing as genic SSRs tend to be more conserved between species (Kalia et al., 2011).

In this study, we demonstrated the effectiveness of the Illumina platform for SSR development in species for which no genomic data was previously available. While only 215 primer pairs were synthesized and screened in our laboratory, over 5,000 additional contigs containing SSRs with 50bp flanking sequences were generated and represent a resource from which additional SSR can be developed. The 190 SSR primer pairs developed represent a significant advance in the development of genomic tools for St. Augustinegrass because they are the first set of SSR markers developed for the species. These markers can be used in the future for genetic and genomic studies in *Stenotaphrum* such as linkage map construction, assessment of genetic diversity and germplasm characterization, and marker-assisted selection.

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Table 3.1. List of *Stenotaphrum* germplasm, including their ploidy level, used for assessing molecular diversity with simple sequence repeat (SSR) markers. Accessions of other genera tested in the present study for SSR transferability are also included.

No.	Identity	Ploidy†	Species	No.	Identity	Ploidy†	Species
1	Amerishade	Diploid	*S. secundatum*	28	PI 410353	Diploid	*S. secundatum*
2	Bitterblue	Triploid	*S. secundatum*	29	PI 410355	Diploid	*S. secundatum*
3	Captiva	Diploid	*S. secundatum*	30	PI 410357	Diploid	*S. secundatum*
4	Classic	Diploid	*S. secundatum*	31	PI 410361	Diploid	*S. secundatum*
5	Delmar	Diploid	*S. secundatum*	32	PI 410363	Diploid	*S. secundatum*
6	Deltashade	Diploid	*S. secundatum*	33	PI 410364	Diploid	*S. secundatum*
7	Eclipse	Diploid	*S. secundatum*	34	PI 414079	Diploid	*S. secundatum*
8	Floralawn	Aneuploid	*S. secundatum*	35	PI 509038	Diploid	*S. secundatum*
9	Floratam	Aneuploid	*S. secundatum*	36	PI 509039	Diploid	*S. secundatum*
10	Floratine	Triploid	*S. secundatum*	37	PI 647924	Diploid	*S. secundatum*
11	Floraverde	Diploid	*S. secundatum*	38	PI 647925	Diploid	*S. secundatum*
12	FX-10	Aneuploid	*S. secundatum*	39	PI 289729	Tetraploid	*S. dimidatum*
13	Jade	Diploid	*S. secundatum*	40	PI 365031	Hexaploid	*S. dimidatum*
14	Majestic	Diploid	*S. secundatum*	41	Aloha	Diploid	*P. vaginatum*
15	Mercedes	Diploid	*S. secundatum*	42	Seaspray	Diploid	*P. vaginatum*
16	Palmetto	Diploid	*S. secundatum*	43	Sea Isle	Diploid	*P. vaginatum*
17	Polaris	Diploid	*S. secundatum*	44	Common Centipede	Diploid	*E. ophiuroides*
18	Raleigh	Diploid	*S. secundatum*	45	Tifblair	Diploid	*E. ophiuroides*
19	Sapphire	Diploid	*S. secundatum*	46	PI414363	Diploid	*E. ophiuroides*
20	Seville	Diploid	*S. secundatum*	47	PI452430	Diploid	*E. ophiuroides*
21	Sunclipse	Diploid	*S. secundatum*	48	PI647882	Diploid	*E. ophiuroides*
22	TX Common	Diploid	*S. secundatum*	49	PI647883	Diploid	*E. ophiuroides*
23	PI 290888	Aneuploid	*S. secundatum*	50	PI647884	Tetraploid	*E. ophiuroides*
24	PI 291594	Triploid	*S. secundatum*	51	Alamo	Tetraploid	*P. virgatum*
25	PI 300129	Triploid	*S. secundatum*	52	Performer	Tetraploid	*P. virgatum*
26	PI 300130	Aneuploid	*S. secundatum*	53	Colony	Tetraploid	*P. virgatum*
27	PI 365032	Diploid	*S. secundatum*				

† Ploidies were obtained from: for *Stenotaphrum* accessions, Milla-Lewis et al. (2013); for *P. vaginatum*, Fedorov (1974); for *E. ophiuroides*, Harris-Shultz et al. (2012); for *P. virgatum*, Hopkins et al. (1996)

Table 3.2. Summary statistics for Illumina sequencing results and SSR primer screening.

Illumina sequencing	
Number of Reads used for Assembly	23,270,975
Total Number of Contigs	405,673
Contigs with SSRs	30,895
with 30bp flanking info (%)	25,011 (81)
with 50bp flanking info (%)	5,729 (19)
-with a three-base motif	1,418
Longest Repeat Length	43bp
SSR screening	
No. of designed primer pairs	215
No. amplified (%)	192 (93)
No. polymorphic	190
Average # Alleles	8.47
Product Size Range	60-250

Table 3.3. Summary statistics for 94 St. Augustinegrass SSRs used to assess molecular diversity and population structure between accessions of Stenotaphrum and related genera.

No.	SSR	Repeat	Size Range (bp)	No. of Alleles	DI†	PIC†	D†
1	SAM00276*	$(TG)_{16}$	119 - 162	7	0.33	0.30	0.88
2	SAM00325	$(GA)_{17}$	89 - 117	8	0.40	0.37	0.91
3	SAM00491*	$(CT)_{13}$	106 - 128	8	0.35	0.32	0.89
4	SAM00557	$(AG)_{15}$	131 - 151	10	0.69	0.67	0.91
5	SAM01084	$(TC)_{15}$	126 - 140	8	0.36	0.33	0.89
6	SAM01646	$(GA)_{13}$	123 - 159	12	0.53	0.50	0.93
7	SAM01681	$(GT)_{19}$	91 - 126	7	0.50	0.48	0.64
8	SAM01709*	$(GA)_{14}$	123 - 185	19	0.75	0.71	0.95
9	SAM01754	$(AC)_{13}$	125 - 162	12	0.61	0.58	0.92
10	SAM01952*	$(TC)_{17}$	87 - 121	10	0.53	0.51	0.91
11	SAM02503*	$(GA)_{15}$	120 - 143	11	0.44	0.42	0.84
12	SAM03084	$(TG)_{18}$	77 - 118	7	0.41	0.39	0.84
13	SAM03453*	$(AG)_{13}$	100 - 126	13	0.37	0.33	0.90
14	SAM03497	$(TC)_{13}$	105 - 137	10	0.26	0.23	0.87
15	SAM03784	$(GA)_{16}$	78 - 130	16	0.70	0.67	0.90
16	SAM04041	$(CT)_{15}$	70-145	14	0.79	0.76	0.92
17	SAM04126	$(CT)_{14}$	124 - 155	6	0.72	0.70	0.79
18	SAM04327	$(AG)_{13}$	107 - 140	12	0.74	0.71	0.92
19	SAM04343*	$(AG)_{15}$	122 - 250	17	0.64	0.61	0.94
20	SAM04444	$(GA)_{13}$	118 - 144	8	0.55	0.53	0.86
21	SAM04930	$(CT)_{18}$	63 - 125	17	0.68	0.64	0.93
22	SAM05258*	$(AC)_{22}$	85 - 136	10	0.52	0.49	0.88
23	SAM05295	$(CT)_{21}$	102 - 141	8	0.51	0.48	0.89
24	SAM05455*	$(CT)_{19}$	86 - 124	11	0.46	0.43	0.85
25	SAM06099*	$(GA)_{18}$	83 - 112	8	0.63	0.60	0.90
26	SAM06321	$(CA)_{18}$	97 - 128	7	0.76	0.74	0.85
27	SAM06818*	$(GA)_{19}$	92 - 126	12	0.62	0.60	0.92
28	SAM06868	$(GA)_{19}$	88 - 117	8	0.56	0.53	0.93
29	SAM07483*	$(GA)_{18}$	83 - 119	7	0.66	0.64	0.84
30	SAM07637	$(AG)_{19}$	99 - 158	13	0.78	0.74	0.95
31	SAM08101*	$(GA)_{19}$	85 - 124	7	0.47	0.44	0.90
32	SAM08347*	$(AG)_{14}$	121 - 144	9	0.56	0.53	0.90
33	SAM08674	$(AG)_{19}$	98 - 126	8	0.74	0.72	0.89

†TA: total number of alleles (min 5, max 19, average 10.17); DI: diversity index (min 0.1, max 0.84, average 0.57); PIC: polymorphic information content (min 0.08, max 0.81, average 0.54); D: power of discrimination (min 0.64, max 0.97, average 0.90)

* SSRs show segregation within a mapping population for gray leaf spot (Magnaporth grisea) resistance (data unpublished).

Table 3.3 continued

No.	SSR	Repeat	Size Range (bp)	No. of Alleles	DI†	PIC†	D†
34	SAM09654*	$(CT)_{14}$	87 - 222	11	0.50	0.47	0.88
35	SAM09747*	$(TC)_{15}$	116 - 159	14	0.76	0.73	0.91
36	SAM10023*	$(TG)_{19}$	85 - 106	5	0.48	0.46	0.76
37	SAM10241*	$(AC)_{14}$	111 - 168	12	0.64	0.62	0.89
38	SAM10626	$(AC)_{18}$	78 - 115	7	0.73	0.71	0.79
39	SAM11067*	$(TC)_{19}$	84 - 116	10	0.62	0.59	0.93
40	SAM11220*	$(AC)_{16}$	96 - 192	13	0.84	0.81	0.94
41	SAM11389*	$(TC)_{14}$	118 - 143	8	0.59	0.57	0.92
42	SAM11402*	$(TG)_{18}$	70 - 140	7	0.10	0.08	0.90
43	SAM11435*	$(GA)_{19}$	88 - 132	12	0.54	0.51	0.92
44	SAM11492	$(TC)_{17}$	94 - 132	10	0.50	0.46	0.94
45	SAM11597*	$(CA)_{17}$	75 - 111	7	0.83	0.80	0.87
46	SAM11655	$(TC)_{17}$	90 - 122	5	0.68	0.66	0.83
47	SAM12178*	$(CT)_{15}$	137 - 196	17	0.45	0.42	0.91
48	SAM12183*	$(GA)_{20}$	80 - 115	9	0.55	0.51	0.93
49	SAM12233	$(CT)_{14}$	96 - 148	12	0.55	0.52	0.88
50	SAM13136	$(CA)_{18}$	88 - 108	7	0.69	0.67	0.82
51	SAM14644	$(AG)_{20}$	93 - 149	8	0.51	0.49	0.88
52	SAM14859*	$(GA)_{14}$	130 - 171	12	0.76	0.74	0.94
53	SAM14922	$(CA)_{15}$	120 - 136	9	0.39	0.36	0.91
54	SAM15826*	$(GA)_{19}$	88 - 140	8	0.53	0.50	0.92
55	SAM16015*	$(GA)_{17}$	87 - 162	18	0.51	0.47	0.96
56	SAM16574*	$(AG)_{14}$	81 - 141	14	0.55	0.52	0.88
57	SAM17002*	$(TC)_{17}$	91 - 126	7	0.55	0.53	0.85
58	SAM17792*	$(TC)_{15}$	127 - 196	15	0.85	0.83	0.92
59	SAM18460*	$(TC)_{18}$	92 - 116	9	0.16	0.13	0.90
60	SAM19467*	$(GT)_{19}$	72 - 127	10	0.12	0.09	0.92
61	SAM19552*	$(GT)_{22}$	88 - 143	13	0.57	0.54	0.92
62	SAM20662*	$(GA)_{22}$	82 - 108	8	0.58	0.55	0.93
63	SAM20919*	$(CT)_{14}$	121 - 156	11	0.22	0.18	0.90
64	SAM21056*	$(CT)_{17}$	83 - 116	9	0.50	0.47	0.93
65	SAM21225	$(TC)_{21}$	83 - 130	13	0.83	0.81	0.92
66	SAM21266*	$(TC)_{18}$	85 - 111	9	0.57	0.54	0.90

†TA: total number of alleles (min 5, max 19, average 10.17); DI: diversity index (min 0.1, max 0.84, average 0.57); PIC: polymorphic information content (min 0.08, max 0.81, average 0.54); D: power of discrimination (min 0.64, max 0.97, average 0.90)

* SSRs show segregation within a mapping population for gray leaf spot (Magnaporth grisea) resistance (data unpublished).

