ABSTRACT

KIMBALL, JENNIFER ANN. Breeding for Cold Tolerance in St. Augustinegrass using Conventional and Molecular Methods. (Under the direction of Drs. Susana Milla-Lewis and Thomas G. Isleib).

St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntz) is a warm-season grass species commonly utilized in the turfgrass industry for its superior shade tolerance, stoloniferous growth habit, and moderately low input requirements. However, winter survival is a major limiting factor for the species. Additionally, the most cold-tolerant cultivars often lack the aesthetic characteristics, such as a semi-dwarf growth habit and fine leaf texture that are desirable in the market. Therefore, new St. Augustinegrass cultivars with improved cold tolerance and desirable turf quality are needed for the turfgrass industry, especially in the transitional climatic region of the United States.

In order to efficiently utilize sources of cold tolerance in a breeding program, an understanding of the genetic control of this trait and its relationship to important turf quality traits is required. Six diploid genotypes of St. Augustinegrass were selected as parents for a diallel mating design without reciprocals. Combining ability analysis revealed that both general and specific combining abilities were significant across years and locations. Specific combining ability was the largest source of genetic variation for winterkill, genetic color, turf density, and end-of-season cover indicating that non-additive gene effects play a key role in the inheritance of these traits. Lines identified as parental selfs generally showed lower winter survival and inferior turf quality than the original parental lines indicating that inbreeding depression can occur in St. Augustinegrass.

In addition to field studies, lab-based freeze tests mimicking field winter survivability can contribute to the selection of cold hardy lines. A whole plant freeze method was used to

evaluate four freezing temperatures and two data collection systems in freeze tests of nine St. Augustinegrass genotypes. Results indicated -3°C and -4°C to be more suitable evaluation temperatures than -5°C and -6°C. Survival and regrowth were correlated with one another over a six week evaluation period post-freeze. Digital imaging techniques utilized in turfgrass field studies were shown to be useful in estimating survival and recovery in lab-based freeze tests. Additionally, the effects of cold acclimation and deacclimation on genotypes were evaluated. Accounting for all levels of acclimation provided excellent cultivar separation at -3 and -4°C freezing temperatures and supports the hypothesis that the inclusion of different acclimation responses offers the best overall assessment of freeze tolerance in St. Augustinegrass. Results also indicated that cold acclimation and deacclimation both play crucial roles in the winter survivability of St. Augustinegrass.

The first complete linkage map for St. Augustinegrass was constructed for cultivars ‘Raleigh’ and ‘Seville’ using a pseudo-F_2 strategy. A total of 178 simple sequence repeat markers were mapped to nine linkage groups covering a total distance of 1299.95 cM. Quantitative trait loci (QTL) controlling winter survival, freeze tolerance, and turf quality traits were mapped. Putative QTL were identified for all traits across multiple environments with the exception of winter survival, where QTL were only identified in single environments. Specific genetic regions were found to have overlapping QTL between winterkill and spring green-up, as well as overlapping QTL between cold tolerance traits collected in the field, and survival and recovery traits collected in the laboratory post-freeze. These results provide strong support for cold tolerance-related QTL in these regions. A large region on LG5 was also identified as a possible region for a large sweep of freeze tolerance and cold acclimation QTL.

Breeding for Cold Tolerance in St. Augustinegrass using Conventional and Molecular Breeding Methods

by
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BIOGRAPHY

Jennifer Ann Kimball was born in 1984 in Corning, NY, a small town in the Finger Lakes region of the state. After high school, Jenny attended Ithaca College to study biology. Upon graduation from Ithaca College, Jenny worked for Dr. Susan McCouch at Cornell University as a research assistant and eventually, as her lab manager. In 2011, Jenny received her Master of Science in the Department of Crop Science at North Carolina State University under the direction of Dr. Susana Milla-Lewis.

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Literature Review

ST. AUGUSTINEGRASS

St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze) is commonly utilized for its superior shade tolerance, stoloniferous growth habit, and moderately low input requirements when compared to other more popular turf species, *i.e.*, tall fescue (*Festuca arundinacea* Shreb.), bermudagrass (*Cynodon* spp.), and zoysiagrass (*Zoysia* Willd.). Conversely, St. Augustinegrass is limited in its northern range of adaptation due to its inferior cold tolerance in comparison to zoysiagrass and the cool-season species commonly grown in North Carolina. Development of cold-tolerant St. Augustinegrass varieties would expand the market for this type of grass leading to an increase in more sustainable management practices as St. Augustinegrass requires less input (i.e. water and fertilizer) than cool-season grasses and several warm-season grasses. Aside from a need to improve cold tolerance in St. Augustinegrass, aesthetics are another important issue for this species; homeowners find the grass's long internodes, thick stolons, and coarse leaf texture somewhat undesirable. St. Augustinegrass cultivars are typically classified by their mowing height (growth habit) and their leaf texture. A dwarf growth habit, finer leaf texture, improved cold and drought tolerances, and resistance to pests are highly desired in new cultivar releases. There is also a need within the breeding community for a better working knowledge of the underlying genetics, including causal polymorphisms, conferring cold tolerance as well as other agronomical important morphological traits in St. Augustinegrass.

Indigenous to the coastlines of east Africa and south Pacific islands, the genus *Stenotaphrum* is comprised of seven species (Sauer, 1972; Busey, 1995) though only *S. secundatum* has been widely cultivated for turf. While species genome compositions are

largely unknown, five different ploidy levels have been reported for *S. secundatum*, including diploids (2n=2x=18), triploids (2n=3x=27), tetraploids (2n=4x=36), hexaploids (2n=6x=54), and aneuploids (2n=28-32) (Long and Bashaw, 1961; Milla-Lewis et al., 2013a). St. Augustinegrass cultivars, 'Floratam' and 'FX-10', were the first reported instances of aneuploidy within the species (Busey, 1979). More recently, additional aneuploid genotypes with chromosome numbers ranging from 2n=28 to 2n=32 have been identified using flow cytometry (Milla-Lewis et al., 2013a; Mulkey et al., 2013b). Busey (2003) hypothesized that polyploidy in *Stenotaphrum* originated sporadically and by different mechanisms. Milla-Lewis et al. (2013a) hypothesized polyploidy originated by interspecific hybridization between the African diploids and Pembagrass (*S. dimidatum* (L.) Brongn.), a species closely related to St. Augustinegrass (Sauer, 1972). Introgression between the species has been reported (Busey, 1993; 1995) and would explain the various levels of aneuploidy, abnormal chromosome pairing behavior during metaphase (Busey, 1979; Milla-Lewis et al., 2013a), and self-incompatibility within the species.

Within St. Augustinegrass, germplasm has been classified into "groups" and "races" based on morphological and performance traits including stolon and stigmata characteristics and leaf color (Busey, 1986; 1995; 2003; Busey et al., 1982), as well as the geographical distribution of the species. Other descriptors such as tolerances to temperature (Philley et al. 1998; Reynolds et al. 2009; Li et al. 2010), drought (Sifers and Beard 1999), diseases, *e.g.*, gray leaf spot (Busey et al. 1982; Milla-Lewis et al., 2010), and pests, *e.g.*, chinch bugs (Busey 1986, Chandra et al., 2013) have all been used to evaluate St. Augustinegrass germplasm. Recent genetic diversity analyses of St. Augustinegrass germplasm using amplified fragment length polymorphism markers (AFLPs) (Milla-Lewis et al., 2013a; Mulkey et al., 2013b) and

simple sequence repeat markers (SSRs) (Mulkey et al., 2013a) confirmed previous morphological classification. Ploidy levels largely determine the grouping within this classification system. Diploid genotypes belong to the Breviflorous and Longicaudatus races and polyploids are classified into the Bitterblue and Floratam groups. Also, Mulkey et al., 2013b found the number of observed AFLP fragments increased with ploidy level, indicating higher levels of genetic diversity is present within higher ploidy levels in St. Augustinegrass. While most present-day cultivars are diploid, new successful tissue culture methods for embryo rescue in St. Augustinegrass (Genovesi et al., 2009) could increase the use of polyploids in cultivar development offering new sources of genetic diversity.

TURFGRASS BREEDING

Trait Improvement. Warm-season turf species are grasses that grow well in warmer climates, tolerate relatively low mowing heights, and are generally considered to have lower input requirements. The species vary in morphology, ploidy level, and tolerance to abiotic and biotic stresses. They also differ in their utilization across different sectors of the industry, which drives the focus of breeding efforts for each species. Bermudagrass *(Cynodon dactylon* (L.) Pers.) is widely grown on athletic fields and golf courses as the species has fine leaf-texture, is able to withstand very low mowing heights, and quickly recovers from wear injury. Zoysiagrass *(Zoysia japonica* Steud. and *Zoysia matrella* (L.) Merr.) is commonly used for athletic fields, golf courses, and home lawns. It is particularly popular within the transition zone of the United States because of its good cold tolerance relative to other warm-season turf species. Seashore paspalum (*Paspalum vaginatum* Swartz) has excellent salinity tolerance and

is frequently used on golf courses and home lawns along coastal regions of the southeastern United States. Coarser-textured grasses like St. Augustinegrass *(Stenataphrum secundatum* (Walt.) Kuntze) and centipedegrass *(Eremochloa ophiuroides* (Monro) Hack.) are commonly grown in home lawns, public parks, and commercial landscapes due to lower maintenance requirements and ability to outcompete weeds.

The transition zone is a particularly unique geographical location in the United States with weather extremes in both summer and winter months. Though both cool- and warm-season turfgrass species can be economically grown here, neither type is completely adapted to year-round conditions, making this zone one of the most difficult regions in the U.S. to grow quality turf. Variable seasonal temperatures consistently pose a large problem for turf in the transition zone. Cool-season turfgrasses are not adapted to the southern-like summers which can cause drought and heat stress, leading to watering and maintenance issues. Despite these limitations, appealing leaf texture and color make cool-season turfgrasses widely popular in North Carolina (47.8% of total turf area, single family dwellings contributing 44.5%) (North Carolina Turfgrass Survey, 1999). Warm-season turfgrasses often suffer during cold winters which can cause dormancy, injury, and death. However, these grasses often thrive during summer months while having lower water and fertilizer requirements. Despite these strengths, many warm-season species lag far behind cool-season species in production and popularity in North Carolina mainly due to the lack of seed based production, slow establishment rates, and limited cold tolerance.