Table 3.3 continued

No.	SSR	Repeat	Size Range (bp)	No. of Alleles	DI†	PIC†	D†
67	SAM21834*	$(AG)_{20}$	89 - 131	5	0.45	0.43	0.82
68	SAM21846*	$(GA)_{15}$	107 - 140	7	0.73	0.71	0.75
69	SAM21927*	$(CT)_{19}$	110 - 160	12	0.64	0.61	0.92
70	SAM22025*	$(CA)_{17}$	115 - 130	8	0.80	0.77	0.93
71	SAM23060	$(TC)_{15}$	109 - 143	12	0.41	0.38	0.89
72	SAM23374*	$(GA)_{19}$	91 - 119	5	0.45	0.43	0.90
73	SAM23382*	$(CT)_{16}$	108 - 144	15	0.74	0.71	0.96
74	SAM23965*	$(TC)_{19}$	88 - 158	23	0.73	0.69	0.96
75	SAM24198	$(AG)_{19}$	86 - 130	14	0.51	0.47	0.92
76	SAM24882	$(AC)_{19}$	104 - 142	5	0.63	0.61	0.85
77	SAM25120	$(GA)_{19}$	83 - 144	9	0.70	0.68	0.89
78	SAM25306	$(AG)_{13}$	120 - 145	7	0.17	0.15	0.89
79	SAM25882*	$(AG)_{19}$	90 - 147	10	0.58	0.55	0.94
80	SAM27103*	$(AG)_{18}$	80 - 126	7	0.61	0.59	0.89
81	SAM27205*	$(CA)_{18}$	89 - 135	12	0.68	0.65	0.92
82	SAM27269*	$(TC)_{19}$	80 - 137	14	0.56	0.52	0.94
83	SAM27477*	$(GT)_{18}$	80 - 120	13	0.63	0.59	0.94
84	SAM27743*	$(CA)_{18}$	86 - 141	17	0.59	0.55	0.93
85	SAM28482*	$(TC)_{18}$	90 - 149	16	0.65	0.60	0.97
86	SAM28514*	$(GA)_{19}$	79 - 98	9	0.63	0.60	0.94
87	SAM28896	$(GA)_{19}$	92 - 122	6	0.55	0.53	0.87
88	SAM29093	$(CT)_{19}$	95 - 132	7	0.54	0.52	0.86
89	SAM29233*	$(GT)_{17}$	111 - 140	11	0.47	0.44	0.92
90	SAM29269*	$(CA)_{13}$	114 - 135	10	0.57	0.54	0.95
91	SAM29522	$(TC)_{17}$	88 - 120	10	0.69	0.66	0.92
92	SAM29525*	$(GA)_{17}$	88 - 124	11	0.70	0.67	0.94
93	SAM29919*	$(TC)_{13}$	101 - 134	16	0.60	0.56	0.94
94	SAM30308	$(AG)_{19}$	101 - 136	6	0.66	0.64	0.86
Min	-	-	-	5.00	0.10	0.08	0.75
Max	-	-	-	23.00	0.85	0.83	0.97
Avg.	-	-	-	10.45	0.58	0.55	0.90

†TA: total number of alleles (min 5, max 19, average 10.17); DI: diversity index (min 0.1, max 0.84, average 0.57); PIC: polymorphic information content (min 0.08, max 0.81, average 0.54); D: power of discrimination (min 0.64, max 0.97, average 0.90)
* SSRs show segregation within a mapping population for gray leaf spot (Magnaporth grisea) resistance (data unpublished).

Table 3.4. Average genetic similarity values (S_{ij}) within and between groups of *Stenotaphrum* accessions based on 94 SSR markers, organized according to ploidy level or STRUCTURE-defined groups.

By germplasm type					
	No. Individuals	Cultivar	PI†	*S. dimidatum*	
Cultivar	22	0.45			
PI	16	0.20	0.26		
S. dimidatum	2	0.15	0.13	0.29	
By ploidy level and species					
	No. Individuals	Diploid	Triploid	Aneuploid	*S. dimidatum*
Diploid	29	0.35			
Triploid	4	0.20	0.51		
Aneuploid	5	0.16	0.40	0.38	
S. dimidatum	2	0.14	0.17	0.15	0.29

†PI = plant introduction.

Table 3.5. Results of the analysis of molecular variance for *Stenotaphrum* accessions based on 94 SSRs, organized according to ploidy level or STRUCTURE defined groups.

Source of variation	**df**	**Sums of squares**	**Variance components**†	**Variation accounted for (%)**
By ploidy level and species (diploid, triploid, aneuploid, and *S. dimidatum*)				
Among ploidy levels (V_a)	3	615.05	23.86 (V_a)***	27.46
Within ploidy levels (V_b)	36	2269.1	63.03 (V_b)***	72.54
Total	39	2884.15	86.89	
By groups identified with Structure				
Among groups (V_a)	5	1407.27	38.58 (V_a)***	47.04
Within groups (V_b)	34	1476.86	43.44 (V_b)***	52.96
Total	39	2884.15	82.01	

***Significant at the 0.001 probability level.

† V_a, among variance component; V_b, within variance component.

Table 3.6. Pairwise differences between *Stenotaphrum* groupings according to Fst values based on 94 SSR markers.

By ploidy level and species						
	Diploid	Triploid	Aneuploid	*S. dimidatum*		
Diploid	-					
Triploid	0.31***	-				
Aneuploid	0.27***	0.07	-			
S. dimidatum	0.27*	0.30	0.20	-		
By groups identified with *Structure*						
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Group 1	-					
Group 2	0.40***	-				
Group 3	0.67***	0.51***	-			
Group 4	0.40***	0.20***	0.43***	-		
Group 5	0.50***	0.30***	0.51**	0.22**	-	
Group 6	0.72***	0.53*	0.94*	0.54*	0.49*	-

*, **, ***: Significant at the 0.05, 0.01, and 0.001 probability levels, respectively.

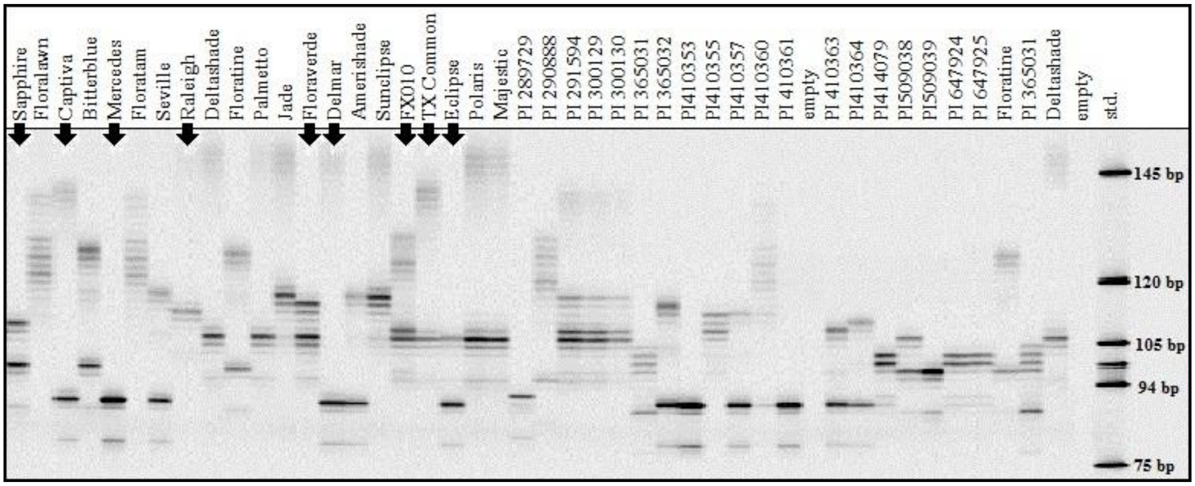
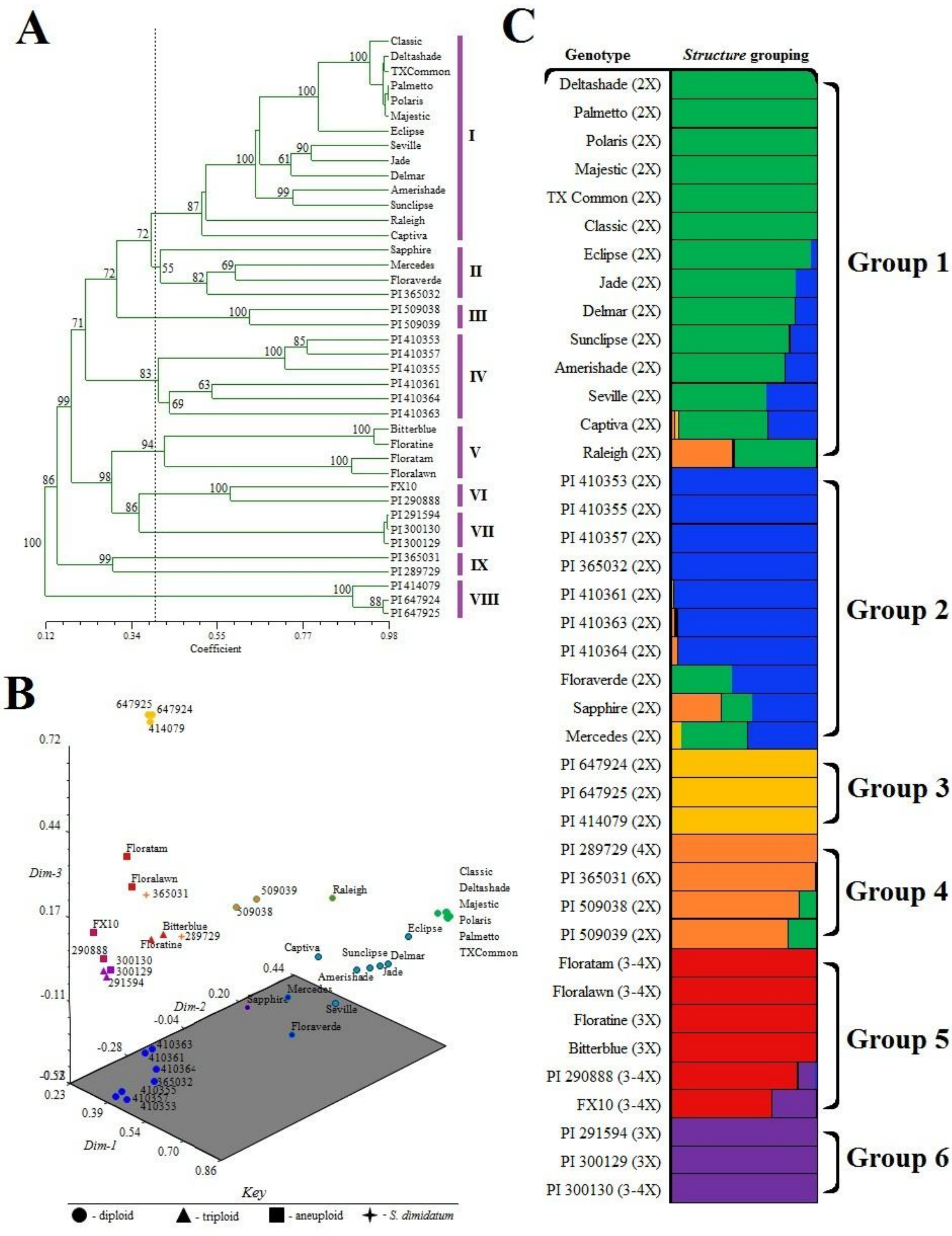


Figure 3.1. Polyacrylamide gel image of *Stenotaphrum* genotypes amplified using SSR marker SAM28482. This primer amplified a total of 16 alleles and had the highest discriminatory power (D=0.97). As observed on the picture, nine cultivars (indicated with arrows) produced unique banding patterns.

Figure 3.2. Genetic analysis of 40 *Stenotaphrum* accessions based on 94 SSR markers. A) UPGMA cluster analysis with bootstrapping. Bars on the right indicate cluster number. Dotted line denotes genetic similarity at which clusters were called. B) Principal coordinate (PCO) plot of 40 *Stenotaphrum* genotypes for the first three principal coordinates estimated with 982 SSR alleles. Colors correspond to STRUCTURE groupings and shapes to ploidy levels. C) Separation of *Stenotaphrum* accessions intro six subpopulations by Structure v2.3. Ploidy levels are indicated in parenthesis after genotype names.



A
Classic
Deltashade
TXCommon
Palmetto
Polaris
Majestic
Eclipse
Seville
Jade
Delmar
Amerishade
Sunclipse
Raleigh
Captiva
Sapphire
Mercedes
Floraverde
PI 365032
PI 509038
PI 509039
PI 410353
PI 410357
PI 410355
PI 410361
PI 410364
PI 410363
Bitterblue
Floratine
Floratam
Floralawn
FX10
PI 290888
PI 291594
PI 300130
PI 300129
PI 365031
PI 289729
PI 414079
PI 647924
PI 647925
I
II
III
IV
V
VI
VII
IX
VIII
Coefficient
B
Dim-1
Dim-2
Dim-3
Key
- diploid
- triploid
- aneuploid
- S. dimidatum
C
Genotype
Structure grouping
Deltashade (2X)
Palmetto (2X)
Polaris (2X)
Majestic (2X)
TX Common (2X)
Classic (2X)
Eclipse (2X)
Jade (2X)
Delmar (2X)
Sunclipse (2X)
Amerishade (2X)
Seville (2X)
Captiva (2X)
Raleigh (2X)
PI 410353 (2X)
PI 410355 (2X)
PI 410357 (2X)
PI 365032 (2X)
PI 410361 (2X)
PI 410363 (2X)
PI 410364 (2X)
Floraverde (2X)
Sapphire (2X)
Mercedes (2X)
PI 647924 (2X)
PI 647925 (2X)
PI 414079 (2X)
PI 289729 (4X)
PI 365031 (6X)
PI 509038 (2X)
PI 509039 (2X)
Floratam (3-4X)
Floralawn (3-4X)
Floratine (3X)
Bitterblue (3X)
PI 290888 (3-4X)
FX10 (3-4X)
PI 291594 (3X)
PI 300129 (3X)
PI 300130 (3-4X)
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6

-CHAPTER IV-

Resistance to Gray Leaf Spot in St. Augustinegrass: Characterization and QTL Mapping

ABSTRACT

St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] is a warm season turfgrass adapted to the tropical and subtropical climates found in the southeastern United States. Genetic linkage maps of St. Augustinegrass were constructed for quantitative trait loci (QTL) analysis of resistance to gray leaf spot using a total of 107 AFLP fragments and 36 SSRs on a pseudo F_2 population of 77 progeny. Twenty two linkage groups were identified, covering 1216.8 cM. Ten linkage groups were ascribed to the susceptible maternal cultivar, 'Raleigh', nine linkage groups to the resistant paternal accession, PI410353, and three groups were found to be homologous. The Raleigh (67 markers) and PI410353 (39 markers) maps covered 525.3 cM and 394.8 cM, respectively, while the homologous linkage groups consisted of 37 markers covering 296.5 cM. The average marker density across all groups was 8.5 cM/marker. Four putative resistance QTL were identified through interval and composite interval mapping. These results present the first linkage map produced for St. Augustinegrass, providing a template for further genetic mapping. Additionally, markers linked to the QTL identified in this study can be used by breeders to transfer resistance to gray leaf spot from unadapted germplasm to elite breeding lines and cultivars.