Newly improved commercial varieties developed by turf breeders include finer textured turfgrass and scores of improved tolerances to environmental stresses. For golf and sports facilities, improved varieties have lower mowing heights and better wear tolerance. For home

lawns and commercial landscapes, improved varieties are more cosmetically appealing to home owners and businesses. Of course, to ensure that variety improvement continue, genetic diversity is key. Natural existing variation found in turfgrass species can be exploited by turf breeders to improve varieties for commercial use. However, capturing natural variation can be problematic in certain turf species. For example, many species, *e.g.*, St. Augustinegrass, have limited available public germplasm. Other species, such as centipedegrass, have very limited genetic variation within germplasm stocks. Collection trips and other means of obtaining genetic diversity, *e.g.*, mutagenesis, will need to be undertaken for these types of turfgrass species to ensure adequate diversity for breeding improvements.

Molecular Marker Use in Turf Breeding. A number of DNA-based molecular marker systems have been applied towards breeding purposes, 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, characterize germplasm, and analyze diversity within a germplasm pool. Additionally, molecular markers can be used to construct 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.

There has been a push in the last two decades to begin utilizing molecular markers in turfgrass breeding. A greater pool of molecular markers for different turf species could aid in QTL identification, marker-assisted selection, and association mapping. Despite the wide range

of molecular markers available for use in crop species, only a few are commonly applied to turfgrass. Cool-season grasses have been leading the way in genetic analysis and molecular marker development, particularly SSRs (Mian et al. 2005; Saha et al. 2005; Wang et al. 2009). Tall fescue (*Festuca arundinacea* Schreb.), perennial ryegrass (*Lolium perenne* L.), Kentucky bluegrass (*Poa pratensis* L.), and creeping bentgrass (*Agrostris stolonifera* L.) are cool-season grasses that have been evaluated using molecular markers to assess their genetic diversity, phylogeny, and genetic makeup as well as for QTL mapping and marker-assisted selection (MAS). Molecular markers have also been used in warm-season turfgrasses, particularly in bermudagrass, seashore paspalum (*Paspalum vaginatum*), and zoysiagrass (*Zoysia* sp.). Other warm-season turfgrasses, such as centipede and St. Augustine grasses, have limited available genetic information and little is known about the genetic relationships and diversity of these turfgrasses.

Germplasm Characterization and Diversity Assessments. One of the first steps in developing a breeding program is understanding the genetic diversity and population structure in available germplasm collections. Common molecular marker types for these types of studies in turfgrass include AFLPs, RAPDs, RFLPs, and SSRs. For example, tall fescue's phylogeny, genetic diversity, and population structure have been well-studied using RFLPs (Xu et al. 1994; Busti et al. 2004), AFLPs (Mian et al. 2002; Flajoulot et al. 2010), and SSRs (Xu et al. 1994; Mian et al. 2005; Flajoulot et al. 2010). In perennial ryegrass, SSRs from cereal crops and tall fescue have also been successfully transferred for use in perennial ryegrass (Jones et al. 2001; Saha et al. 2004; Saha et al. 2006; and Sim et al. 2009). Characterization of available cultivar germplasm stocks using AFLPs, RAPDs, and SSRs have revealed strong relationships between cultivars implying that diversity within perennial ryegrass may be limited (Huff 1997;

Roldan-Ruiz et al. 2000; Guthridge et al. 2001; Kubik et al. 2001). In Kentucky bluegrass, genetic diversity studies using RAPDs revealed high levels of diversity within and among cultivars (Curley et al. 2004). In creeping bentgrass, numerous molecular marker techniques, including RAPDs, AFLPs, EST sequences, and SSRs have been used for germplasm identification (Golembiewski et al. 1997; Caceras et al. 2000; Vergara et al. 2004; Rotter et al. 2007; Kubik et al. 2009).

Bermudagrass is one of the most thoroughly genetically studied warm-season turfgrasses. Many techniques have been used to evaluate bermudagrass germplasm diversity, including DNA fingerprinting (Caetano-Anolles 1998; Anderson et al. 2001; Yerramsettuy et al. 2005), and AFLP (Zhang et al. 1999), RAPD (Roodt et al. 2002), and most recently SSR analysis (Karaca et al. 2002). Two AFLP studies showed that bermudagrass samples cluster by ploidy level and geographical distribution (Zhang et al. 1999; Wu et al. 2005), which is similar to results found for St. Augustinegrass using AFLPs (Milla-Lewis et al., 2013). St. Augustinegrass and centipedegrass have an extremely limited amount of genetic information available. Both AFLPs and SSRs have been used to evaluate St. Augustinegrass (Mulkey et al., 2013a; Mulkey et al., 2014) and SCRAs have been used in centipedegrass (Milla-Lewis et al., 2013a). In zoysiagrass, phylogenetic relationships of the different *Zoysia* species have been disputed in several publications using several different molecular marker systems (Yaneshita et al. 1997; Caetano-Anolles et al. 1998; Anderson 2000; Tsuruta et al. 2008; Chen et al. 2009; Tsuruta 2011; Kimball et al., 2012, Kimball et al., 2013). The continuum of overlapping morphological variation and the lack of genetic structure between the different *Zoysia* species seems to indicate a high level of admixture within zoysiagrass.

Marker-Assisted Selection and Association Mapping. To date few turfgrass breeding

programs have made use of molecular markers for marker assisted selection (Roldan-Ruiz and Kolliker, 2010). While MAS is commonly mentioned in QTL mapping studies, its application has not been well documented. For most turfgrass species, there is a lack of available molecular markers to conduct large-scale association mapping studies. However, as sequencing technologies become cheaper, it is to be expected that more molecular markers, particularly SNPs, can be developed and these types of studies can proceed.

LINKAGE AND QTL MAPPING

The application of molecular markers towards linkage and QTL mapping in turfgrass species has been gaining ground in recent years. Mapping studies using molecular markers leading to the development of genetic maps can serve as important tools for breeding and population development. The majority of mapping 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).

Classical Linkage Mapping. Classic linkage analysis has been used for over a century to ascertain gene arrangement on individual chromosomes of an organism. Deviations from independent assortment reveal linkage and can be used to calculate genetic distances based on co-inherited traits or genetic markers and ultimately, generate a genetic-linkage map. Independent assortment deviations are determined by calculating recombination frequencies, which determines the magnitude of linkage within a particular species. For example, maize (*Zea mays* L.) has considerably high rates of recombination resulting in high linkage

disequilibrium (LD). Soybeans (*Glycine max* (L.) Merr.), on the other hand, have lower rates of recombination resulting in lower LD, which reduces a geneticist's ability to determine the location of gene(s) affecting a particular trait with linkage mapping.

Molecular marker-based genetic linkage maps have become a very important aspect of crop improvement, predominantly in economically important agronomic species. Cereal crop breeders and geneticists, in particular, utilize molecular markers to identify genes of interest. For most warm-season turfgrass species, linkage maps are just beginning to be developed. Bermudagrass and seashore paspalum are at the forefront of this type of research with zoysiagrass following them. For St. Augustinegrass, one molecular-based linkage map has been developed in a population segregating for gray leaf spot resistance (Mulkey et al., 2013). Warm-season turfgrasses are generally vegetatively propagated and therefore, a pseudo-F2 strategy is commonly employed to combat issues within these species.

Pseudo-F_2 Testcross Strategy. Linkage maps are typically constructed using inbred populations derived from an F_1 of the cross between two highly inbred, putatively homozygous, parents. In outcrossing plants, such as St. Augustinegrass, segregating F_2 or backcross populations are rarely available due to high rates of self-incompatibility within the species, significant genetic load, and the laborious nature and time requirements for population development. Linkage mapping in outcrossing species is further complicated as non-inbred parents are highly heterozygous, markers may be co-dominant or dominant, and the linkage phase of the marker alleles is rarely known (Maliepaard et al. 1997).

The pseudo-F_2 mapping strategy employs F_1 crosses developed from heterozygous parents to construct individual linkage maps for each parent and then utilizes information from both maps to create a consensus linkage map (Grando et al., 2003). Unlike inbred populations

developed from homozygous parents whose progeny will typically segregate in a 1:1 fashion, F_1 progeny derived from an outbreeding species will segregate genetically based on the meiotic recombination from both parents may they be heterozygous or homozygous (Zhang et al., 2012). This strategy has been employed in many turfgrass species including bermudagrass (Bethel et al., 2006), creeping bentgrass (Chakraborty et al., 2005; Bonos et al., 2011; Zhang et al., 2012), ryegrass (*Lolium* spp.) (Jones et al., 2002; Warnke et al., 2004; Studer et al., 2010), and tall fescue (Saha et al., 2005).

Co-dominant molecular markers, particularly simple sequence repeats (SSRs), are very popular for linkage mapping studies because of their ease of assay, high polymorphic rates, and their ability to detect heterozygosity at a locus. However, SSR development requires sequence information, which is expensive and time consuming to generate. In St. Augustinegrass, approximately 600 markers designed from the sequencing of 'Raleigh' using Illumina platform technology are currently available (Mulkey et al. 2013). While this is a valuable resource for St. Augustinegrass, there are still an insufficient number of polymorphic SSR markers for linkage map construction. Other species with little sequence information have employed the use of both co-dominant and dominant molecular markers. Dominant molecular markers lack the ability to detect heterozygosity and are scored for the presence or absence of a particular locus. Amplified Fragment Length Polymorphisms (AFLPs) (Vos et al., 1995) are commonly used in linkage mapping to increase the marker coverage between SSRs as well as build a framework for integrating both parental linkage maps into one consensus map for the entire population. The AFLP marker system is efficient for such tasks due to the marker system's ability to generate a large number of markers within a short time (Saha et al., 2005). However, the ambiguity of dominant markers increases the complexity of linkage analysis and

consequently, the accurate estimation of recombination frequencies within and between the two different marker systems becomes very important (Alves et al., 2010).

Recombination frequencies are utilized to test for linkage and to construct both parental linkage maps or to build an integrated map for the cross. Unlike the typical segregation of a diploid, full-sib family descendent from two fully inbred parents, the segregation of co-dominant markers within a pseudo-F_2 population has the possibility of up to four alleles at a particular locus. When both parents are heterozygous at a locus, the expected segregation ratio is 1:1:1:1. In this case, the differences between the male and female recombination events can be estimated directly, rather than assumed to be equal as in F_2 populations from inbred lines (Maliepaard et al., 1997). When only one parent is heterozygous at a locus, the expected segregation ratio is 1:1 and the recombination frequencies can only be used to construct that parent's linkage map. For dominant markers, the expected segregation ratio should be 3:1 and the recombination frequencies can be used to build both parental linkage maps.