Abbreviations: AFLP, amplified fragment length polymorphism; AUDPC, area under the disease progress curve; AULEC, area under the lesion expansion curve; GLS, gray leaf spot; MAS, marker assisted selection; PCR, polymerase chain reaction; PDA, potato dextrose agar; SSR, simple sequence repeat; QTL, qauntitative trait loci

INTRODUCTION

St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] is a warm season turfgrass adapted to the tropical and subtropical climates found in the southeastern United States. The broad leaf type and rapid stoloniferous growth that characterize the species allow it to effectively outcompete weeds (Busey, 2003a; Long and Bashaw, 1961), while its tolerance to a wide range of soil types and shade (Busey, 1995; Peacock and Dudeck, 1993) make it a versatile option for home lawns and commercial landscapes. Previous work in St. Augustinegrass has primarily focused on phenotypic evaluation and classification within the species (Atilano and Busey, 1983; Busey, 1986; Flor et al., 2010). More recently, progress has been made in identifying pools of variation within the germplasm at the genomic level (Milla-Lewis et al., 2013; Mulkey et al., 2013a). Development of new cultivars has largely focused on the incorporation of cold tolerance (Busey et al., 1982; Busey, 2003b), resistance to chinch bugs (Busey, 1990; Reinert and Dudeck, 1974), growth type (Riordan et al., 1980), and turf quality. Despite these efforts, St. Augustinegrass remains susceptible to a number of pests and diseases, including gray leaf spot [GLS, causal agent: *Magnaporthe grisea* (T.T. Hebert) Yaegashi & Ugadawa]. Sources of resistance to GLS have been identified within St. Augustinegrass germplasm (Milla-Lewis et al., 2011). Breeders hoping to incorporate this resistance into elite varieties would be aided by a set of molecular markers linked to resistance genes or quantitative trait loci (QTL) which could be utilized in a marker assisted selection (MAS) scheme.

Gray leaf spot is a major disease of St. Augustinegrass, capable of rapidly destroying whole swathes of turf under warm, humid conditions. *Magnaporthe* is a common pathogen throughout the grass family, causing blast disease in rice, wheat, and barley, as well gray leaf spot in tall fescue and ryegrass. Control of GLS in most crops relies on the use of fungicides, making management of this disease costly and difficult for most home lawn and public use settings. Incorporation of host resistance into planted cultivars would present an ideal alternative for the management of GLS is St. Augustinegrass. Because of its widespread prevalence in important food crops, the disease has been widely researched as a model for host-pathogen interactions in rice (Liu et al., 2010). Consequently many resistance genes have been identified in this species (Yang et al., 2009). In addition to these single genes which typically confer complete but race specific resistance, QTL conferring partial resistance have been identified in rice (Fukuoka and Okuno, 2001; Xu et al., 2008), ryegrass (Curley et al., 2005), and barley (Sato et al., 2001). Quantitative trait loci are commonly associated with durable modes of resistance (Boyd, 2006; Kou and Wang, 2010) and present an ideal alternative to reliance upon fungicide for control of the disease. Active breeding efforts in rice have managed to produce varieties with durable resistance (Skamnioti and Gurr, 2009; Wang et al., 1994), and provide proof of concept for application within St. Augustinegrass.

Incorporation of resistance genes and QTL into elite varieties can be expedited through the use of closely linked molecular markers. Linkage mapping provides an important foundation for QTL mapping and the identification of linked markers which can be used in breeding for resistance. Linkage maps have been used in rice (Zenbayashi et al., 2002), barley (Sato et al., 2001), and ryegrass (Curley et al., 2005) to identify resistance-linked markers for *Magnaporthe grisea*. Commonly linkage mapping relies on F_2 or recombinant inbred line populations to analyze marker segregation patterns (Young, 2000). Unfortunately, St. Augustinegrass experiences low seed setting rates resulting from self incompatibility, making the generation of F_2 populations extremely difficult. This problem can be overcome through the use of a 'pseudo F_2' population, an F_1 population generated from two highly heterozygous parents. Practical theory for linkage mapping in these situations has been discussed (Alves et al., 2010; Jansen, 2005; Lu et al., 2004; Maleipaard et al., 1997), and numerous examples of successful mapping experiments in other crops exist (Billotte et al., 2010; Jones et al., 2002; Maleipaard et al., 1998; Okogbenin et al., 2008).

Though recent progress has been made in our understanding of diversity within St. Augustinegrass germplasm, genetic resources for this species remain limited. In other turfgrass species a variety of molecular markers have already been developed and employed for linkage analysis. For example, simple sequence repeat markers (SSRs) and amplified fragment length polymorphism markers (AFLPs) have been used to create linkage maps for perennial ryegrass (Jones et al., 2002), bermudagrass (Harris-Schultz et al., 2010), Zoysiagrass (Cai et al., 2004; Li et al., 2009), and colonial bentgrass [*Agrostis capillaris* L.] (Rotter et al., 2009). To date no such studies have been conducted in St. Augustinegrass, though recently a set of highly polymorphic SSRs were developed (Mulkey et al., 2013b). In conjunction with a pseudo-F_2 population these SSRs could be used for linkage mapping and provide a powerful tool for the identification of resistance QTL. The goals of this study were therefore to: (i) construct and evaluate a pseudo-F_2 mapping population for resistance to gray leaf spot; (ii) characterize this population using a combination of AFLPs and SSRs in order to construct a linkage map; and (iii) identify makers linked to QTL conferring resistance to the disease.

MATERIALS AND METHODS

Plant Materials. A total of 131 pseudo initial F_2 progeny were produced from crosses between 'Raleigh' and PI 410353, diploid accessions which were previously identified as being highly susceptible and resistant, respectively, to GLS (Milla-Lewis et al, 2011). Crosses were performed following the procedures described by Genovesi et al. (2009). Out of the initial 131 progeny, a total of 77 were found to be true progeny (not selfs) following analysis with SSR markers. Mature plants were maintained in the greenhouse in plastic containers containing Fafard potting mix (Conrad Fafard Inc., Agawam, MA). Plants were fertilized at a rate of one pound of nitrogen per 1000 square feet every month using Scotts® Starter® Fertilizer (The Scotts Company LLC, Marysville, OH) and mowed to a height of 5.5-7.5 cm. Roughly two months prior to phenotypic assessment 5 to 6 stolons from each plant were propagated in 16oz. Styrofoam cups containing Profile Field & Fairway™ PPC (Profile Products LLC, Buffalo Grove, IL) in a controlled growth chamber at the

Southeastern Environment Laboratory (Raleigh, NC). Plants were kept at 30°C, with a 12 hr photoperiod with the same mowing and fertilizer regimes as described above.

Fungal strains and production of conidia. Five isolates collected from the Lake Wheeler Turfgrass Field Laboratory (Raleigh, NC) were selected for inoculation based on vigor and sporulation in culture. Isolates were initially maintained on potato dextrose agar (PDA). To initiate the production of conidia, isolates were transferred to an oatmeal ryegrass agar (Ma et al., 2009) for 20-25 days prior to harvesting. For conidial suspension preparation, 5 mL of water were added to five healthy petri dishes of each isolate and spores were scraped free with a brush. Conidial suspensions of each isolate were filtered through cheesecloth and pooled together. Subsequently, the suspension's spore concentration was measured using a hemacytometer. This solution was then diluted to a concentration of 2×10^5 conidia mL^{-1}.

Inoculation and phenotypic analysis. The experiment was conducted following a randomized complete block design. For a single rep the 129 progeny along two copies of each parent were randomly distributed within three plastic bins (39.5 x 20.25 x 7 in.). Additionally, two copies of each of the susceptible cultivars, 'TX Common' and 'Classic', as well as the resistant PI 290888 were included as controls within each bin. Bins were filled with just enough water to cover the bottom and wrapped in transparent plastic bags (International Plastics Inc., Greenville, SC) for the duration of the experiment in order to maintain 100% humidity. The day prior to inoculation total leaf numbers for each cup were counted in order to obtain a baseline for the calculation of disease incidence. Inoculations were performed by applying 5ml of conidial suspension evenly over all blades of grass within a cup using a Badger® 350® airbrush (Badger Air-Brush Co., Franklin Park, IL). For 48 hours following inoculation the plants were held at 24°C in darkness, after which a 12 hour photoperiod was initiated. Though the ambient temperature was held constant, greenhouse effects raised the temperature within the bags to 29-30°C. Measurements were made daily for five days 48 hours after inoculation. Incubation period (number of days until first observed lesion), disease incidence (number of infected leaves divided by the total number of leaves per cup), and mean lesion length were measured for each genotype. Mean lesion length was calculated based on the length of five randomly selected lesions per cup, measured using a handheld digital caliper (Series 500, Mitutoyo Corp., Aurora, IL). For the first run of the experiment the total number of leaves for each cup was counted one day prior to and eight days after inoculation. From these measurements an average daily increase in leaf number was calculated for each cup and used to determine the total number of leaves for each day. To increase the accuracy of daily leaf totals in the second run of the experiment, counting was performed on the second and seventh day following inoculation. Additionally, two derived parameters, area under the disease progress curve (AUDPC) and area under the lesion expansion curve (AULEC) were calculated following Shaner and Finney (1977). Azoxystrobin and chlorothalonil fungicides were applied at 2 and 3 fl. oz. per 1000 square feet, respectively, immediately after and two weeks following the first run of the experiment. Plants were allowed to recover for a month in order to eliminate any residual fungicide and then reinoculated for a second run of the experiment.

Genotypic Analysis. DNA was extracted from six to eight fresh, young leaves for each genotype using the CTAB method described by Stein et al. (2001), with the exception that samples were ground using a *Fast Prep* (Qbiogene Inc., Montreal, Canada). Following extraction, samples were quantified using a Hoefer DQ 300 fluorometer (Hoefer Inc., San Francisco, CA) and stored at 4°C.

To create a linkage map backbone 215 SSR markers (Mulkey et al., 2013b) were screened for polymorphism using parental DNA. One hundred and one of these were amplified across the progeny following the PCR conditions described in Mulkey et al. (2013b). Markers were subsequently selected for linkage mapping based on their clarity of amplification, and ease of scoring. To fill in the backbone and add power to QTL analysis, AFLPs were generated following Milla et al. (2005). Fragments showing segregation among the progeny were selected for scoring. For both dominant (AFLP) and codominant (SSR) marker systems, data was scored according to segregation type (Jansen, 2005) as outlined in JoinMap v.3.0 (van Ooijen and Voorrips, 2001). Chi-square tests for segregation distortion were performed for each locus at a threshold of P=0.05 and P=0.01.

Data Analysis. For each trait data (days to incidence, day seven mean lesion length, day seven disease incidence, AUDPC, and AULEC) were analyzed using the PROC GLM function of SAS v9.2 (SAS Institute Inc., 2008). The data was tested for normality, and in the case of AULEC a square root transformation was performed. A linear model with interaction effects between genotypes, containers, reps, and runs was initially tested using the controls. Container effects were not found to be significant and were excluded from further analysis (data not shown). Subsequently a model with interactions testing genotypes, reps and runs was tested on the whole population. Single factors and two factor interactions were compared against the three factor interaction to conduct F-tests. This model (Appendices 3) was used for the calculation of least square (LS) means for AUDPC and AULEC.

Linkage Mapping. Simple sequence repeat markers with at least three alleles and AFLPs present in both parents segregating with a 3:1 ratio were used to produce linkage map backbones using JoinMap v.3.0 (van Ooijen and Voorrips, 2001). The population was treated as a 'cross pollinator' (CP) in JoinMap. The rest of the markers, segregating separately for each parent, were then analyzed using the backbones to produce a set of linkage groups for each parent. A logarithm of odds ratio (LOD) of 3.0 was selected to identify linkage groups. Linkage groups sharing the same backbone markers were merged together using the 'combine groups for map integration' function. Map distances were calculated using the Kosambi mapping function (Kosambi, 1994).

QTL Analysis. Quantitative analysis was carried out using composite interval mapping via Windows QTL Cartographer v.2.5 (Wang et al., 2012). Because WinQTL Cartographer requires genotypic data to be in bi-allelic form for each locus, but pseudo-F_2 populations can have up to four segregating alleles, SSRs were rescored as dominant (1,0) for each allele when more than two alleles were present. Parental linkage group sets were analyzed individually and simultaneously. Single marker analysis was first performed to quickly identify regions strongly correlated with resistance. Following this, interval mapping (IM) and composite interval mapping (CIM) procedures were used to identify putative QTL. Default settings for CIM were used, and a permutation test was conducted to simulate estimates for unknown parameters and establish thresholds for the detection of putative QTL (Churchill and Doerge, 1994). Permutations were performed 1000 times at a significance level of $P<0.05$.

RESULTS

Phenotypic Analysis. For all five controls, average values of each parameter analyzed across both runs are shown in Table 4.1. Minimum, maximum, and average values for the pseudo-F_2 population are also shown. Continuous variation was observed for all traits other than days to infection, which was largely uniform across both the progeny and controls and not considered for further analysis. A final model using genotypes, reps, and runs was used for the final evaluation. For both AUDPC and AULEC, significant differences were observed between reps, runs, genotypes, and all interactions of these except genotype by rep. Results for F-tests conducted using AUDPC and AULEC can be seen in Table 4.2. While estimates of disease incidence and mean lesion length for day seven evaluations were indicative of resistance/susceptibility within the progeny, AUDPC and AULEC were chosen as more comprehensive phenotypic variables for QTL analysis, as they reflect an individual's change in response over the course of the whole evaluation period. After testing for normality, a square root transformation was performed for AULEC data to provide a better fit. Deviation from normality was not significant enough for AUDPC to warrant transformation. Histograms displaying the distribution for each trait are shown in Figure 4.1.