Another complicating factor in linkage analysis is that while *a priori* knowledge of a marker's linkage phase is required to detect recombination events, for crosses with outbreeders the linkage phase is unknown. Four different scenarios have to be considered: (1) alleles linked in coupling phase in one parent and unknown in the other, (2) alleles linked by repulsion in one parent and unknown in the other, (3) alleles linked by coupling in both parents, and (4) alleles linked by repulsion in both parents. The particular linkage phase combination has to be deduced using the segregation of alleles in the population itself *a posteriori* by comparing LOD scores obtained for each combination (Bhering et al., 2008).

QTL Mapping. Quantitative trait loci (QTL) mapping for important agronomic traits has been extensive in major agricultural species such as maize, rice (*Oryza sativa*), sorghum

(*Sorghum bicolor*), soybean, and wheat for numerous phenotypic characteristics associated with yield as well as tolerances to both abiotic and biotic stresses (Forster et al., 2004). In turfgrass, the majority of QTL mapping has been in the cool-season grasses, particularly tall fescue (Xu et al., 1995; Saha et al., 2005; Fribourg et al., 2009) and ryegrass (Hayward et al., 1994; Bert et al., 1999; Jones et al., 2002; Warnke et al., 2004; Yamada and Forster, 2005; Xiong et al., 2007). QTL mapping in cool-season grasses has mainly focused on disease resistance, abiotic stress tolerance, reproductive development, *i.e.*, self-incompatibility, and morphological characteristics (Forster et al., 2004). In tall fescue, forage digestibility, biomass yield, drought tolerance, and some morphological QTL have been identified using genetic maps (Fribourg et al. 2009; Saha et al. 2009). The success of molecular marker use and development in tall fescue has impacted research on other species of turfgrass as well. Molecular markers developed for tall fescue have been successfully transferred to several cool-season turfgrass species including meadow fescue, tetraploid fescue, and ryegrass for use in genetic studies (Saha et al. 2004; Saha et al. 2006). In perennial ryegrass, high-density genetic molecular marker maps have been developed and QTLs for morphological traits, winter-hardiness, and disease resistance have been identified (Jones et al. 2002a; Jones et al. 2002b; Faville et al. 2004; Yamada et al. 2004; Jensen et al. 2005; and Myulle et al. 2005). Molecular markers such as AFLPs have been used to build a linkage map in Kentucky bluegrass as well (Porceddu et al., 2002). In creeping bentgrass, several linkage maps have been built using EST sequences and SSR markers, and QTLs for disease resistance have been identified (Rotter et al. 2009; Bonos et al. 2011).

Only very recently have molecular markers begun to be implemented in genetic mapping and the identification of QTL in many warm-season turfgrass, particularly in

bermudagrass (Bethel et al., 2006; Harris-Schultz et al. 2010) and zoysiagrass (Jessup et al., 2011). Several genetic linkage maps of zoysiagrass have been constructed using RFLPs (Yaneshita et al., 1999), AFLPs (Cai et al., 2004), and SSR markers (Cai et al., 2005; Li et al. 2009). Yaneshita et al. (1999) reported five pairs of linkage groups shared a series of ordered, duplicated loci in a *Zoysia japonica* x *Zoysia matrella* mapping population indicating homology between Zoysia species. A significant number of SSRs have been developed for zoysiagrass as well (Cai et al. 2004; Tsuruta et al. 2005). Recently, the first linkage map was developed for St. Augustinegrass using AFLPs and SSRs, and markers linked to QTL conferring gray leaf spot resistance were identified (Mulkey et al., 2014).

One of the key obstacles for QTL mapping in warm-season turfgrasses is an insufficient number of Mendelian markers (Leonards-Schippers et al., 1994). The ability to predict the expression of simply inherited traits is based on the expectation of Mendelian segregation of alleles at a locus. However, segregation distortion, the deviation from Mendelian segregation, has been observed in mapping studies across all species (Li et al., 2011). Estimates of genetic distance between markers as well as the order of markers may be affected by segregation distortion. A χ^2 test can be performed for each marker to test for segregation distortion. In a study comparing an F_1 mapping population and an F_2 mapping population of diploid alfalfa (*Medicago sativa* L.), lower levels of segregation distortion were found in the F_1 mapping population; however, the authors hypothesized that segregation distortion levels were lower because molecular markers were less informative in the F_1 generation (Li et al., 2011). In creeping bentgrass, a study found segregation distortion ratios ranged from 5% to 20.5% in individual linkage groups (Chakraborty et al.,2005). While segregation distortion adds complexity to analyses, it needs to be accounted for in QTL studies by removing the distorted

markers from subsequent analyses once they are identified.

IMPORTANT FACTORS AFFECTING COLD TOLERANCE

Environmental stresses, such as drought and extreme temperatures, can have severe effects on plant growth and survival. Many factors affect the cold hardiness of a species including their response to cold acclimation, deacclimation, chilling injury, and freeze injury. Cool-season and warm-season species of turfgrass are largely characterized based on their ability to survive and thrive in a varying range of temperatures. Evaluation of St. Augustinegrass cultivar's response to winter stress in Indiana, measured as percent winterkill, revealed a range of tolerance from zero to 100 percent survival (Moseley et al. 2010). Raleigh, one of the most cold tolerant St. Augustinegrass cultivars, does consistently well in the upper range of St. Augustinegrass' northern geographic limitations. Somatic mutants of Raleigh were identified, which showed similar if not better tolerance to cold temperatures in comparison to Raleigh (Reynolds et al., 2009). Two lines, GF and GF2, had comparable cold tolerance to Raleigh and improved leaf texture. 'Seville' showed some of the lowest cold tolerance of all the genotypes assessed in the study.

Cold acclimation. Cold acclimation is a natural process whereby plant cells upregulate and downregulate various cellular components when exposed to low, non-freezing temperatures (Fowler and Thomashow, 2002). As a result, cold acclimation improves a plant's freezing tolerance. During the beginning stages of winter, seasonal changes, particularly low temperatures, signal many plant species to induce cold acclimation or hardening (Fry and Huang, 2004; Stavang et al., 2008). Metabolic modifications, including physiological and

biochemical changes, increase a plant's ability to minimize freeze injury and ultimately enhance winter survival. Overall, plants typically suffer from dehydration and have smaller, darker leaves upon cold acclimation (Ebdon et al., 2002). However, different regions of a cold-acclimated plant can be affected differently. Young leaves, for example, are hardier than old leaves or the leaf apex and typically respond better to cold-acclimation (Beard, 1973).

Cool-season turfgrasses are well known for their ability to cold acclimate and greatly improve their ability to resist lower freezing temperatures than non-acclimated plants. In perennial grasses, cold acclimation is induced by decreases in temperature, light intensity, photoperiod, and water availability (Bertrand et al., 2013). The amount of water-soluble sugars and carbohydrates maintained during cold acclimation play an important role in survival. Warm-season turfgrasses appear to vary in their capacity to cold acclimate. Studies in saltgrass (Shahba et al, 2003), buffalograss (Qian et al, 2001), zoysiagrass (Patton-Reicher, 2007) and bermudagrass (Anderson et al, 1988; Anderson et al, 1993, Anderson et al, 2003) have indicated that warm-season grass species respond well to cold-acclimation resulting in better freezing tolerance. In St. Augustinegrass, several studies have revealed variability of individual genotype's response to cold-acclimation. Maier et al. (1994b) found that Raleigh acclimates well to cold and has greater freezing tolerance than 'FX-332' and 'Floratam'. Li et al. (2010) confirmed this finding and also developed an acclimation protocol based on Raleigh's freezing response. Milla-Lewis et al. (2013a) also found differences between Raleigh and Seville's cold acclimation response. 'Floratam', however, appears not to respond to cold acclimation (Fry et al., 1991; Busey, 2003).

Deacclimation. The process of deacclimation, referring to the de-hardening of cold acclimated plants, is an integral part of cold tolerance and winter survival. Deacclimation

relates to fluctuating temperatures during winter or early spring. Warm temperatures can deacclimate plants within hours or days and the return to cold temperatures can cause significant damage to the deacclimated plant. This is particularly important in the transition zone as 22°C days are not uncommon in the middle of the winter. Svenning et al (1997) and Taulavuon (2004) showed that deacclimation is regulated by environmental factors interacting with gene expression. During deacclimation, a significant increase of water content in rye crowns was observed, and it was hypothesized that water increase also occurs in other grass species (Gusta, 1976).

Previous studies in cool-season turf species have reported both temperature and duration of the deacclimation event can affect a plant's ability to retain freeze tolerance gained from cold acclimation (Gay and Eagles, 1991; Jørgensen et al., 2010; Hoffman et al., 2013). Specifically, plants exposed to the highest deacclimation temperatures for the longest periods of time experience the greatest decreases in freezing tolerance (Gay and Eagles, 1991; Hoffman et al., 2013). Although relatively few studies have investigated the effects of deacclimation in warm-season turf species, available research in bermudagrass suggests that deacclimation adversely affects freezing tolerance and can rapidly cause significant physiological and metabolic changes (Chalmers and Schmidt, 1979; Zhang et al., 2011a; Zhang et al., 2011b).

Chilling Injury. Low temperatures that induces chilling injury is any temperature above freezing that causes damage to membranes, chlorophyll loss, photosynthesis and respiration inhibition, and changes in enzymatic activity. Prolonged chilling ultimately causes turf to enter a state of dormancy. Occurring at temperatures below 12°C, chilling injury in plants is characterized by wilting, lack of growth, loss of chlorophyll, a reduced photosynthetic

rate, and the presence of leaf lesions (DiPaola and Beard, 1992; Stier and Fei, 2008). Chilling injury can disrupt cell membrane activity, which leads to electrolyte loss from the cytoplasm, the release of vascular substances, and loss of protein activity.

Freezing Injury. Freeze injury in plants involves the dynamics and kinetics of water as it interacts within different tissues of the plant (Livingston et al., 2006***).*** In grasses, freezing begins in the roots, travels to the crown, and finally progresses upwards into shoots and leaves (Stier et al., 2003). Successful recovery from freezing stress depends upon the meristematic tissues inside crowns and nodes to survive freezing damage (Harrison, 1997; Livingston et al., 2005). Stoloniferous and rhizomatous turfgrass species have meristematic regions located within the nodes of stolons or rhizomes, which have the ability to develop new leaves, roots, and lateral shoots (Duble, 1996). In St. Augustinegrass for example, Milla-Lewis et al 2013b found that despite severe damage to all surrounding tissue 14 days after freezing, new shoots were produced within the meristematic regions of St. Augustinegrass nodes suggesting that these are the most freezing tolerant regions of a St. Augustinegrass plant. Milla-Lewis et al. (2013b) also discovered significant differences between the basal (oldest), mid, and apical (youngest) regions of individual stolons of St. Augustinegrass after freezing tests. The mid region of the stolon recovered significantly better than either basal or apical regions. Concentrating on these specific regions of turfgrass plants during freeze testing could provide accurate estimates of the freeze tolerance of St. Augustinegrass genotypes.