Rankings for the three susceptible controls varied depending on the parameter analyzed, but each was consistently rated as highly susceptible, in congruence with previous observations (Milla-Lewis et al., 2011). Similarly both resistant controls showed low levels of disease across all parameters. In comparison to the progeny, Raleigh consistently showed higher rates of infection and disease severity than the most susceptible progeny. Conversely, PI410353 showed higher levels of resistance than the most resistant progeny, except when considering AUDPC. Among the progeny LS means for AUDPC ranged from 32.30 to 182.13 (avg. 97.51), compared to 70.92 and 127.90 for PI410353 and Raleigh, respectively. Estimates of AULEC ranged from 1.64 to 3.99 (avg. 2.78) in comparison to 1.85 for PI410353 and 3.19 for Raleigh. Rankings among the progeny changed significantly between runs, though this was much less pronounced among the highly resistant/susceptible varieties (data not shown).

Markers Analysis. Amplification of AFLPs using 11 selective primer combinations produced a total of 226 polymorphic fragments which could be scored across the progeny. A χ^2 test for the expected 1:1 or 3:1 ratios ($P<0.05$) identified 108 fragments displaying extreme segregation distortion, and were not used for linkage analysis. Six of the remaining 118 markers were present in both parents and showed the expected 3:1 segregation ratio. Sixty nine and 43 markers segregated 1:1 for a Raleigh and PI410353 allele, respectively.

Of the 215 SSR markers initially screened, 101 were initially identified as being polymorphic between the parents. Eight of these were found to be difficult to score, and another eight showed strange banding patters such as high rates of non-parental alleles or more than two alleles in the progeny (indicating multiple loci). Nine loci appeared to identify numerous 'selfs' among the progeny in disagreement with most other markers and need further assessment before they can be used for linkage analysis. Similarly, 18 markers could not be utilized due to too much missing data or conflicts in scoring among the controls, and require further assessment before they can be used. Expected segregation ratios for SSRs can be 1:2:1, or 1:1:1:1 in a pseudo-F_2 population depending on the number of number of alleles and whether or not one of them is shared (Jansen, 2005). A χ^2 test ($P<0.05$) of the remaining 65 markers found eight to have extreme segregation distortion and were not used for linkage analysis.

Linkage Mapping. Out of 171 markers analyzed, 107 AFLPs and 36 SSRs were successfully mapped to 22 linkage groups (LGs) covering a total distance of 1,216.8 cM for an average density of 8.5 cM/marker. Table 4.3 lists the number of fragments mapped from each selective AFLP primer combination, as well as the 36 SSR markers successfully mapped. Specific primer information for the SSRs is reported in Mulkey et al. (2013). Linkage groups ranged in size from 10.8 to 214.8 cM, with an average of 55.3 cM. A LOD of 3.0 was used to determine linkages, though typically most groups were significant at much greater stringencies.

Initially markers segregating for one parent or the other were used to produce maternal and paternal LGs. This method produced 13 and 12 LGs for Raleigh and PI410353, respectively. The presence of SSRs and AFLPs segregating for both parents allowed three LGs from each parental set to be merged. While the R13 and P6 LGs share a marker in common (SAG29919), JoinMap was unable to successfully combine the two groups. The final 22 LGs are shown in Figure 4.2. The Raleigh map is composed of 67 markers covering 525.5 cM, while the PI410353 map is composed of 39 markers covering 394.8 cM. The merged LGs cover 296.5 cM based on 37 markers.

QTL Identification. Windows QTL Cartographer 2.5 was used to identify putative QTL for resistance to gray leaf spot based on the genotypic and phenotypic data from 77 progeny. Analysis were performed three times, once for each parental set of LGs, and once with all groups together. Because SSR data was scored as dominant (1,0) to meet WinQTL Cartographer's format requirements, unmerged linkage groups from the two parental maps had to be used for analysis. Interval mapping (IM) and composite interval mapping (CIM) identified the same putative QTLs, though CIM tended to detect a stronger signal for each. Very similar results were obtained when analyzing each parental set of LGs alone and together. A LOD threshold of 2.5 was determined as significant by JoinMap, at which four QTL were identified through IM and CIM. One QTL was based on AULEC data while three were found for AUDPC (Table 4.4). Locations of putative QTL in the unmerged WinQTL Cartographer parental maps were compared against the merged linkage groups. All QTL were located on the combined linage group, R7/P2 (Figure 4.1b), and accounted for 10.3%, 11.5%, 15.3%, and 30.7% of the phenotypic variation. Logarithm of odds peaks for each putative QTL are shown in Figure 4.3. Similar peaks were observed for AUDPC where QTL for AULEC were identified, though they did not cross the threshold LOD.

DISCUSSION

Successful linkage mapping is dependent upon two primary factors: population size and marker coverage. Though an initial population size of 131 individuals were developed and phenotyped, genetic analysis identified numerous selfed-Raleigh individuals, reducing the total true progeny numbers down to 77. Small population sizes can result in skewed linkage maps, with false marker orders and distances arising from artifacts of the population and limited recombination events (Zeng, 1994). These in turn can have significant effects for QTL analysis, resulting in false QTL-marker associations, overestimation of QTL effects, or the failure to identify any correlation at all (Jansen et al., 2003). These effects are exacerbated when a linkage map has poor marker coverage. Despite an initial screening of 215 SSRs, only roughly 50 could be used in this study for linkage analysis. An addition 20-25 may yet be added upon further investigation, which could significantly improve the current map due to the highly informative nature of these codominant markers. In this study, much of the distance covered in this map was due to 107 AFLPs, which helped to mitigate some of the problems discussed above. AFLPs are cheap and can be quickly produced/analyzed to provide an additional source of markers to fill in gaps in the linkage map. The addition of more markers may expand map coverage and improve accuracy, allowing for the correct identification of QTL and an accurate assessment of their effects. It should be noted, however, that there are limits to the extent to which additional markers can improve the situation without a concurrent increase in mapping population size.

The linkage analysis performed in this study was able to identify 22 linkage groups between both parental maps. The 13 LGs corresponding to the Raleigh map (10 plus the 3 merged groups) and the 12 LGs of the PI410353 map (9 plus the 3 merged maps) are more than the ideal nine groups expected (diploid St. Augustinegrass cultivars have nine homologous pairs of chromosomes). Given the population size and the number of markers analyzed, this is not unexpected. Both parental maps, and especially the PI410353 map, contain linkage groups with few markers covering small distances. An expansion of this study would likely be able to merge many of these and bring the number of LGs down.

An additional challenge of this study is the pseudo F_2 structure of the mapping population. Because of the highly heterozygous nature of the parents as many as four alleles can be segregating in the progeny for each locus, as opposed to just two alleles in a traditional F_2 or backcross population. This poses a challenge for linkage phase determination and limits the ability to identify homologous linkage groups. Because codominant markers provide genotypic information from both parents, linkage phases can be determined for both parental maps (Maliepaard et al., 1998). This is essential for identifying homologous chromosomes from both parents. For a pseudo F_2 population a codominant marker must have three or four alleles to provide linkage phase information for both parents. Out of the thirty six SSRs mapped in this study, 31 had at least three alleles.

Additionally, 21 of these were used to identify the three homologous chromosomes from the parental maps. It is clear that such highly polymorphic codominant markers are essential for map construction in crops depending on pseudo F_2 mapping populations.

This study identified four putative QTL for resistance to gray leaf spot, three for AUDPC and one for AULEC. All four QTL were located on the shared R7/P2 linkage group. Though non-significant, peaks were observed for AULEC where the QTL for AUDPC were identified, which was expected as both traits were derived from observations of disease severity on the same individuals. It is likely that the same underlying mechanisms control resistance for both parameters. Together these QTL appear to explain roughly 11.5% and 66% of the phenotypic variation for AUDPC and AULEC derived parameters, respectively. The QTL associated with markers SAG21056 and SAG23374 are located just 6.6 cM from each other which is consistent with QTL mapping results in both rice and perennial ryegrass where a number of resistance genes or small effect QTL formed small clusters sparsely scattered throughout the genome (Chauhan et al., 2002; Curley et al., 2005; Tabien et al., 2002). This should be taken with a measure of caution, however. The fact that SAG21056 and SAG23374 are located so close to each other may indicate that the two QTL detected here are identical and the exact location could not be clearly delineated due to the limitations mentioned above. Additionally, small linkage populations have a tendency to overestimate the effects of QTL, and it seems unlikely that these four QTL truly explain such a large portion of the phenotypic variance (Laird and Lange, 2011). Despite these issues, the fact that strong signals were detected at these loci through multiple forms of analysis provides good evidence that there are true QTL in these general regions of R7/P2. Further phenotypic evaluation would aid the QTL analysis by providing a clearer picture of the amount of variation resulting from environmental influences. Being able to accurately account for this source of variation allows QTL analysis to more reliably ascribe the remaining variation to individuals based on their genotype, and hence increases the chance of identifying the underlying genes controlling a trait.

Compared to other turfgrasses, St. Augustinegrass remains limited in terms of genetic resources for the dissection of genomic structure and identification/selection of genes controlling useful traits. Though a combination of comprehensive germplasm analysis for key traits and the development of SSR markers has expanded breeders' capacity to conduct cultivar improvement, this study highlights a number of obstacles that still remain, and points the way towards future research. The generation of more informative SSRs will be essential for future linkage mapping and QTL analysis projects. The parental linkage maps generated in this study can be used as a rough template for further mapping studies, and the QTL identified here can be used to facilitate the transfer of resistance to GLS from unadapted germplasm into commercially important cultivars.

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Table 4.1. Average values of disease incidence, mean lesion length, AUDPC, and AULEC across both experimental evaluations.

Genotype†	Incidence (%)††	Lesion Length (mm)††	AUDPC*	AULEC*
Classic	39.02	9.14	94.57	3.38
Raleigh	43.91	7.41	127.90	3.19
TXCommon	40.89	8.02	103.33	3.37
PI290888	15.27	0.90	64.12	1.33
PI410353	17.45	1.65	70.92	1.85
Progeny Min	10.98	1.88	32.30	1.64
Progeny Max	57.14	11.90	182.13	3.99
Progeny Avg	32.66	5.57	97.51	2.78

† For the five controls values across all reps and runs were averaged

†† Disease incidence and mean lesion length listed above are based on day seven evaluations, the final day of observations.

*Values for AUDPC and AULEC are LS means calculated using the PROC MIXED command in SAS 9.2.

Table 4.2. Analysis of variance for AULEC and AUDPC.

AULEC					
Source	DF	Type III SS	Mean Square	F-Value	Pr > F
genotype*rep	256	47.23	0.18	1.06	0.3326
genotype*run	128	52.56	0.41	2.35	<.0001
run*rep	2	4.19	2.10	12.00	<.0001
genotype	128	174.30	1.36	7.79	<.0001
rep	2	26.42	13.21	75.60	<.0001
run	1	115.95	115.95	663.49	<.0001
AUDPC					
Source	DF	Type III SS	Mean Square	F-Value	Pr > F
genotype*rep	256	197186.92	770.26	0.88	0.8439
genotype*run	128	312676.10	2442.78	2.79	<.0001
run*rep	2	95485.64	47742.82	54.62	<.0001
genotype	128	804249.84	6283.20	7.19	<.0001
rep	2	310789.51	155394.75	177.77	<.0001
run	1	20508.65	20508.65	23.46	<.0001

Table 4.3. List of AFLP primer combinations producing mappable fragments, as well as mapped SSRs.

AFLP Selective Primer Combinations [†]		
E41M60 (17)	E38M55 (9)	E35M52 (4)
E37M47 (16)	E39M49 (7)	E44M47 (4)
E37M56 (14)	E46M57 (7)	E36M59 (6)
E37M53 (12)	E35M49 (6)	
SSR IDs [††]		
SAG01466 (3)	SAG12183 (4)	SAG21927 (4)
SAG01745 (4)	SAG15446 (3)	SAG22025 (3)
SAG02590 (3)	SAG15826 (4)	SAG22114 (3)
SAG03453 (3)	SAG16015 (4)	SAG23374 (4)
SAG03957 (2)	SAG16154 (3)	SAG23382 (3)
SAG06099 (3)	SAG16574 (3)	SAG27743 (4)
SAG08347 (2)	SAG17356 (3)	SAG28482 (3)
SAG09654 (4)	SAG18530 (2)	SAG28514 (4)
SAG09747 (4)	SAG19467 (2)	SAG29269 (4)
SAG10050 (2)	SAG20662 (3)	SAG29525 (3)
SAG11597 (4)	SAG21056 (3)	SAG29919 (4)
SAG12178 (3)	SAG21266 (3)	SAG30021 (4)

† Numbers in ( ) indicate how many scored fragments were mapped.

†† Numbers in ( ) indicate how many alleles were identified for each SSR.

Table 4.4. Characteristics of the QTL detected for gray leaf spot resistance.

QTL #	Parameter	Linkage group	Closest marker	R^2 (%)	LOD threshold*
1	AULEC	R7/P2	E37M53M355	10.3	2.5
2	AUDPC	R7/P2	E37M53M233	11.5	2.5
3	AUDPC	R7/P2	SAG23374	15.3	2.5
4	AUDPC	R7/P2	SAG21056	30.7	2.5

*Thresholds determined through Windows QTL Cartographer 2.5 iterative permutation testing.

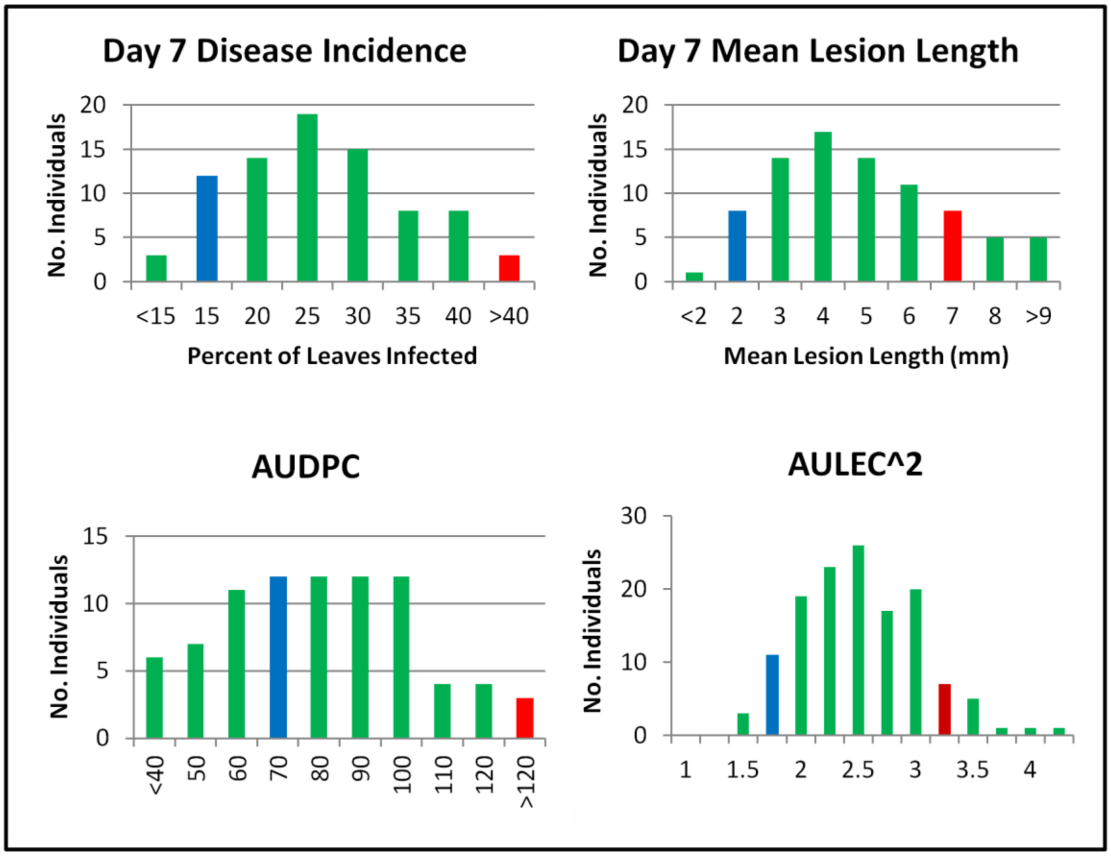
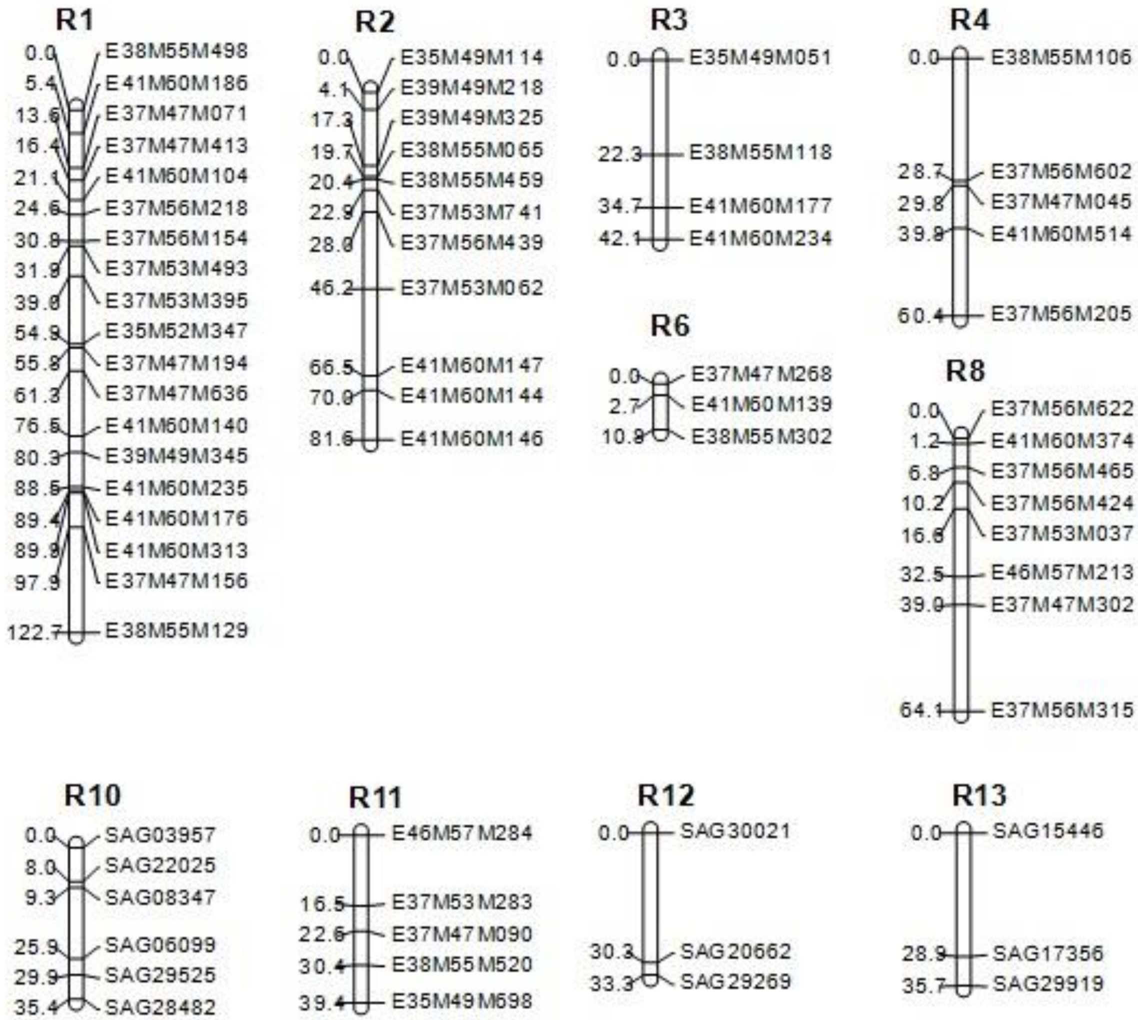
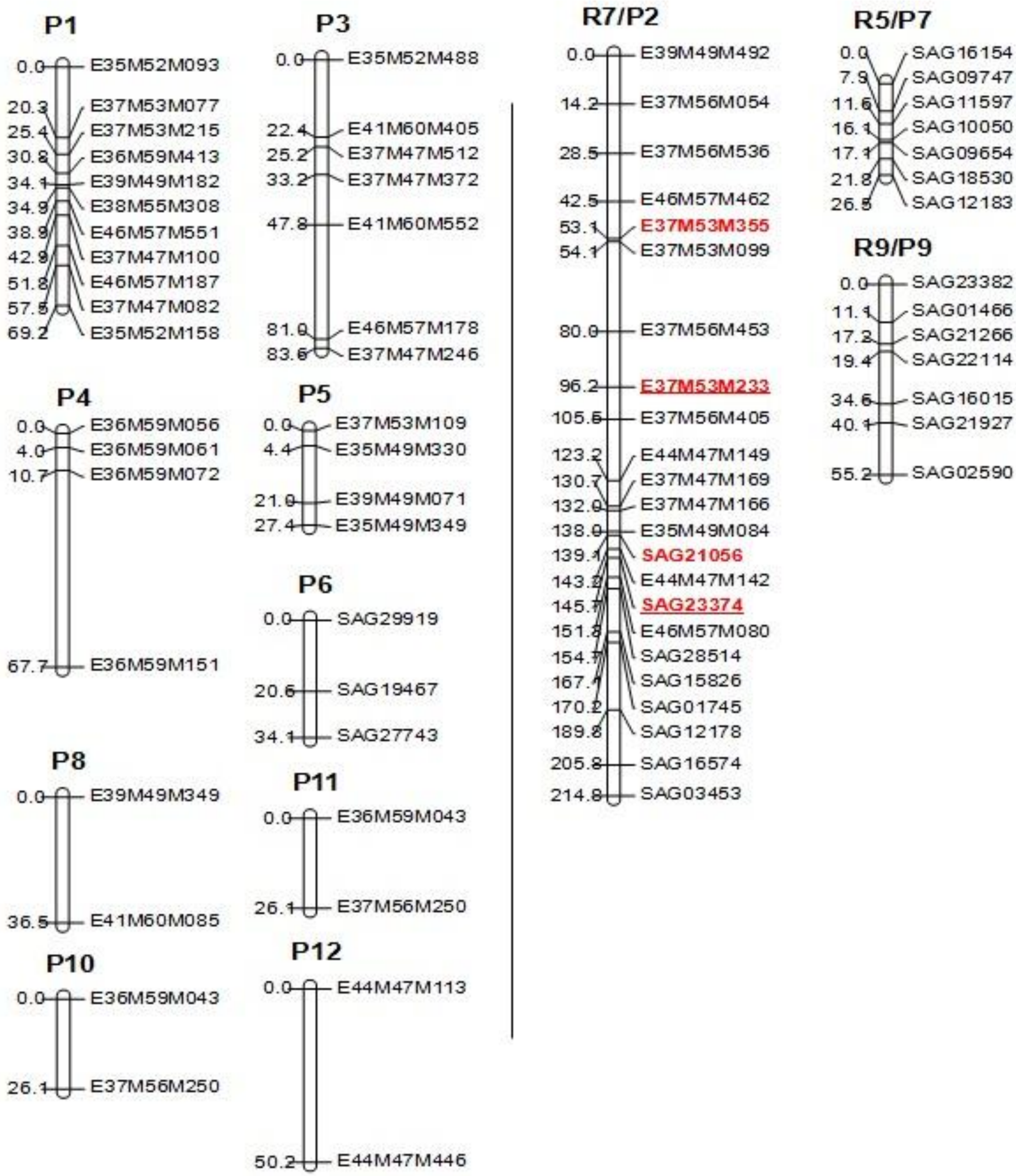


Figure 4.1. Histograms showing the population distribution for each of the four measured parameters: day seven disease incidence, day seven mean lesion length, area under the disease pressure curve (AUDPC), and area under the lesion expansion curve (AULEC). Blue and red lines indicate average values for the resistant (PI410353) and susceptible (Raleigh) parents. For day seven disease incidence and mean lesion length, values used were the average across all reps and runs. LS means calculated with SAS were used for AUDPC and AULEC. A square root transformation was applied to the AULEC data.

Figure 4.2. A genetic linkage map of the female parent (R: Raleigh), male parent (P: PI410353), and homologous linkage groups (R/P) generated from 77 pseudo F_2 progeny. Numbers on the left indicate genetic distances in centiMorgans (cM), calculated using the Kosambi function. Numbers on the right indicate marker names. Markers underlined and in red indicate correlation with putative quantitative trait loci.


R1
0.0 E38M55M498
5.4 E41M60M186
13.6 E37M47M071
16.4 E37M47M413
21.1 E41M60M104
24.6 E37M56M218
30.8 E37M56M154
31.9 E37M53M493
39.8 E37M53M395
54.9 E35M52M347
55.8 E37M47M194
61.3 E37M47M636
76.5 E41M60M140
80.3 E39M49M345
88.5 E41M60M235
89.4 E41M60M176
89.9 E41M60M313
97.9 E37M47M156
122.7 E38M55M129
R2
0.0 E35M49M114
4.1 E39M49M218
17.3 E39M49M325
19.7 E38M55M065
20.4 E38M55M459
22.9 E37M53M741
28.8 E37M56M439
46.2 E37M53M062
66.5 E41M60M147
70.0 E41M60M144
81.6 E41M60M146
R3
0.0 E35M49M051
22.3 E38M55M118
34.7 E41M60M177
42.1 E41M60M234
R4
0.0 E38M55M106
28.7 E37M56M602
29.8 E37M47M045
39.9 E41M60M514
60.4 E37M56M205
R6
0.0 E37M47M268
2.7 E41M60M139
10.9 E38M55M302
R8
0.0 E37M56M622
1.2 E41M60M374
6.8 E37M56M465
10.2 E37M56M424
16.6 E37M53M037
32.5 E46M57M213
39.0 E37M47M302
64.1 E37M56M315
R10
0.0 SAG03957
8.0 SAG22025
9.3 SAG08347
25.9 SAG06099
29.9 SAG29525
35.4 SAG28482
R11
0.0 E46M57M284
16.5 E37M53M283
22.6 E37M47M090
30.4 E38M55M520
39.4 E35M49M698
R12
0.0 SAG30021
30.3 SAG20662
33.3 SAG29269
R13
0.0 SAG15446
28.9 SAG17356
35.7 SAG29919


P1
0.0 E35M52M093
20.3 E37M53M077
25.4 E37M53M215
30.8 E36M59M413
34.1 E39M49M182
34.9 E38M55M308
38.9 E46M57M551
42.9 E37M47M100
51.8 E46M57M187
57.5 E37M47M082
69.2 E35M52M158
P3
0.0 E35M52M488
22.4 E41M60M405
25.2 E37M47M512
33.2 E37M47M372
47.8 E41M60M552
81.0 E46M57M178
83.6 E37M47M246
R7/P2
0.0 E39M49M492
14.2 E37M56M054
28.5 E37M56M536
42.5 E46M57M462
53.1 E37M53M355
54.1 E37M53M099
80.0 E37M56M453
96.2 E37M53M233
105.5 E37M56M405
123.2 E44M47M149
130.7 E37M47M169
132.0 E37M47M166
138.0 E35M49M084
139.1 SAG21056
143.2 E44M47M142
145.7 SAG23374
151.8 E46M57M080
154.7 SAG28514
167.1 SAG15826
170.2 SAG01745
189.8 SAG12178
205.8 SAG16574
214.8 SAG03453
R5/P7
0.0 SAG16154
7.9 SAG09747
11.6 SAG11597
16.1 SAG10050
17.1 SAG09654
21.8 SAG18530
26.5 SAG12183
R9/P9
0.0 SAG23382
11.1 SAG01466
17.2 SAG21266
19.4 SAG22114
34.6 SAG16015
40.1 SAG21927
55.2 SAG02590
P4
0.0 E36M59M056
4.0 E36M59M061
10.7 E36M59M072
67.7 E36M59M151
P5
0.0 E37M53M109
4.4 E35M49M330
21.0 E39M49M071
27.4 E35M49M349
P6
0.0 SAG29919
20.6 SAG19467
34.1 SAG27743
P8
0.0 E39M49M349
36.5 E41M60M085
P11
0.0 E36M59M043
26.1 E37M56M250
P10
0.0 E36M59M043
26.1 E37M56M250
P12
0.0 E44M47M113
50.2 E44M47M446

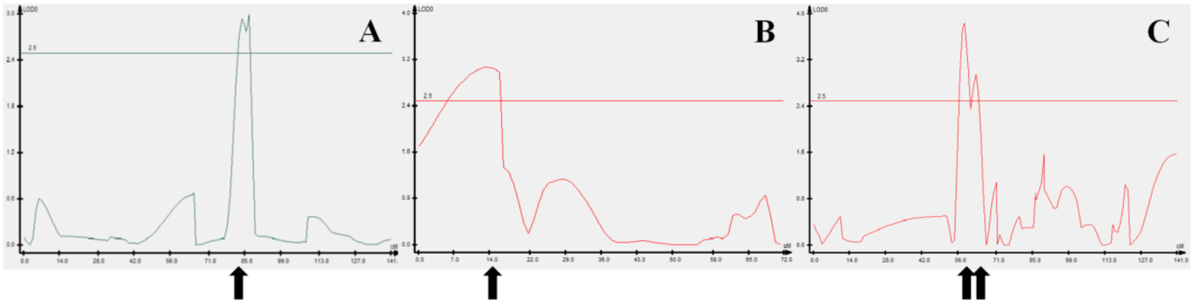


Figure 4.3. Logarithm of odds (LOD) peaks for putative quantitative trait loci identified through interval mapping and composite interval mapping. Black arrows indicate relative positions of associated molecular markers. For 'A' and 'C', peaks were identified using the PI410353 linkage map; 'B' was identified on the Raleigh map. Four putative QTL were identified, one based on the area under the lesion expansion curve parameter (A), and three for the area under disease progress curve parameter (B, C).