The freeze tolerance of several warm-season turfgrasses have been evaluated including bermudagrass, centipedegrass, St. Augustinegrass, and zoysiagrass. Distinct morphological traits of some turfgrasses, such as internode length and rhizome depth, can influence the freeze tolerance of St. Augustinegrass (Ahring et al., 1975). Specifically, plants with shorter internode

lengths and deeper rhizomes have the best freeze tolerances. Based on lethal temperature and winter survival measurements, Raleigh and Seville have some of the best and worst freezing tolerances of St. Augustinegrass cultivars (Philley et al., 1995, 1998).

Post-freeze survival of rhizomes, single stolon nodes, whole stolons, and whole plants has been used to estimate freeze tolerance in turf species (Bush et al., 2000; Dione et al., 2001; Qian et al., 2001; Anderson et al., 2003; Sahba et al., 2003; Patton et al., 2007). In St. Augustinegrass, single-node (Philley, 1995; Li et al., 2010), four-node (Maier et al., 1994b), and whole stolons (Milla-Lewis et al., 2013a) have been utilized. Differences between these experiments resulted in a fifty percent survival rate when freezing Raleigh at -4^{o}C (Li et al, 2010) and sixty percent survival at -6.0^{o}C (Maier et al, 1994a). Moreover, Milla-Lewis et al. (2013a) reported differences between survival of the basal, mid, and apical regions of St. Augustinegrass stolons indicating node age is an important factor in freeze tests. Therefore, based on previous research, differences in tissue-type and freezing methodologies are important factors to consider when comparing results across studies.

Ratings based on regrowth have been used as a measure of survival (Anderson et al., 2002; Anderson et al., 2003; Hinton et al., 2012) as well as a combination of surviving green tissue and regrowth (Maier and Lang, 1994; Cardona et al., 1997; Dunn et al., 1999; Espevig et al., 2011). Independent scores have been created for surviving green tissue and regrowth, yet ultimately both were included in an overall survival rating (Li et al., 2010; Milla-Lewis et al., 2013). Therefore, the relationship between surviving green tissue and recovery and whether one rating is more useful or predictive than the other in freezing tests is unclear.

PHYSIOLOGICAL ASPECTS OF COLD TOLERANCE

Environmental abiotic stresses, such as cold temperatures, induce various biochemical and physiological responses in plants (Bray et al., 2000), particularly in their cellular membrane. Accumulation of sugars, proline, and other compounds is observed during these stresses and thought to play a role in osmotic adjustment.

Physiological Adaptations of Cell Membrane Composition in Response to Cold Stress. A primary target of freezing injury in plants is their cellular membrane, which primarily consists of lipids, proteins, sterols, and carbohydrates. The lipid bilayer is a gateway for material transfer, while the proteins within the membrane allow for selective transport and accrual of solutes such as carbohydrates. Physiological changes in the cellular membrane, particularly alterations of phospholipid and fatty acid composition, are important factors in cold acclimation and freezing tolerance. At low temperatures, cellular membranes undergo a phase transition from a fluid liquid crystalline phase to a more rigid gel phase where lipids are tightly packed together. Lipid compaction impedes normal physiological functions and can render the membrane more permeable and prone to rupture, which results in the loss of cytoplasmic components. During periods of low, non-freezing temperatures, new compounds are sometimes synthesized in an effort to circumvent or diminish the deleterious effects of cellular dehydration (Gusta et al., 1996), also known as cold acclimation.

Phospholipids, the primary type of lipid in the cellular membrane, have a hydrophilic, polar head group and two hydroxyl groups esterified to long, hydrophobic, fatty acid chains. These saturated (single carbon bond) or unsaturated (one or more double bonds in cis or trans configuration) fatty acids regulate cell membrane fluidity based on their composition and

saturation levels. Tolerance to some abiotic stresses, such as cold temperatures, is largely dependent on maintaining osmotic homeostasis within cells as well as the capacity of the cells to retain water molecules. Increased desaturation of fatty acid chains aids in maintaining the fluidity of the cellular membrane by increasing the number of double bonds, hence the number of kinks in the chain, which ultimately prevents the unsaturated fatty acids from packing together as tightly as saturated fatty acids.

The number of double bonds in membrane fatty acids is an indirect measure of the overall fluidity in the cells. Several unsaturated fatty acids have been shown to be important in cold tolerance in many plant species. Linolenic acid appears to play an important role in maintaining a fluid, liquid state in the cellular membrane. It is synthesized by a series of desaturase enzymes where stearic acid (C18:0) is desaturated to oleic acid (C18:1), then to linoleic acid (C18:2), and then to linolenic acid (C18:3), each converting a single to a double bond. The role of carbohydrates in response to low temperatures has been extensively evaluated in the grass family. Typically, an increase in soluble sugar concentrations in the autumn can be seen with a decrease in tissue starch concentration (Levitt, 1980). Sucrose, glucose, fructose, and raffinose have all shown changes in levels before and after the onset of low temperatures.

SCREENING METHODOLOGIES FOR FREEZING TOLERANCE

Lab-based Cold Tolerance Screening Methodologies. While field evaluations for screening cold tolerance provides plant breeders with the most accurate assessment of genotype's winter survivability, environmental conditions are unpredictable and difficult to

reproduce (Anderson and Taliaferro, 2002). Field observations commonly vary from location to location and from year to year, and also require extensive resources including personnel, adequate field space, and significant monetary investment. In order to circumvent these issues and to expand testing year-round, laboratory-based experiments can be a reliable and reproducible method to evaluate freeze tolerance. Moreover, screening in a controlled environment has generally corresponded well with field screenings (Anderson and Taliaferro, 2002; Qian et al., 2001).Thus, laboratory-based experiments could aid in the improvement of cold tolerance within a species in an efficient, inexpensive manner.

Two main laboratory-based methods have been developed to assess freeze tolerance in turfgrass species including measurement of electrolyte leakage (EL) (Gusta et al, 1980; Fry et al, 1991; Maier et al, 1994; Ebdon et al, 2002) and evaluation of tissue re-growth after freezing (Maier et al, 1994a; Li et al, 2010, Milla-Lewis et al., 2012b). Inconsistencies in studies implementing the EL procedure, including the underestimation of freeze tolerances in seashore paspalum (Cardona et al., 1997) and centipedegrass (Fry et al., 1993) as well as the overestimation of freeze tolerance in St. Augustinegrass (Maier et al., 1994a), imply the re-growth tests may be a better option to determine freeze tolerance in grasses (Patton and Reicher, 2007).

Different protocols focusing on specific tissue types and a range of freezing temperatures have been used to evaluate tissue re-growth of turfgrass after freezing in laboratory-based tests. Rhizomes and whole-plants have been used to evaluate freeze tolerance in bermudagrass (Ahring and Irving, 1969; Anderson et al., 2003) through a triphenyl tetrazolium chloride (TTC) test. Single node cuttings, stolons, and rhizomes wrapped in moist paper have also been used to evaluate bluegrass (Dione et al., 2001), buffalograss (Qian et al.,

2001), carpetgrass (Bush et al., 2000), saltgrass (Sahba et al., 2003), and zoysiagrass (Patton et al., 2007). In the case of St. Augustinegrass, Philley et al. (1995) used the electrolyte leakage (EL) method with single-node stolons, and found that the cultivars Raleigh, 'Texas Common' and 'Delmar' could survive lower freeze temperatures than 'Seville', 'Floratam' and 'FX-33'. Maier et al (1994b) evaluated the freezing response of four-node stolons placed in plastic bags and Li et al (2010) did the same using single-node stolons in plastic containers. Differences in technique between these experiments resulted in LT_{50}'s as high as -4^{o}C (Li et al, 2010) and as low as -6^{o}C (Maier et al, 1994b).

Milla-Lewis et al. (2013b) compared different cold-acclimation and freezing temperature effects on two St. Augustinegrass cultivars using a whole-stolon rolled method (WSRM). Significant differences between Raleigh and Seville were found between all the different cold-acclimation and freezing temperatures. However, the 3°C cold-acclimation temperature and the -2^{o}C freeze temperature seemed to provide the best separation of St. Augustinegrass cultivars using the WSRM (Milla-Lewis et al., 2013b). While the WSRM is effective for comparing a small number of cultivars, this method would not be appropriate for surveying a whole breeding population mainly due the space required for this procedure, including greenhouse and freezer space.

HERITABILITY OF COLD TOLERANCE

Knowledge of the genetic and non-genetic factors influencing cold tolerance or lack thereof in St. Augustinegrass will aid breeders in implementing appropriate selection methods ultimately shifting the mean of breeding populations towards improved cold tolerance. Despite

the identification of cold tolerant germplasm, limited progress has been made in breeding for cold tolerance in St. Augustinegrass. This is in part due to the complexity of the trait, insufficient genetic knowledge of tolerance components, lack of efficient selection criteria, and limited breeding efforts. Previous investigations in other crops (Bohnert et al 1995, Lyons et al 1979) have indicated that plant response to cold stress is a complex phenomenon, controlled by more than one gene and is highly influenced by environmental variation. These factors confound the separation of genetic control from non-genetic factors, both of which are essential in deciding the selection methods of tolerant materials. Similar situations were observed in studies of other traits. A study assessing the heritability of brown patch disease found a strong environmental influence in tall fescue progeny (Bokmeyer et al., 2009). A stability analysis to identify the most stable genotypes based on performance over multiple years and locations also revealed a significant environmental effect (Bokmeyer et al., 2009).

Diallel analyses offer breeders genetic information regarding quantitative traits along with a wide recombination of genomes with greater chances of generating superior cultivars in segregant generations (Barelli et al., 1999). Diallel crosses are traditionally used to evaluate the general and specific combining abilities (GCA and SCA) of parent plants (Griffing, 1956). Information in combining abilities is not only helpful to determine the potential contribution of specific plants when used as parents in a breeding program, but it can also be used to estimate what genetic components (additive, non-additive, and maternal effects) and in what proportions control the trait. Significant GCA can indicate substantial diversity among the parents, which suggests that genetic gain through selection is achievable. Specific combining ability values can provide information regarding the performance of hybrid progeny relative to their parents. Philley et al. (1998) evaluated the inheritance of cold tolerance in St. Augustinegrass with eight

diploid genotypes using a diallel analysis. General combining ability was the largest source of variation for lethal temperature and winter survival. Narrow-sense heritabilities for winter survival ranged from 0.70 to 0.95 over a three year period.

Plant breeders use heritability estimates to determine the influence of the environmental and genetics factors affecting the trait of interest and what selection procedure to implement. Broad-sense heritability is the ratio of total genetic variance to phenotypic variance, which gives plant breeders an understanding of to what extent a trait is influenced by the genotype as opposed to the environment. Estimation of narrow-sense heritability, the proportion of a trait that is controlled by additive genetic effects, can give an estimate of the gain from selection or how much progress can be accomplished towards improving the trait through breeding. Narrow-sense heritability can also be calculated using data from both parents and progenies (Bokmeyer et al., 2009).

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-CHAPTER II-

Combining Ability for Winter Survival and Turf Quality Traits in St. Augustinegrass

ABSTRACT

New elite St. Augustinegrass varieties with improved cold tolerance and desirable turf quality are needed for the turf industry in the transition zone of the United States. In order to efficiently utilize sources of cold tolerance in a breeding program, an understanding of the genetic control of this trait and its relationship to important turf quality traits is required. Therefore, the objective of this study was to estimate general and specific combining abilities for cold tolerance and turf quality traits. Six diploid genotypes of St. Augustinegrass were selected as parents for a diallel mating design without reciprocals. Hybrid progenies and self-pollinated populations were evaluated over three years at two locations. The true hybridity of crosses was confirmed using molecular markers. Combining ability analysis revealed that both general and specific combining abilities were significant across years and locations. Specific combining ability was the largest source of genetic variation for winterkill, genetic color, turf density, and end-of-season cover indicating that non-additive gene effects play a key role in the inheritance of these traits. The parental genotype GF2 was identified as a promising parent for future breeding efforts as it provided positive GCA effects for both cold tolerance and turf quality traits, which were not significantly correlated with one another. Lines identified as parental selfs generally showed lower winter survival and inferior turf quality than the original parental lines indicating that inbreeding depression can occur in St. Augustinegrass. This study provides information regarding the combining ability of cold tolerance and turf quality traits in St. Augustinegrass, which will ultimately aid in parental selection for breeding efforts in the species.

INTRODUCTION

St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntz) is a warm-season, perennial grass species commonly utilized in the turfgrass industry for its superior shade tolerance and stoloniferous growth habit. However, winter survival is a major limiting factor for the species affecting its area of adaptation and marketability, specifically in the transition zone of the United States. Additionally, the most cold-tolerant cultivars currently available often lack the aesthetic characteristics, such as a semi-dwarf growth habit and fine leaf texture that are desirable in the market. Therefore, new St. Augustinegrass cultivars with improved cold tolerance and desirable turf quality are needed for the turfgrass industry in the transition zone of the United States.

Studies have reported significant variability in winter survival and freezing tolerance among present-day varieties (Moseley et al., 2010, Reynolds et al., 2009, Milla-Lewis et al., 2013, Kimball et al., 2015 a and b). A cold hardy cultivar, 'Raleigh', performs consistently well in the upper range of the species' geographic limits. However, Raleigh's coarse leaf texture and long internodes when compared to other St. Augustinegrass cultivars as well as other warm-season turfgrasses are often undesirable (Reynolds et al., 2009). Somatic variants and gamma-ray mutants of Raleigh were developed in 2004 (Li, 2007). In comparison to Raleigh, they had similar cold hardiness as well as improved aesthetic characteristics (Reynolds et al., 2009). On the other hand, available commercial cultivars with good turf quality often have limited cold tolerance. Seville, a popular cultivar well liked for its semi-dwarf habit, fine-leaf texture, and dark green color, exhibits high levels of winterkill (Riordan et al., 1980; Philley et al., 1996; Moseley et al., 2010).

Despite the identification of cold tolerant germplasm, limited progress has been made

in breeding for cold tolerance in St. Augustinegrass. This is in part due to the complexity of the trait, insufficient genetic knowledge of tolerance components, lack of efficient selection criteria, and limited breeding efforts. For example, Raleigh is still considered an industry standard for cold tolerance hardiness in the species thirty years after its release. Previously, Philley et al. (1998) evaluated the inheritance of cold tolerance in St. Augustinegrass with eight diploid genotypes using a diallel analysis. General combining ability was the largest source of variation for winter survival and lethal temperature. The large additive variance indicated that St. Augustinegrass should respond to selection (Philley et al., 1998). However, field trials were conducted in Mississippi and may not reflect genotype performance in the upper transition zone. Additionally, turf quality was not reported in the study and knowledge of the transferability of turf quality traits from parents to progeny in combination with cold tolerance in St. Augustinegrass is important in the development of new, elite cultivars.

A set of Raleigh variants selected for high levels of cold tolerance as well as improved turf quality (Reynolds et al., 2009) and collections from home lawns in Raleigh, NC selected for early spring green-up were identified as potential parents for cold tolerant population development. In order to efficiently utilize these sources in a breeding program, an understanding of the genetic control of cold tolerance and its relationship to important turf quality traits is required. Therefore, the objective of this study was to estimate the general and specific combining ability for cold tolerance and turf quality traits.

MATERIALS AND METHODS

Six diploid (2*n*=2x=18) genotypes of St. Augustinegrass were selected as parents for a diallel mating design without reciprocals based on their high levels of cold tolerance and/or turf quality (Table 2.1). Reciprocals were not utilized in this study as Philley et al (1998) found no maternal effects in their St. Augustinegrass heritability study. The six genotypes included: cultivar Raleigh, the industry's standard for cold tolerance in St. Augustinegrass; experimental line GF2, a gamma radiation-derived Raleigh mutant; experimental line 106T3, a somaclonal variant of Raleigh; accessions C1 and 1800s, collections from old home lawns in Raleigh, NC selected for early spring green-up (Li, 2007); and the cultivar Seville, which has poor levels of winter survival in the field but excellent turf quality and is characterized by a semi-dwarf growth habit. Crosses were made according to the protocol of Genovesi et al (2009) during the fall of 2009. Three to four simple sequence repeat (SSR) markers developed by Mulkey et al. (2013) were used to validate that products from crosses were true hybrids following the protocols outlined in that study. Parental selfs identified by SSR markers were included in this study.

Three replications of each single cross, parental self, and the six parents were planted in a randomized complete block design (RCBD) in $1m^2$ plots at the NCSU Lake Wheeler Turfgrass Field Lab in Raleigh, NC, and at the NCDA Upper Mountain Research Station in Laurel Springs, NC in June 2011. Plots were mowed weekly at a height of 6.35 cm, fertilized monthly at a rate of 0.23 kg ha^{-1} from May to October to accumulate 1.36 kg ha^{-1} for a year, and irrigated to avoid drought stress.

Traits were grouped into three categories: winter survival including winterkill and spring green-up; turf quality traits, including overall turf quality, leaf texture, genetic color, and

turf density; and establishment, including mid-season cover and end-of-season cover. Traits were evaluated over a three year period from 2011-2014. Spring green-up ratings were taken in April and winterkill ratings were collected in May. Turf quality traits and mid-season cover were rated in July, while end-of-season cover was rated in October of each year. Mid-season and end-of-season cover of individual plots were rated on a 0-100% scale. The remaining traits were evaluated visually on a 1 to 9 scale according to the National Turfgrass Evaluation Program (NTEP, 2012) as follows: (1) spring green-up, 1=straw brown color and 9=completely green turf stand; (2) winterkill, 1=100% leaf injury and 9=no injury; (3) turf quality, 1=poor quality and 9=excellent quality where 5= minimum acceptable quality; (4) leaf texture, 1= coarsest texture and 9=finest texture; (5) genetic color, 1=light green/yellow and 9=dark green; (6) turf density, 1=lowest density and 9=maximum density. Note that for each of these ratings scales, a higher score indicates superiority.

An analysis of variance, using the following general linear models, tested for genotypic differences using the MIXED model (PROC MIXED) in SAS statistical software version 9.4 (SAS Institute, 2015). For parents: $\bar{Y}_{ii} = \mu_p + p_i + \bar{\varepsilon}_{ii}$, for selfs: $\bar{Y}_{jj} = \mu_c + 2g_j + s_{jj} + \bar{\varepsilon}_{jj}$, for crosses: $\bar{Y}_{ij} = \mu_c + g_i + g_j + s_{ij} + \bar{\varepsilon}_{ij}$ where $\bar{Y}_{ii}$ is the mean for the ith parent of the six parents, μ_p is the mean of all parents, p_i is the effect of the ith parent, $\bar{Y}ij$ is the mean for the cross between the ith and jth parents, μ_c is the mean of all crosses, g_i is the general combining ability (GCA) effect of the ith parent, s_{ij} is the specific combining ability (SCA) effect on the cross of the ith and jth parents (s_{ji}=s_{ij}), and $\bar{\varepsilon}_{ii}$ and $\bar{\varepsilon}_{ij}$ are the mean error terms associated with the respective means. Griffing's (1956) approach, Method 2 Model 1, was used to analyze general and specific combining abilities for all traits. Fisher's protected t-test was used to compare

parental vs. family means as well as parental vs. self means.

RESULTS AND DISCUSSION

The interaction of location by year was significant for winterkill, spring green-up, and genetic color (Table 2.2). The significant interaction resulted largely from differences in magnitude between years and locations. For example, winterkill was minimal at Lake Wheeler in 2012 and severe at Laurel Springs in 2013 and 2014. However, moderate shifts in winter survival as well as genetic color rankings were identified, and both location and year accounted for a large amount of variation in their models. Environmental variation contributed less than genetic effects to the overall variation in the majority of turf quality traits as expected for qualitative characteristics. Similar results were reported in zoysiagrass (*Zoysia* spp.) where large environmental influences were identified for stress-related traits but not for turf quality traits (Schwartz et al., 2009). Additionally, large environmental and error effects were also identified for genetic color in Schwartz et al (2009) who suggested that the use of a handheld normalized difference vegetation index (NDVI) sensor, which has been shown to improve the calculation of color heritability (Kenworthy et al., 2006), could increase realized heritability of genetic color by reducing error variation.