-APPENDICES-

Appendices 1. List of simple sequence repeat (SSR) forward and reverse primers developed for St. Augustinegrass.

Pair No.	Primer Name	Sequence	Length (bp)	Tm (°C)	Expected product size (bp)
1	SSR19552_F	TGTCAGTGGCAAGAAATGGA	20	60.2	95
	SSR19552_R	CAGGGTGGGGAGATGGTTA	19	60.7	
2	SSR20662_F	CCACCATCTTTGTCTTGAGCA	21	61.2	91
	SSR20662_R	CTCACGCTGCCACCACAT	18	62	
3	SSR5258 _F	CTGCCATCTGGAGCACAAC	19	60.4	95
	SSR5258 _R	TGCTCTACTTCTTGATCCCAAAA	23	60.2	
4	SSR12053_F	ACCTCATCGGCCATTTTGA	19	61.4	90
	SSR12053_R	GATCCAGGCTGTGTCGATG	19	60.2	
5	SSR21225_F	GGATGCCATGCACGAGTAAT	20	60.9	90
	SSR21225_R	AATTGTGTATTTCATGCCAGCTC	23	60.4	
6	SSR5295_F	GCTCTCTTCCCCTTCCTCTT	20	59	101
	SSR5295_R	CCTTCGTCTTCGTCTTCTCC	20	58	
7	SSR9481_F	CCTTCCCAGGCGTTTGTG	18	62.6	99
	SSR9481_R	GTAGACTCACACAACACCACCA	22	58.5	
8	SSR21834_F	ATCACCGACGACACACACA	19	59.5	94
	SSR21834_R	CTAGCGAGACCCAATGACG	19	59.4	
9	SSR12183_F	AATGTCTGTGCTGCCTGCT	19	59.6	86
	SSR12183_R	GTCGTCGGCAACAAGTGC	18	61.5	
10	SSR14644_F	CATCTGTTAGGGGTGAAGCTG	21	59.7	97
	SSR14644_R	TGTTTGTCCTCTCTCTCATGTCA	23	59.9	
11	SSR11067_F	CCCTGATCCATTCTCTCTACCA	22	60.5	90
	SSR11067_R	AACCGAGCTCTGCCACCT	18	61	
12	SSR15826_F	CGTTGACAGGCAGTCGAG	18	59.1	93
	SSR15826_R	GGACTCCGTTACGACGTG	18	57.6	
13	SSR1681_F	CCATTGTTGCTGTGTGGAAT	20	59.4	96
	SSR1681_R	CAGGAACCAAACACACAAGC	20	59.2	
14	SSR19467_F	TGTATCTATCTCTCGTTCAGCTTCA	25	59.6	92
	SSR19467_R	TTTTTCTCTTATCTTCCAAACACA	24	57.2	
15	SSR21927_F	TTGTTACCACAACTGCTTCGTC	22	60.2	90
	SSR21927_R	GCACACATGCACACACACG	19	62	
16	SSR22548_F	TCCCCAAGTGGAGGTCCTT	19	61.8	90

	SSR22548_R	ACCCCAACCGACCAGAAC	19	60.8	
17	SSR23374_F	GATCGTTCGCATGAGGTGAT	20	61	93
	SSR23374_R	AGTGGTAAACAGCCATAAATGAA	23	57.8	
18	SSR23965_F	CACGAGGCTGTACTACCACTCA	22	60.4	94
	SSR23965_R	TGAAATGTTAAGTTTGTGCGTGT	23	59.6	
19	SSR24198_F	AGAGAGAGATCATCATCAAGTCCA	24	59.4	92
	SSR24198_R	CTGAAAGCACACACGGTTCT	20	58.9	
20	SSR24882_F	AAAGCTCATGAATTTGACAGAAGA	24	59.4	95
	SSR24882_R	CTTTTGAGCAACGTACTCCGTA	22	59.5	
21	SSR25120_F	TTACGACAAACATTTCATGAGG	22	57.7	86
	SSR25120_R	GAGCCAAGCATGACCACA	18	59.3	
22	SSR29093_F	CCTAGCATCTCCAGGTTTGG	20	59.7	96
	SSR29093_R	CCACTTTACATGTTGTTGTTACCTG	25	59.8	
23	SSR8054_F	TGTTATCTGAACATAGCAAATTGTC	25	57.5	93
	SSR8054_R	AAACAACGTCTAATGCAATTTTT	23	57.1	
24	SSR10023_F	TGTAAGCTTGGGTGCAAGG	19	59.8	96
	SSR10023_R	GAACCAGGGCCTCACTCTTA	20	59.3	
25	SSR10879_F	GGAGGGAGGGGCAGTACAAA	20	63.5	75
	SSR10879_R	CGGACGACCGGTGAAAGAT	19	63.4	
26	SSR11435_F	GGGACCCGCCTAAGCTAAA	19	61.4	89
	SSR11435_R	GGCCGTGTCATGGCTAGTAA	20	61.1	
27	SSR25882_F	CATTTAGGTACTGGGAAAACACG	23	59.8	92
	SSR25882_R	TGTATCGCTATCCAAGAATATCACA	25	59.9	
28	SSR27269_F	CCCCTGCCATGCTTGTTT	18	62	90
	SSR27269_R	ATCAGCATGTGCCCCAACT	19	61.5	
29	SSR28514_F	CACGGAAATGTGGTTCATGTTT	22	61.9	80
	SSR28514_R	CTTGAATCGCACGGTCCATA	20	62	
30	SSR28896_F	ACCGACCTCATTTTCCTGTC	20	59	93
	SSR28896_R	CACTGCTCAAGACTGAAGAGC	21	58	
31	SSR30308_F	GCTCACGAGAAGGAAAAGGTT	21	59.9	
	SSR30308_R	AACCAAGGTGCATTTCTTTCA	21	59.6	
32	SSR5455_F	GTGGGCGTAGGTCCGTCT	18	61.1	
	SSR5455_R	GCACAGGAGAGAGATGGTGTG	21	60.9	
33	SSR6818_F	TGTACTACCATGGAGATCAACTTGT	25	59	94
	SSR6818_R	TGGTATGGTGTGACTACCTTGTG	23	59.8	
34	SSR6868_F	CGATACGATGGTTTTGTTTCG	21	60.4	87
	SSR6868_R	AAACCAAAGCCAGGTAACACC	21	60.3	
35	SSR7637_F	TCGGTGTTCATGGGGATT	18	59.7	90
	SSR7637_R	GAAGGTACCCCGCCATCT	18	59.9	

36	SSR8101_F	GCCCGCTAACGCACTCT	17	60.1	82
	SSR8101_R	CCACCATTCCTCAACCGTAT	20	59.7	
37	SSR8674_F	CCTCTTGACAAGTAAACAATCCA	23	58.3	91
	SSR8674_R	ACTACTAGTCCTTTGCCTTATTTGA	25	57	
38	SSR11402_F	AATTTGTTGTGTTTGTTATGTAATCTC	27	57	93
	SSR11402_R	TCTCTATTCGTTAGATTCCCAAAT	24	57.5	
39	SSR21266_F	ATTCCAGCGGGCAGCATA	18	62.7	83
	SSR21266_R	CGCCGTCGTCTTTACATGAG	20	61.8	
40	SSR27205_F	TCTCTCAACGCATCCTCCTC	20	60.5	96
	SSR27205_R	CGCCTATTCCCTTCACTTACC	21	60	
41	SSR6321_F	TCGAACTAGCTAGCCAAAAACA	22	59.2	96
	SSR6321_R	TCCACACTCCACAGCTTACAA	21	59.4	
42	SSR10626_F	ATCATTGTATTAAAAGGAGAAGAAGAG	27	57.1	80
	SSR10626_R	GCGTCCCTCCACCACTAT	18	58.5	
43	SSR13136_F	CATCTGATGACGCATCTACGAG	22	60.8	88
	SSR13136_R	GTGAGTGGCTGGCGATG	17	59.9	
44	SSR18460_F	GACCAAGTTGATCGGTATGC	20	58	95
	SSR18460_R	CATCAACATTGATAAACAAGGAGA	24	58.2	
45	SSR21183_F	CCAATTTTAGGTTTTCAATGGGTA	24	60.3	94
	SSR21183_R	TTTTCATCAGACGACAGAATCG	22	60.2	
46	SSR21721_F	GGCCTGCAGGGTATAATTAGTG	22	59.9	91
	SSR21721_R	ACAAAACATTATTCAAGAATCACCA	25	58.9	
47	SSR27103_F	AATAGCCTCAGCAATATACCCACT	24	59.5	80
	SSR27103_R	CTTCCTTGCTGGCTGGAC	18	59.5	
48	SSR27477_F	GAGTGCTAGGCCCATGGTAA	20	60.1	77
	SSR27477_R	TATGCTACGCCGGCACTAA	19	60.4	
49	SSR27743_F	ATGTTAAATTTTCTATGTGGCAACT	25	57.5	90
	SSR27743_R	GCATAAGAAAGGATGGGCATA	21	59	
50	SSR28482_F	TCATTGTTGTACCTTTTTAGTTCC	24	56.6	86
	SSR28482_R	TCTGAAAGAAAGGTAACAAGAGC	23	57	
51	SSR3084_F	TCGATGCATCAAACCAAATAAT	22	59.3	81
	SSR3084_R	TTGTTTGTTCATTGCTATCCATT	23	58.6	
52	SSR4930_F	AAGTGATCGATCCCATCCTA	20	57	79
	SSR4930_R	CCTCAAAGCACGCACGTA	18	59.6	
53	SSR6099_F	AACGGGACATCCGGAGAG	18	61	86
	SSR6099_R	TCGAAGACGACGCACTTG	18	59.7	
54	SSR7483_F	GACGTGGCAGCCTGTACTCT	20	60.5	86
	SSR7483_R	ATTCCGGACGAGCACATCT	19	60.6	
55	SSR11492_F	ATGTGAAGATCGCCGTTTC	19	58.7	94

	SSR11492_R	CGAAAATGAAGGGAGAGAATG	21	58.8	
56	SSR11597_F	AGGCTCCGATCCCCTTC	17	60.1	75
	SSR11597_R	GAGTGCTCATGCAGCCTACC	20	61	
57	SSR11655_F	GGAGCATTCACATACAAACTCCT	23	59.5	93
	SSR11655_R	CATGCACGCACCTCACTC	18	60	
58	SSR15463_F	CTTCCGGCAAGTCACCTC	18	59.3	85
	SSR15463_R	GCCGATCCTAGGCTACAACA	20	60.2	
59	SSR16015_F	TGAGGTGGTTCTCACCTAATGA	22	59.6	91
	SSR16015_R	GCCTTCCACCTCCAGTTCC	19	62	
60	SSR17002_F	AGTTGACCCCAATGGACG	18	59.3	93
	SSR17002_R	ATCAAGGGAGAGAAGCCCTC	20	59.8	
61	SSR1952_F	CATGCATCCGTGTTACATGC	20	61	90
	SSR1952_R	GCAGGAGGAGGAGGAGAGG	19	61.4	
62	SSR21056_F	TCATTACGGGCCCAAATTC	19	60.7	82
	SSR21056_R	TTGTGCAACTTTGGACCTTG	20	59.7	
63	SSR29522_F	CCGCGACCATTTATTCAATC	20	60.3	91
	SSR29522_R	CTCCTCCTCCACGCTCAC	18	59.5	
64	SSR29525_F	AGCACGTTAGGCTTCCACAC	20	60.3	84
	SSR29525_R	GGTATGGCTGCTCGTGTCA	19	60.9	
65	SSR325_F	GAAGGAGACGACAGGACACG	20	60.9	90
	SSR325_R	GGTGGGTGCTTCTTGGTTC	19	60.5	
66	SSR5334_F	ACGTGTGTGTAGAGCATCACAA	22	59.3	90
	SSR5334_R	AGTTTCCACGGACATCTCTGA	21	59.7	
67	SSR7250_F	CTCTTGTACATGCCAATGCTCT	22	59.4	90
	SSR7250_R	ACGCTAGCTCTTTCCCGACT	20	60.5	
68	SSR8109_F	TGCCTGCATCCACTAACATT	20	59.2	86
	SSR8109_R	ACTTTCCAACCTGCCTGTATG	21	59.1	
69	SSR15826-50_F	CGTTGTTTTCCTCCGTTGAC	20	60.5	127
	SSR15826-50_R	GATAGAGGACGGCCTTGGA	19	60.2	
70	SSR21927-50_F	GCACGGGATGTCTTGTTACC	20	60.4	110
	SSR21927-50_R	ACACACATGCACACATGCAC	20	60.1	
71	SSR22548-50_F	ACAAATCCCCTCCCCAAGT	19	60.6	122
	SSR22548-50_R	TCTTTGGACCCCTCTCCTTT	20	60	
72	SSR28896-50_F	TAAATTGCCTCCACCGACCT	20	61.7	111
	SSR28896-50_R	ACGTACCACTGCTCAAGACTGA	22	60	
73	SSR17375_F	TTGGTATAAATGCTAACTGCCTAGT	25	57.7	116
	SSR17375_R	CATATGCATGCCAAGCTTCA	20	60.8	
74	SSR29525-50_F	AGGCGCCTGCAGACATAGTA	20	61	124
	SSR29525-50_R	GCCCACAGCGATGAATACC	19	61.4	