Winter Survival. Significant variability for winter survival was identified among the parental lines in this study (Table 2.2). According to means separation using Fisher's protected LSD for winterkill, Raleigh's winter survival was significantly better than all other parental lines (Table 2.3). The remaining five parental lines could not be significantly distinguished from one another in terms of their winterkill. Raleigh was also the only parent with a significant parental effect for winterkill (Table 2.4). These findings are consistent with

previous studies documenting Raleigh's high level of cold tolerance (Maier et al., 1994; Philley et al., 1988; Li et al., 2010; Moseley et al., 2010). C1 and 106T3 had the highest levels of winterkill across locations and years. While C1 is a germplasm collection and thus little is known about its genetic makeup, previous studies have reported varying levels of cold tolerance for 106T3 (Reynolds et al., 2009; Moseley et al., 2010) indicating that this genotype may not be stable across different environments. Somaclonal variants such as 106T3 can be unpredictable in nature and genetically unstable (Dennis et al., 1987; Lee and Phillips, 1988; Kaeppler and Phillips, 1993), and results suggested that further evaluation of the line is needed. Likewise, Seville had lower winterkill than expected for a known cold sensitive cultivar. Previous studies (Philley et al., 1995; Moseley et al., 2010; Kimball et al., 2015a; Kimball et al., 2015b) have reported varying winterkill and freeze damage in Seville and suggest that Seville is another genotype whose performance is not consistent across environments.

For spring green-up GF2, a mutant of Raleigh, was the best performer followed by Raleigh and 1800s. Seville was the slowest to green-up. As the best and worst performers, respectively, GF2 and Seville had significant parental effects for spring green-up (Table 2.4). A significant, positive correlation (0.63) between winter surival and spring green-up was detected between parental lines as well as between crosses in this study (Table 2.5) indicating that a genotype able to withstand cold temperatures in winter will green up faster in the spring than a genotype severely affected by winterkill. Little has been reported on St. Augustinegrass' rate of spring green-up in the literature. However, according to the NTEP, St. Augustinegrass is slower than bermudagrass (*Cynodon dactylon*) and zoysiagrass (NTEP, 2012).

Across all locations and years, the general combining ability (GCA) and specific combining ability (SCA) effects were both significant sources of variation for winterkill and

spring green-up (Table 2.2). Significant GCA effects indicated parents of genotypes contributed differently to responses in hybrid combinations as well as the involvement of additive gene effects in response to winterkill and spring green-up. However for winterkill, SCA effects accounted for three times (~60%) more of the total genotypic variance than GCA effects (~20%). The high SCA effects for winterkill suggested non-additive gene effects (i.e. dominance and epistasis) play an important role in winter survival in the species. For example, while Raleigh had the largest parental effect for winterkill (0.82), it had a significant negative GCA effect (-0.72) and a non-significant, mainly neutral self effect (-0.05) (Table 2.4). Because Raleigh was no longer distinguishable from other parents upon selfing for winterkill, these results suggest that while dominance could contribute to winter survival, strong heterotic effects and epistatic interactions could also play a key role in the genetic control of this trait. Conversely, Philley et al (1998) found the largest portion of variance was additive for lethal temperature and winter survival, suggesting that St. Augustinegrass should respond well to selection. Large environmental differences between testing sites may reflect the differences between these studies as well as the germplasm used. Additionally, this study has demonstrated (see below under selfing rates section) the importance of confirming true hybridity in crosses of St. Augustinegrass as selfing is an important issue to consider in these types of studies.

There was a large range of significant positive and negative SCA effects for winterkill and spring green-up for the majority of crosses (Table 6). In general, SCA estimates indicated that crosses with good winter survival also had faster spring green-up, which is consistent with the high correlations identified between family means for these traits (Table 2.5). The cross Seville x GF2 had larger SCA effects for winterkill and spring green-up than any other cross (Table 2.6). The effect of this particular cross largely contributed to the significantly high SCA

effects identified for winterkill and spring green-up and provide evidence that a more complex type of inheritance than additive gene effects may be involved.

Turf Quality. Significant variability for turf quality traits was identified among the parental lines in this study (Table 2.2). While 1800s had the finest leaf texture, Seville outperformed all parental lines in turf quality, turf density, and genetic color (Table 2.3). Seville is known for its semi-dwarf growth habit, fine-leaf texture, and dark green color, which provides excellent turf quality in St. Augustinegrass (Trenholm et al., 2006). Turf quality provided excellent separation of the genotypes (Table 2.3) with the most cold-tolerant genotypes forming two separate groups based on Fisher's LSD values. Raleigh, 1800s, and GF2 appeared to have an intermediate turf quality between Seville and the lowest parental performers, C1 and 106T3. Similar results can be seen for genetic color and turf density, while leaf texture had the lowest amount of variability between the parental lines (Table 2.3).

Across all locations and years, GCA and SCA effects were significant sources of variation for turf quality traits (Table 2.2). Additive gene effects appear to play a larger role than non-additive effects for turf quality and leaf texture, while SCA or non-additive effects contributed twice as much to the overall variation than GCA effects for genetic color (Table 2.2). The magnitude of SCA effects were slightly higher than GCA effects for turf density as well. These results indicated that while turf quality traits can be improved through selecting parents with significant GCA values, progeny testing of specific parental combinations could provide more value to a breeding program. For example, the cross Seville x GF2 had the largest SCA values for turf quality, green color, and turf density along with high SCA values for winterkill and spring green-up (Table 2.6), and has proven to be an excellent St. Augustinegrass cross producing progenies with superior performance.

Seville had the highest positive GCA effect for all turf quality traits (Table 2.4). The majority of cold tolerant genotypes provided a negative or neutral GCA effect, with the exception of GF2 on overall turf quality, genetic color, and turf density. GF2 also had a positive GCA effect for both winterkill and spring green-up. Previous studies have reported better establishment rates, qualitative characteristics, and similar winter survival of GF2 when compared to Raleigh (Li, 2007; Reynolds et al., 2009). This genotype showed the most promise as a parental line to transfer both cold tolerance and turf quality traits into new breeding lines, especially with the low correlations identified between these two types of traits (Table 2.5).

Establishment. Significant variability for mid-season and end-of-season cover ratings was identified among the parental lines in this study (Table 2.2). For mid-season cover, Seville had significantly better cover than other parental lines at 100%, which dropped to ~87% at the end of the season (Table 2.3). This may indicate that low temperature injury was already beginning to occur in Seville during the fall, especially as it was the only genotype whose cover ratings dropped between rating times. Raleigh had the second significantly highest cover rating during mid-season and the best cover rating at the end of the season (Table 2.3). Interestingly, significant positive correlations were found between winterkill and both mid-season (0.55) and end-of-season (0.72) cover ratings when comparing crosses but non-significant, negative correlations between these traits were identified when comparing parental lines (Table 2.5). It appears these negative correlations between parental traits were most likely attributed to Seville's high cover ratings and intermediate winterkill (Table 2.3). The positive correlations between winterkill and cover ratings when comparing crosses indicated that a full turf stand, which is indicative of good overall plant health, appeared to aid a

genotype's ability to withstand cold temperatures in winter. Additionally, full mid-season cover indicates fast establishment rates leading to reduced soil exposure for weed competition in newly established turf stands (Busey and Myers, 1979), which is important for new varieties in the turfgrass industry.

Across all locations and years, GCA and SCA effects were both significant sources of variation for both cover ratings (Table 2.2). Large positive and negative differences in GCA effects were identified for parental lines. Seville had the largest GCA effect for end-of-season cover followed by 106T3. 1800s, 106T3, C1, and Raleigh all had significantly large GCA effects on mid-season cover but not for end-of-season cover (Table 2.4).

Overall Cross Performance. As mentioned above, the cross Seville x GF2 proved to be an excellent cross with high performance (Table 2.3) and significantly high SCA effects (Table 2.6) for a majority of traits. The crosses 106T3 x 1800s, 106T3 x GF2, and 106T3 x Seville also had low winterkill, fast spring green-up, good turf quality, and fast establishment rates (Table 2.3). Additionally, the cross C1 x 1800s had very good spring green-up and turf quality. Cross combinations with poor performance were identified as well. The cross Raleigh x GF2 produced low performing progenies across the cold tolerance and turf quality traits. As GF2 is a gamma-ray derived mutant of Raleigh, it is possible that these two parents are highly similar to one another genetically resulting in high levels of inbreeding depression within crosses between the two.

Selfing Rates and Inbreeding Depression. Although St. Augustinegrass is widely known as a predominately outcrossing species, this research identified high rates of selfing (~40-60%) using SSR markers to confirm parentage. This reflects the importance of using molecular markers to validate the true hybridity of crosses which more turfgrass breeders are

currently doing. Studies in common bermudagrass (*C. dactylon* (L.) Pers. var. *dactylon* (Tan et al., 2015) and zoysiagrass (*Zoysia* spp.) (Guo et al., 2014) have identified high outcrossing rates in these species with the use of molecular markers. However, crosses in these studies were generated in open-pollinated fields where pollen was not limited. The high selfing rates observed in controlled crosses in the current study may be the result of a controlled crossing environment but could also indicate an underestimation in the levels of self-pollination occurring in the species, especially when pollination is limited to a turf stand of the same genotype. It is important to ascertain the cause of these high selfing rates of controlled crosses as they can negatively affect the progress and efficiency of a breeding program.

Previous studies have reported the negative impacts of inbreeding in St. Augustinegrass (Atilano and Busey 1983; Busey, 2003). Comparison of the parental and self means and effects indicate that, in general, inbreeding depression impacted most genotypes negatively across traits (Figure 2.1 and Supp. Figure 2.1). However depending on the trait, some parental lines showed little to no change, and a few actually showed improvement. For winterkill, all parental means were significantly higher than their selfed counterparts with the exception of C1, indicating that selfing had a negative impact on winter survival for most parental lines (Figure 2.1a). Similar results were seen for spring green-up, turf quality traits, and establishment (Supp. Figure 2.1). Little change was seen in the leaf texture of parental lines and their selfs.

Interestingly, C1 and 106T3 effects after selfing improved in comparison to their parental effects for all traits except for leaf texture in 106T3 selfs (Table 2.6). Neither parental genotype was severely affected by selfing and the selfed progenies performed better for several traits (Figure 2.1 and Supp. Figure 2.1). While C1 is a collection and little is known about its historical pedigree, as a somaclonal variant of Raleigh, 106T3's improvement upon selfing

provides further evidence that the genotype is genetically unstable. This also indicates that selfing may help stabilize somaclonal variants in St. Augustinegrass and improve their performance in the field for important turf quality traits.

CONCLUSIONS

Significant variation was observed between parental lines and the progeny means of crosses for cold tolerance and turf quality traits. The GCA and SCA effects were both significant for all traits, while SCA effects were greater for winterkill, genetic color, turf density, and end-of-season cover indicating that non-additive gene effects may be more important than additive gene effects for these traits. While Raleigh had a negative GCA effect for winterkill, GF2 had a positive GCA effect for both cold tolerance and turf quality traits and shows promise as a parental line for future breeding efforts.

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Table 2.1. List of St. Augustinegrass cultivars and experimental genotypes used in the U.S. and their breeding information and release date.

No.	Parent	Type	Experimental notation	Source/Breeder	Year Released
1	Raleigh	Cultivar	NA	North Carolina State Univ., Raleigh, NC	1983[†]
2	1800s	NCSU Collection	1800s	North Carolina State Univ., Raleigh, NC	NA[‡]
3	GF2	'Raleigh' Mutant	GF2	North Carolina State Univ., Raleigh, NC	NA[‡]
4	Seville	Cultivar	S-6-68-516	Pursely, Inc.; T.P. Riordan; O.M. Scotts & Sons	1978[§]
5	C1	NCSU Collection	C1	North Carolina State Univ., Raleigh, NC	NA[‡]
6	106T3	'Raleigh' Somaclonal Variant	106T3	North Carolina State Univ., Raleigh, NC	NA[‡]

[†] Bateman, D.R. 1980. Notice to sod producers and growers relative to the naming and release of the new St. Augustinegrass cultivar 'Raleigh'. N. C. Ag. Res. Serv. Raleigh, NC

[‡] NA, not applicable. Experimental germplasm under development.

[§] Riordan, T.P., V.D. Meier, J.A. Long, and J.T. Gruis. 1980. Registration of Seville St. Augustinegrass. Crop Sci. 20:824-825.

Table 2.2. Mean squares for winterkill, spring green-up, turf quality, green color, leaf texture, turf density, mid-season cover, and end-of-season cover.

Source	df	Winterkill	Spring Green-up	Turf Quality	Genetic Color	Leaf Texture	Turf Density	Mid-season Cover	End-of-season Cover
Location	1	577.19***	714.50***	16.43**	13.39	6.79**	66.53***	11058.01***	32792.54***
Year	2	746.73***	325.45***	56.92***	21.85**	6.43**	39.73***	60.33	57.54
Replication	2	13.08***	7.27	3.43	1.06	1.01	23.50***	1980.84*	8612.05***
Among Parents	6	45.62***	69.49***	32.67***	23.72***	2.45*	54.45***	5534.68***	6904.26***
Among Selfs	5	3.40	5.30	20.75***	13.69***	10.68***	31.62***	6613.43***	5339.67
Among Crosses	14	23.49***	17.29***	14.87***	9.24***	5.31***	20.36***	2393.88***	4892.72***
GCA	5	19.36***	43.09***	27.76***	1.65	22.56***	29.81***	4243.47***	5214.35***
SCA	9	30.476***	39.75***	23.48***	6.57***	18.24***	31.83***	1905.71***	5425.18***
Parents vs Crosses	1	0.09	0.07	4.21	0.3	0.01	5.21	1374.97	2632.53
Parents vs Selfs	1	107.64***	257.86***	79.84***	0.08	139.77***	116.48***	7967.81***	12549.01**
Location x Year	2	141.34***	157.94***	0.25	29.32***	0.91	4.08	200.23	221.68
Location x Replication	2	1.66	0.41	0.64	13.82**	0.38	9.83*	62.80	2688.82
Location x Among Parents	6	22.21***	37.18***	11.82***	11.75***	0.77	19.82***	5413.33***	5711.20***
Location x Among Crosses	14	6.03***	5.19	5.58**	2.41	3.06***	7.31**	681.03	3745.14***
Year x Replication	4	1.48	3.86	0.85	1.71	1.06	0.34	203.46	212.16
Year x Among Parents	12	17.63***	18.58***	2.70	2.25	6.97***	1.76	2210.54*	2323.79*
Year x Among Crosses	28	6.51***	5.13	2.75	2.35	0.82	3.2	1149.62	1325.51
Location x Year x Replication	4	4.69*	2.48	0.44	1.6	1.48	15.81**	2348.05	2437.01
Location x Year x Among Parents	12	8.30***	20.46***	6.47**	1.7	1.73	2.16	1578.49	1696.39
Location x Year x Among Crosses	28	4.67***	2.53	2.11	2.63	0.58	1.52	795.35	847.25

* p>0.05, ** p>0.01, *** p>0.0001

Table 2.3. List of six St. Augustinegrass parents and their crosses used in this study along with mean separation for winterkill, spring green-up, turf quality, genetic color, leaf texture, turf density, mid-season cover, and end-of-season cover using Fisher's protected LSD.

No.	ID	#	Winterkill	Spring Green-up	Turf Quality	Genetic Color	Leaf Texture	Turf Density	Mid-Season Cover	End-of-Season Cover
1	Raleigh	4	4.86 a	2.96 ab	6.39 bcd	6.61 bc	5.9 efg	6.27 bcd	78.96 cdef	93.96 ab
2	1800s	4	3.56 cde	2.79 ab	6.64 b	6.87 b	6.58 ab	6.62 bc	64.58 ghi	85.42 abcd
3	GF2	4	3.46 cde	3.75 a	6.26 bcde	6.45 bcd	6.11 cdef	5.87 cdef	54.79 ij	80.94 bcde
4	Seville	4	3.46 cde	1.64 cde	7.56 a	7.43 a	6.27 abcdef	7.58 a	98.96 a	87.29 abcd
5	C1	4	3.24 defg	2.31 bcd	5.48 fghi	5.97 defg	5.89 efg	5.05 fghi	57.93 hij	71.35 efgh
6	106T3	4	3.08 efgh	2.18 bcd	5.06 ghi	6.01 def	6.51 abc	4.62 ghi	44.79 j	64.17 gh
7	106T3 x 1800s	3	3.61 cd	2.29 bcd	5.65 efg	5.96 defg	5.87 fg	5.28 efg	89.44 abc	80 cde
8	106T3 x C1	3	2.66 hij	1.5 cde	4.88 hi	5.64 fg	5.33 h	4.48 hi	73.33 defgh	63.47 gh
9	106T3 x GF2	4	3.57 cde	2.32 bc	5.93 def	6.4 bcd	6.55 ab	5.59 def	79.49 cdef	76.88 def
10	106T3 x Seville	3	3.8 bc	2.29 bcd	6.54 bc	6.65 bc	6.17 bcdef	6.83 b	90.28 abc	95.69 a
11	C1 x 1800s	4	4.22 b	2.68 b	6.68 b	6.73 bc	6.57 ab	6.55 bc	79.79 cde	90 abc
12	C1 x GF2	4	2.28 j	1.35 ed	-	4.85 h	5.67 gh	4.29 i	76.46 defg	61.67 h
13	C1 x Seville	3	2.65 hij	1.04 e	5.69 efg	5.39 g	6.06 defg	5.21 efgh	95.83 ab	75.83 defg
14	GF2 x 1800s	4	2.81 ghi	1.76 cde	5.35 gh	6.08 def	5.98 efg	5.12 fgh	66.75 fghi	66.17 fgh
15	Raleigh x 106T3	3	3.31 cdef	1.93 cde	5.97 cdef	5.88 efg	6.4 abcd	5.75 def	83.06 bcd	86.81 abcd
16	Raleigh x 1800s	4	2.92 fghi	1.63 cde	5.13 gh	5.76 fg	5.44 h	4.73 ghi	69.58 efgh	75.42 defg
17	Raleigh x C1	4	3.08 efgh	2.13 bcd	5.02 hi	5.93 defg	6.1 def	4.85 ghi	72.71 defgh	68.75 efgh
18	Raleigh x GF2	4	2.74 hi	1.54 cde	5.32 gh	5.79 efg	5.99 efg	5.24 efgh	56.41 ij	66.46 fgh
19	Raleigh x Seville	3	2.87 gfghi	1.43 cde	6.49 bcd	6.29 cde	6.6 a	5.89 cde	90.83 abc	77.5 def
20	Seville x 1800s	4	2.49 ij	1.49 cde	5.64 fghi	6.02 def	6.31 abcde	5.18 efgh	75.42 defg	69.46 efgh
21	Seville x GF2	3	2.46 ij	1.02 e	6.59 b	6.82 bc	6.66 a	6.03 cd	80.28 cde	68.61 efgh

* Means with the same letter are not significantly different from one another

Table 2.4. Estimates of parental, self, and general combining ability (GCA) effects for winterkill, spring green-up, turf quality, green color, leaf texture, turf density, mid-season cover and end-of-season cover.

a.	Winterkill			Spring Green-up			Mid-Season Cover			End-of-Season Cover		
Line	**P**	**S**	**GCA**	**P**	**S**	**GCA**	**P**	**S**	**GCA**	**P**	**S**	**GCA**
Raleigh	0.82***	-0.05	-0.72***	0.08	-0.02	-1.26***	0.66	7.94**	8.29*	5.76	-2.39	-6.72
106T3	-0.19	-0.09	0.05	-0.34	-0.05	-0.23	-19.62***	-9.73**	10.01***	-10.35*	-8.57*	2.10
C1	-0.33	0.24**	-0.51***	-0.30	0.15	-0.70	-1.06	3.57	9.20**	-5.35	6.33*	-8.96*
Seville	-0.14	0.01	0.96***	-1.25***	-0.20	1.45***	26.77***	17.48***	-2.96	5.35	10.33***	21.79***
GF2	-0.09	-0.06	0.66**	1.56***	-0.22	1.71***	-8.23	3.22	-35.27***	0.63	1.63	-3.40
1800s	-0.06	-0.06	-0.44**	0.25	0.32	-0.97***	1.49	-22.48***	10.73**	3.96	-7.34	-4.81
b.	Turf Quality			Genetic Color			Leaf Texture			Turf Density		
Line	**P**	**S**	**GCA**	**P**	**S**	**GCA**	**P**	**S**	**GCA**	**P**	**S**	**GCA**
Raleigh	-0.23	-0.36*	-0.95***	-0.06	-0.36*	-0.97***	-0.38*	-0.33**	-0.11	-0.20	-0.38*	-0.98***
106T3	-0.69	-0.23	-0.24*	-0.48*	0.05	-0.31***	0.35*	0.12	-0.06	-0.96**	-0.51*	-0.17
C1	-0.53	-0.01	-0.74***	-0.54*	-0.17*	-0.77***	-0.35*	-0.16*	-0.16	-0.64*	-0.11	-0.78***
Seville	1.20***	0.91***	2.07***	1.02***	0.56***	1.67***	0.09	0.68***	0.48**	1.44***	1.04***	2.10***
GF2	-0.09	0.02	0.63*	-0.18	-0.12	1.14***	-0.03	0.06	0.06	-0.14	-0.01	0.67*
1800s	0.35	-0.33	-0.77***	0.24	0.04	-0.76***	0.33*	-0.36*	-0.22	0.50	-0.03	-0.83**

P= parental effect, S=self effect, and GCA=general combining ability
* p>0.05, ** p>0.01, *** p>0.0001

Table 2.5. Pearson correlation coefficients between parental lines and between crosses for winterkill, spring green-up, turf quality, leaf texture, green color, turf density, mid-season cover, and end-of-season cover.