75	SSR11492-50_F	TCGTGCCAGGTCATGTGATA	20	61.1	126
	SSR11492-50_R	AATTGCAGGACTGCGAAAAT	20	59.7	
76	SSR11597-50_F	GAAGATGACTCGCGAAATGG	20	60.7	133
	SSR11597-50_R	CCGCTCTACTCGCAGATGA	19	60.3	
77	SSR15463-50_F	ATCCTGAACGTGGAGGAACTT	21	60	131
	SSR15463-50_R	CCGTGTCAAAAAGAGAGACTCC	22	60.3	
78	SSR17002-50_F	GCAGTTGACCCCAATGGA	18	60.5	112
	SSR17002-50_R	CCCTCCCCCTTTTATACATCA	21	60	
79	SSR21056-50_F	GGTTCGGCAAAATCTGTGTT	20	60	121
	SSR21056-50_R	ATCTTTACCGGGCTGAGAGC	20	60.7	
80	SSR29233_F	AGAAACAGCGAGGGAGTTCA	20	60	116
	SSR29233_R	CCAAAAACCTTTGTTTGTCCA	21	59.9	
81	SSR21696_F	GGAGCAGCAGCTGATGAGA	19	60.4	101
	SSR21696_R	AAGTATCCGCTGCCTCGTC	19	60.4	
82	SSR21789_F	GAGGGAGAAGACAAGCCGTA	20	59.4	102
	SSR21789_R	CAGTTCCGGATGCCTCTAAT	20	59.1	
83	SSR22025_F	TTTTTCCAACTTTGGGACAAATATC	25	61.45	110
	SSR22025_R	CATTCAGCCACCATATAAGTTTCA	24	60.3	
84	SSR3784_F	GACCAGACCACCAAGGTGAA	20	61	100
	SSR3784_R	GCCGGTCACCGTCTCTCT	18	61.4	
85	SSR11107_F	CGACTGCATGGATCCTTACTT	21	59.2	130
	SSR11107_R	GCCACCAAGCTGATAGAGGT	20	59.3	
86	SSR11220_F	TTTGGAGTCTCGATGCACAC	20	59.8	118
	SSR11220_R	TCGTGCTTATATATGGACTTCATCA	25	59.9	
87	SSR276_F	TGTTTCTATGTTTCACGCACA	21	57.8	119
	SSR276_R	TTGATCTATTATTTGTCAACTTTAACC	27	56.4	
88	SSR15122_F	GACCATCAATATACTTGTTTTGCAC	25	58.9	116
	SSR15122_R	GGATCGCTTTGGTTATTGGA	20	59.9	
89	SSR23382_F	TCCTTCCACTCCTCTCCTCTC	21	59.9	111
	SSR23382_R	TGTGAGCATCTATGTGTTGTACTATTT	27	58.4	
90	SSR1084_F	GGCCCCCATCTAAATCAATA	20	58.7	125
	SSR1084_R	AGATGCAAGGGAGAGACTGC	20	59.6	
91	SSR2503_F	ACCTGGTACTGCAACCGTCT	20	59.6	116
	SSR2503_R	TCTCTCTAGTCTCTCTGAACTGAGC	25	57.9	
92	SSR4343_F	CTGAGACGCTGAGAGAGCTG	20	59.2	120
	SSR4343_R	CTTCTTCGGAGTTCCCACTG	20	59.8	
93	SSR16013_F	GAGGCGGCAACAATGATTA	19	59.6	122
	SSR16013_R	CGCTTGATAATGACGGAAGG	20	60.6	
94	SSR17792_F	TCCTACAAGACAATCAAATCTGGA	24	60	115

	SSR17792_R	GGGCACACAATTGAGTTCAGTA	11	60	
95	SSR21846_F	AGTCCTCGCCTGCCTCTC	18	60.7	100
	SSR21846_R	TCCGGCTCTCTCCAGTCC	18	61.5	
96	SSR23060_F	GGCCTCCGACGAGGAATA	18	61.1	110
	SSR23060_R	ACTAGATCCACGGAGCAGGA	20	59.8	
97	SSR285_F	TTGCTGCAAGTCCATATTGC	20	59.8	102
	SSR285_R	CAGAATCGAGTTCTCCCATTTC	22	60.1	
98	SSR557_F	TGCTTGCTTGTCGCAGAG	18	60	122
	SSR557_R	GTACACGCTGCCCCTCTC	18	59.4	
99	SSR4041_F	AATTTGAAGGAGAATAAGCATACACA	26	59.5	110
	SSR4041_R	CTTTTGTATCTCAATTTTGGATGTTG	26	60.2	
100	SSR9747_F	GGACACGCATCGTTCAGAT	19	59.7	116
	SSR9747_R	AACAAGACAATTTGAGATGTGCTC	24	59.7	
101	SSR11688_F	ACCCGGGTGTAGGAAAAACT	20	59.7	100
	SSR11688_R	AGTTAGATGACAACTTTCTTCCCATT	26	59.9	
102	SSR12178_F	CCAGGGTTTATGGTGTCATT	20	57.2	129
	SSR12178_R	GCAGATCCGTTTCCACTATTT	21	58.2	
103	SSR14922_F	CCAGCCAAACTGTTCTGTGT	20	58.8	112
	SSR14922_R	TCGTAATGTTTGCTAGTTCTTCTGT	25	58.6	
104	SSR22821_F	CCCGTCGAATATTTTTCTGTC	21	58.6	118
	SSR22821_R	GGAAGACCCCCACTACAGAC	20	58.4	
105	SSR484_F	CAAATCTACAGAGAATGGGGAGA	23	59.6	112
	SSR484_R	CTCTCTGCCTGAGCCAAAAC	20	60.1	
106	SSR8347_F	CTCCGCCTCCTCTGCTTC	18	61.2	115
	SSR8347_R	CTTCCTCTCTCCTTCGCTGTC	21	60.7	
107	SSR11389_F	GAGCCCCTACACCGCTAGA	19	60.4	118
	SSR11389_R	CCAAATCTCCAAACCCTAATTG	22	59.7	
108	SSR12233_F	GGCTCCTCTTCTCAGTGCTC	20	59.3	122
	SSR12233_R	TTCTGTGCTGCTGCTGCT	18	59.6	
109	SSR14859_F	ATCGAGTGCACCAGGGTTAC	20	60	124
	SSR14859_R	TCTTCCCCCTATAGTCTGGTCA	22	60	
110	SSR16622_F	GCAGCCGGAGGATGGAC	17	62.9	119
	SSR16622_R	TTAGCCGGTTCACCACCTC	19	61	
111	SSR20919_F	TCTTGGGGCAAGAACAATG	19	59.6	113
	SSR20919_R	GGGATGTAAAATGTCATGATGC	22	59.2	
112	SSR23390_F	GCCTCCACATCCAAGAATGA	20	61	112
	SSR23390_R	TTGATGAGGATGGAGGCTTG	20	61.1	
113	SSR30606_F	CACGAACACGCACGAAGG	18	62.5	111
	SSR30606_R	CCAGGGCCGATCGAATAG	18	61.5	

114	SSR642_F	CCTATCTTCTTTCTCTACTCGAGTGTC	27	59.6	111
	SSR642_R	CGAGCACCGCTACGAGTT	18	60.2	
115	SSR1709_F	CTCGCGCCAAAGAAGAGG	18	62.2	118
	SSR1709_R	CCCGGGCTTGAATTGAC	17	60	
116	SSR2902_F	CGTTGCTGGCTGACTACG	18	59.1	100
	SSR2902_R	GGCTTCTGCCTCTCTCCTCT	20	60.2	
117	SSR4126_F	TCTTTGGTCAGCCCTTTCAT	20	59.7	121
	SSR4126_R	AGAGATGGAGTGGTTGTGTCCT	22	60	
118	SSR9654_F	GACGAGCACGGAGCACAT	18	61	92
	SSR9654_R	TACGCGCACAGAGGGAGA	18	61.7	
119	SSR10241_F	GCAACCACGTTCCCATTTAG	20	60.4	121
	SSR10241_R	TGCTTGATGGATTATGCAAGG	21	61	
120	SSR11437_F	GGAGCACGAAGAAGAAGAGG	20	59.2	111
	SSR11437_R	GGTGTCATGGCCTTCTACCT	20	59	
121	SSR16574_F	ACCAGTCCAACCCAGCCTA	19	60.5	101
	SSR16574_R	GGCGTCTCAGCTCATTGC	18	60.7	
122	SSR29919_F	AAGCTGCTGGGGTGAGGT	18	60.8	102
	SSR29919_R	TTGAACAAGTTATTGGTGAAATGC	24	60.3	
123	SSR491_F	CCTGCCACTCGCAAACAC	18	61.5	100
	SSR491_R	CAATAATTCGGGGCCAGTT	19	59.8	
124	SSR1754_F	TCTGTGATGTCAACCTTTTTAGAG	24	57.6	114
	SSR1754_R	TCATACAGAGACCCAAACACG	21	58.6	
125	SSR3453_F	CAAAATACTCCCCACCCTAGAGA	23	60.7	100
	SSR3453_R	CGCAGGAACTGCTTCCAC	18	60.6	
126	SSR3497_F	AGAAGCCACTCTCAGCCACA	20	61.2	110
	SSR3497_R	CGGAGTAAACAGAAAAGGAGCA	22	60.8	
127	SSR4319_F	TGGGTGAGGGAAAGAGAGAG	20	59.4	125
	SSR4319_R	TTCACCTTCCGCGTCTAGTAT	21	58.9	
128	SSR4327_F	AGGATTTGGTTTTCCTCTTCG	21	59.6	111
	SSR4327_R	ACAGCCTCGGAGCGAACT	18	61.1	
129	SSR4444_F	CAGCTTTGAAATCAGTTATCAGTCA	25	59.8	110
	SSR4444_R	AGGGATCGGAATGGGGTC	18	62	
130	SSR9951_F	CGTCTCTGCATCGCCTATC	19	59.5	110
	SSR9951_R	CATCACCTCACCGAGCAAG	19	60.4	
131	SSR10028_F	GGAGGAAGAATCCATGGAGAA	21	60.4	104
	SSR10028_R	CCCGCTCTTTTCCATTCC	18	60.5	
132	SSR10212_F	GTGAAACACGACACTCCTTCC	21	59.6	110
	SSR10212_R	TCTTTCTGAACCATTGTTATCTGAG	25	58.9	
133	SSR14983 _F	AGCCCGGGAAGCTGAACT	18	62.3	116

	SSR14983 _R	GACGCCGTTAGTACCCAACC	20	61.7	
134	SSR16541_F	CCTCCCGATCCCATCTCT	18	60	114
	SSR16541_R	ACCCTCAAATATTCCAGAGAGAAA	24	59.5	
135	SSR17074 _F	ATCTCCTTCACCGCTCTCTG	20	59.6	111
	SSR17074 _R	TTTTAATCTACGGTACCTAATATGTGAT	28	57.1	
136	SSR17201_F	CGACGAAGAACTAGCCAAGG	20	60	119
	SSR17201_R	CGCAGCATGGACAAAAACT	19	59.9	
137	SSR21897_F	TGTATGCAGGTCATCCGTCT	20	59.1	110
	SSR21897_R	ATTTGCGGTGGTTTGCAC	18	60.5	
138	SSR22114_F	CCTTCGGAACGGGAGAAG	18	60.7	91
	SSR22114_R	TCTCCCCCTGCCCTTTCT	18	62.1	
139	SSR25306_F	TGTTGCAGCACAAATATAAATTGAC	25	60.3	112
	SSR25306_R	ACCTTGTCAGCTTGCTAATGC	21	59.5	
140	SSR29269_F	ACCACCAACGGCAACAAC	18	60.4	107
	SSR29269_R	TCATCACATGATTGCATGCTT	20	60.1	
141	SSR1349_F	TGAGACCACATCATCTTCTTGC	22	60.3	116
	SSR1349_R	GCGAGAGGAGTTGAGAGCAG	20	60.4	
142	SSR1646_F	TTCATCTGTTGCCAATATACATCA	24	59.4	111
	SSR1646_R	GGGTAGGAATGAGGAATCCAA	20	60.1	
143	SSR1853_F	GTCTGCTTCCTTGCTTTGCT	20	59.8	100
	SSR1853_R	GCTCTCCCTCTGGATCTGG	19	59.9	
144	SSR2759_F	CAACATTGCCGGTGATATTTT	21	59.7	110
	SSR2759_R	TTCCTATGCATTTGCTTGGTT	21	59.6	
145	SSR2842_F	TTCAGTGATCATGTACCGAATTG	23	59.9	90
	SSR2842_R	AAAAAGATAGAGAACAGAGAAGAGCTA	27	57.2	
146	SSR2995_F	TATTTCTGGGTGCAGGGATT	20	59.4	78
	SSR2995_R	CACAACTCGTACACGCAATG	20	58.8	
147	SSR10050_F	TGTTGCTCAAGTTCTTGCATAC	22	58.1	111
	SSR10050_R	GCCTCCTCCTGAAGAGAACA	20	59.5	
148	SSR10992_F	CAGTCGGCCTTCCATTCTATT	21	60.5	101
	SSR10992_R	GAGGCAGACACCCCCAAC	18	61.5	
149	SSR16288_F	GGTTCATCCGACCCTTTG	18	58.9	110
	SSR16288_R	GTATAGCAGGAAACATTATAAGTAGGG	27	57.3	
150	SSR16906_F	TGCCCAAAGTGAAGATACCA	20	59.1	117
	SSR16906_R	GATTTGACACATGAAAACTCGTTA	24	58.2	
151	SSR16948_F	AGGGGGAGCGGGTTAGAC	18	61.4	91
	SSR16948_R	CCTCCCTACTCTCCACCTCCT	21	61	
152	SSR17857_F	TTTGTATCTCCTTTTGTTTGCTACT	25	57.8	110
	SSR17857_R	GCATGCATACATAATTACACAGATT	25	57.3	

153	SSR19053_F	GCTCATGCAATAGTTTTTATATGTGG	26	60.2	81
	SSR19053_R	CCCTAGAATAGCGTGTGCGTA	21	60.3	
154	SSR22572_F	CTTATCTTCCCGGTCCCTCT	20	59.5	101
	SSR22572_R	AAAAACAAATGGGAAAGTTGC	21	58.2	
155	SSR23133_F	AATGTGACGCCCAGATCC	18	59.4	111
	SSR23133_R	GTGTTGCTTTCCGCGATAC	19	59.3	
156	SSR30021_F	CAATAGATTGGCACGGTTTATG	22	59.4	122
	SSR30021_R	AACAGCATTTTCAAATAATAAAAGGTA	27	58	
157	SSR1828_F	TCATGATCTACATTTAAGAATGAAAAA	27	57.6	112
	SSR1828_R	AACGCTGAACGAACACTCAA	20	59.5	
158	SSR2442_F	CTCGCCTCACCTCCATTC	18	59.3	103
	SSR2442_R	CCATTCGGCTCATCAAGTG	19	60.2	
159	SSR3231_F	CTAGAAATCGCCGGGTAAGA	20	59.3	110
	SSR3231_R	TCAGATCATCGATATGCTCGTT	22	59.7	
160	SSR9943_F	GCCTCCCTCCATCCTCTC	18	59.7	83
	SSR9943_R	CTGGTGGGGAGAGCAGATAA	20	60.2	
161	SSR10668_F	ACCAAAAAGCCATCACAACC	20	59.8	102
	SSR10668_R	ACGGACGTACATGCTCTGG	19	59.7	
162	SSR16601_F	AGCGGACGACAAACGAAA	18	60.4	90
	SSR16601_R	CTTTTATATCTCAAGGGGGAAGG	23	59.4	
163	SSR16755_F	TTCTTATATCAATTGCACCTTGTTG	25	59.5	118
	SSR16755_R	TCAGTCACTCCGAGAAAGCA	20	59.7	
164	SSR19047_F	ACCCTACCACAATCCAGCAC	20	59.9	116
	SSR19047_R	TTTCTTGAACACGTAGGAGAACTG	24	59.9	
165	SSR19246_F	ACAGCACAGGGCTAAAGGAC	20	59.4	115
	SSR19246_R	TTGGGAACGGATAGAGTATCG	21	59.1	
166	SSR23320_F	GCGATGGTGGATCAAGTTTT	20	59.9	111
	SSR23320_R	CGCCACCTATTGGATGCTA	19	59.7	
167	SSR29418_F	TCTCCTGCAGATGAGACCAC	20	58.9	113
	SSR29418_R	AATAAAGTGGAGATGAAGAAACTAGC	26	57.8	
168	SSR1445_F	CGAATGTAGCTGATATGTGAAACC	24	59.9	121
	SSR1445_R	CACGGAATGGTTTGAATGG	19	59.8	
169	SSR2502_F	AAGAACAAGGGTTTCAAAGTCAA	23	59.2	110
	SSR2502_R	TTCCATTCCAACTGAATGTCC	21	59.8	
170	SSR3028_F	CGTGGGGGAAAGAAATTTTG	20	61.5	91
	SSR3028_R	GGAAAGAAAGCGCAATCACA	20	61.3	
171	SSR3033_F	TAATTTGTAGCAGTAAAATGAAATGTG	27	58	110
	SSR3033_R	ATGCAGGGGTGTTTTCGT	18	58.9	
172	SSR3068_F	CGCACCGTATAGGCACCAT	19	61.9	100

	SSR3068_R	ATTCGTGAGAAGGATGGAGTTGT	23	61.3	
173	SSR3790_F	CTTTTAGTTGAGATCACTAGTTTTGG	26	57.1	115
	SSR3790_R	GAATAGCCTCTATGTCTGTATAGGTCT	27	57	
174	SSR15230_F	CAGTATGTGTTGAGGAGATGGTG	23	59.5	112
	SSR15230_R	TACTTAGGGCAGTCCCAACC	20	59.1	
175	SSR15446_F	CAAGCTCGCCACAAGGTC	18	60.6	71
	SSR15446_R	GGGGTTCGCAATTTCTATGAT	21	60.2	
176	SSR16466_F	TCGGTGGGGGTAAGAAGTG	19	60.9	110
	SSR16466_R	GATGCTGGAGCCTCGTAGTG	20	61	
177	SSR17198 _F	CCCCTCTCCCTTTTATACCTCA	22	60.6	100
	SSR17198 _R	CGGACCCCAGAAGTCGAT	18	61	
178	SSR17356_F	ATTGTTGAGCGGCATACACA	20	60.1	113
	SSR17356_R	AGACTCGAGCGATGGTGATT	20	59.8	
179	SSR17841_F	CGCCTTGGATAGCTTGCTT	19	60.5	121
	SSR17841_R	GGACAAATCATACATACCTTTTCCA	25	60.3	
180	SSR18105_F	GCAAGGGAAGGCATGTTAAA	20	60.1	71
	SSR18105_R	TCATCTCCTTTGCATCTCCA	20	59.3	
181	SSR238 _F	GACTTGGACTGTTATATTGGACTGA	25	58.6	110
	SSR238 _R	AAGACTCCAAAGAGATCAAGGAG	23	58.2	
182	SSR629_F	GCGTTGCAGAGAAACAGAGAC	21	60.2	114
	SSR629_R	TACGGGCGGATTGGACTT	18	61.4	
183	SSR933_F	ACAAAAATCAGAGCCGCAAT	20	59.7	111
	SSR933_R	CTCGCTCCCATGAAATAAAAA	21	57.2	
184	SSR1073_F	CCGCTGATTTCAAGATGGAT	20	60	115
	SSR1073_R	AGGCTGCTGCATGATCAAA	19	60.5	
185	SSR2330_F	GTGGCGCGTCTGTTCACT	18	61.1	110
	SSR2330_R	AAATACAACAAGCTAGGCTTAACAGC	26	60.5	
186	SSR2590_F	TTCTACCACGAGACCAGTGTGT	22	59.7	110
	SSR2590_R	TTTGTGTGGTCCAGCTCTCC	20	61.3	
187	SSR2683_F	GAAATGCTCCCTTCTTCACG	20	59.8	115
	SSR2683_R	GCGAAGTTTGTTGCTGAATG	20	59.5	
188	SSR2839_F	GGCGACGGCAACTTGTTT	18	62.2	100
	SSR2839_R	CACCCGGACACCAATGAG	18	61	
189	SSR3298_F	TCACCAGAGGACGCATAAGA	20	59.4	100
	SSR3298_R	TAGTATCTTTTCACAGTAGATGTGTGG	27	58	
190	SSR10269_F	CGGGGGTTAGTCTTTGACAG	20	59.6	100
	SSR10269_R	ATAAATTCCGCCGGTTGAG	19	59.9	
191	SSR12866_F	CAAGGGCAAGATATAAGTGGAAA	23	59.5	91
	SSR12866_R	GGTACCGTGAGGGTGCAG	18	60.1	

192	SSR12940_F	TGGAGGCGCTTTTATCAAATA	21	59.7	111
	SSR12940_R	AGGAGCTAAGGTCGTTGCAC	20	58.5	
193	SSR18495_F	GAGAGGCGAGGCGAGTAAT	19	59.5	93
	SSR18495_R	TTTTGCCCTTTTCGGTTTG	19	61	
194	SSR19284_F	AGCTATTGTTTTTGGGGGAAT	21	58.9	110
	SSR19284_R	ATCAGATAGCAGTGATGGCTCT	22	58.1	
195	SSR22169_F	ATTCCGGTGGATTGTCATTG	20	60.6	116
	SSR22169_R	CACATGGAGTCACAGTCACCA	21	60.6	
196	SSR22609_F	AAAGAGTGCTTGGGCTGGT	19	59.9	100
	SSR22609_R	CAAAAATCCTAAACCTCTCCTCAA	24	60	
197	SSR29056_F	GCTTCTTTTCCTCATGACTATTGTG	25	60.5	112
	SSR29056_R	CCAGCACCTATGCCTTATCAA	21	60.1	
198	SSR404_F	GTGCTGACCCTCGAAAACTT	20	59.3	100
	SSR404_R	TCGAGATTTATCCCCAAATCG	21	61.1	
199	SSR1257_F	GTATCAGAAACACCACAAAGAATACTG	27	59.5	110
	SSR1257_R	AAAGTTATTGTTAATGCAGCGTGA	24	60.1	
200	SSR1466_F	ATCGAGGTCGTGTCGACGTA	20	61.7	102
	SSR1466_R	CGAATTCTTGGAGGCGAAAT	20	61.4	
201	SSR3378_F	ACGCCGCTGGTGAGAACT	18	62	90
	SSR3378_R	ATGCGTCATGTTGTCGGTTT	20	61.4	
202	SSR3957_F	ATTTGCCAACTACTACCTATTTTGG	25	59	90
	SSR3957_R	AAAATTAGCAATAAAGCAATGGAA	24	58.4	
203	SSR4132_F	CGAATTTCTGTCCTCAAACAAG	22	58.9	120
	SSR4132_R	TCGAGCTATGATCTTTTGAACAAC	24	59.8	
204	SSR11476_F	ACTCTATTTGTAGTGCTCCTCACG	24	59	110
	SSR11476_R	GTCCACACAGCCTCACAGG	19	60.3	
205	SSR16154 _F	CCACCATCATCAAACCAAGA	20	59.3	77
	SSR16154 _R	GATGGCACTATGGGATAGAAGG	22	59.8	
206	SSR17022_F	AAGGGTGGAGTGCTGACG	18	59.8	100
	SSR17022_R	GCATTTATGGCACTCATCCA	20	58.5	
207	SSR17987_F	TGCGTATCACTATATTAAGAAGAAAAA	27	57.1	116
	SSR17987_R	TCAGGTACAGAATTTAATCTTTTCG	25	58.1	
208	SSR18530_F	GATGAATGGTCTACGACTCTACGTT	25	60	103
	SSR18530_R	ATGGGCCCTTAGCAAGATG	19	60	
209	SSR21192_F	TAGACCCACTGATGGGGATG	20	60.7	115
	SSR21192_R	TCAGGTTATCAGGGTAACTGGGTA	24	61	
210	SSR22021_F	GCATCATAAGGCTCTGTGGA	20	58.8	111
	SSR22021_R	TCTCCCTTTTGTACCCCAAG	20	59	
211	SSR22317_F	GCCTGTCTACTCCCGCTAGG	20	61.3	110

	SSR22317_R	CAATCACTAAAATTACAAACAATGGTC	27	59.2	
212	SSR29567_F	CCCACGAAGCTACCACTACC	20	59.6	103
	SSR29567_R	AGTCCGAGCTCCACATCATC	20	60.2	
213	SSR266_F	ATTTTGTTTCATTTGGAAGGATT	23	58	110
	SSR266_R	TGGGCTAGAGATGAAAACCA	20	58.3	
214	SSR492_F	AACTGGCCCCGAATTATTG	19	59.8	110
	SSR492_R	TATTCCTGCTGCGTTGGAC	19	59.8	
215	SSR852_F	AGCATGCAACACCCTCGAC	19	62.3	105
	SSR852_R	GGCGATGATTCACAAGCAGA	20	62.3	

Appendices 2. Regression analysis of allele frequency and ploidy level.

```
DATA AlleleFrequency;
    INPUT ploidy freq;
    ploidy_sq=ploidy**2;
    ploidy_cu=ploidy**3;
    ploidy_cua=ploidy**4;
PROC REG data=AlleleFrequency;
    MODEL freq=ploidy ploidy_sq ploidy_cu ploidy_cua;
RUN;
```

Appendices 3. Analysis of variance for AULEC and AUDPC, and calculation of LS Means for each genotype.

```
PROC GLM data=PhenotypicAnalysis;
    CLASS genotype rep run audpc;
        */replace with aulec to compare dependant variables;
    MODEL audpc = genotype run rep genotype*run genotype*rep  run*rep
    genotype*run*rep;
    RANDOM genotype run rep genotype*run genotype*rep  run*rep
    genotype*run*rep;
    TEST h= genotype*rep  e=genotype*rep*run;
    TEST h=genotype*run  e=genotype*rep*run;
    TEST h=rep*run  e=genotype*rep*run;
    TEST h=genotype e=genotype*rep*run;
    TEST h=rep e=genotype*rep*run;
    TEST h=run e=genotype*rep*run;
    LSMEANS genotype;
RUN;
```