	Winterkill	**Spring Green-Up**	**Turf Quality**	**Leaf Texture**	**Green Color**	**Turf Density**	**Mid-Season Cover**
Between Parental Lines							
Spring Green-Up	0.49**						
Turf Quality	-0.49**	0.26					
Leaf Texture	-0.28	-0.17	0.54**				
Green Color	-0.02	0.00	0.50**	0.14			
Turf Density	-0.26	0.21	0.83***	0.56**	0.29		
Mid-Season Cover	-0.46	0.55*	0.80***	0.25	0.67**	0.73**	
End-of-Season Cover	-0.30	0.07	0.52**	0.09	0.54***	0.47**	0.83***
Between Crosses							
Spring Green-Up	0.79***						
Turf Quality	0.11*	0.22***					
Leaf Texture	-0.10*	-0.06	0.30***				
Green Color	0.06	0.06	0.48***	0.13			
Turf Density	0.44***	0.44***	0.79***	0.19***	0.38***		
Mid-Season Cover	0.55***	0.46***	0.44***	0.08	-0.03	0.58***	
End-of-Season Cover	0.72***	0.56***	0.37***	-0.02	0.22***	0.67***	0.55***

* p>0.05, ** p>0.01, *** p>0.0001

Table 2.6. Estimates of the specific combining ability of 15 crosses in St. Augustinegrass for winterkill, spring green-up, turf quality, leaf texture, green color, turf density, mid-season cover, and end-of-season cover traits across two locations over three years.

Cross	Winterkill	Spring Green-Up	Turf Quality	Leaf Texture	Genetic Color	Turf Density	Mid-Season Cover	End-of-Season Cover
106T3 x 1800s	0.43*	0.75**	0.27	-0.04	0.29	0.18	-0.19	2.00
106T3 X C1	-0.99***	-1.03**	-1.23***	-0.75***	-0.90***	-1.51***	-7.00	-19.49***
106T3 X GF2	-0.04	-0.19	-0.21	0.4**	-0.06	-0.36	-0.06	-6.12
106T3 X Seville	0.18	-0.21	0.40	0.02	0.24	0.90**	11.38*	12.89**
C1 x 1800s	0.43*	0.75**	0.27	-0.04	0.29	0.18	-0.19	2.00
C1 x GF2	-1.00***	-0.91**	-0.99***	-0.25	-1.00***	-1.01**	6.63	-12.99**
C1 x Seville	-0.41*	-1.00**	0.05	0.01	-0.57**	-0.08	17.74**	4.09
GF2 x 1800s	-1.65***	-2.63***	-1.33**	-1.13***	-1.62***	-1.57**	21.67*	-14.14
Raleigh x 106T3	0.42*	0.68**	0.78***	0.37*	0.43*	0.80**	-4.14	10.72*
Raleigh x 1800s	0.51**	1.11**	0.51*	-0.40*	0.76**	0.46	-17.06**	6.65
Raleigh x C1	0.78***	1.34****	0.35	0.21	0.94***	0.54*	-11.01*	4.63
Raleigh x GF2	-0.77**	-1.63***	-0.59*	-0.17	-0.99***	-0.47	15.6**	-3.72
Raleigh x Seville	-0.94***	-1.5***	-1.05***	-0.01	-1.15***	-1.33***	16.61**	-18.28**
Seville x 1800s	-1.60***	-1.74***	-1.9***	-0.19	-1.57***	-2.01***	0.03	-26.98***
Seville x GF2	2.77***	4.46***	2.50***	0.18	3.05***	2.53***	-45.76***	28.28**

* p>0.05, ** p>0.01, *** p>0.0001

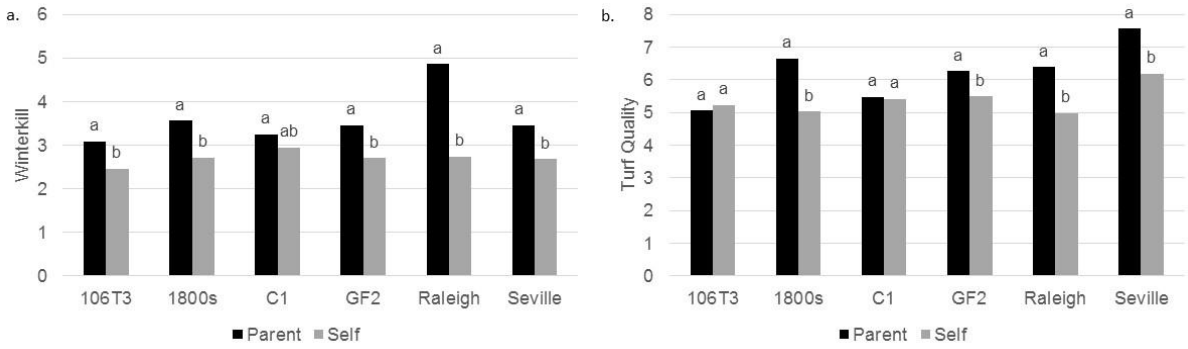
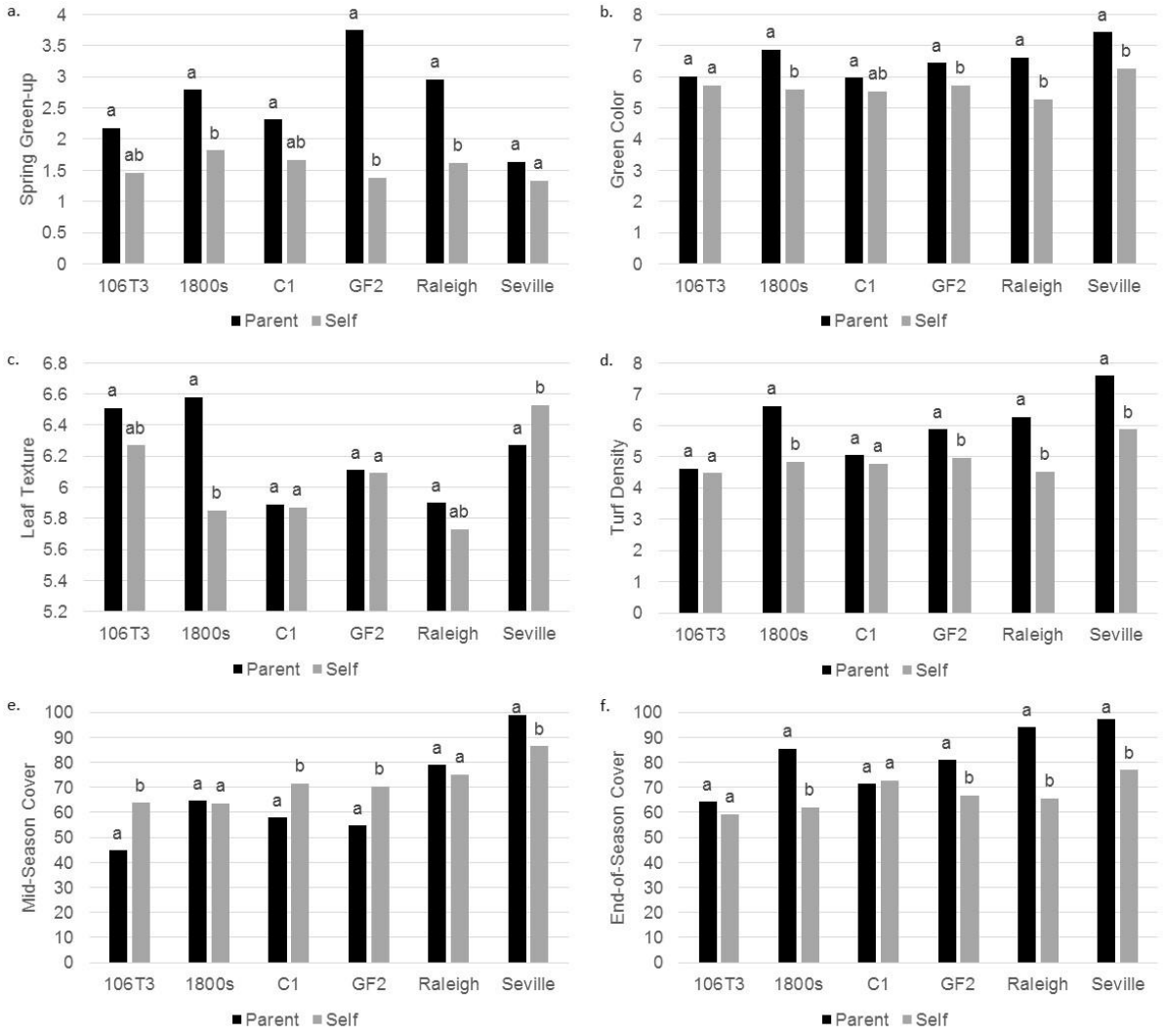


Figure 2.1. Lsmeans and mean separation between parental and selfed lines for a. winterkill and b. turf quality.



Supplementary Figure 2.1. Lsmeans and mean separation between parental and selfed lines for a. spring green-up, b. green color, c. leaf texture, d. turf density, e.mid-season cover, and f. end-of-season cover.

-CHAPTER III-

Freeze-Testing in St. Augustinegrass I: A Methodological Approach

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ABSTRACT

Winter survivability is a major-limiting factor for St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze) grown in the transition zone of the United States as cold winters can result in high levels of winterkill. In addition to field studies, lab-based freeze tests mimicking field winter survivability can contribute to the selection of cold hardy lines and ultimately, aid in breeding for cold tolerance. This study used a whole-container freeze method to evaluate four freezing temperatures and two data collection systems in freeze tests of nine St. Augustinegrass genotypes ranging in their winter survivability. Results indicated -3°C and -4°C to be more suitable temperatures for evaluating freeze survival in St. Augustinegrass using this methodology than -5°C and -6°C. Weekly visual ratings of surviving green tissue and regrowth were correlated with one another over a six week evaluation period post-freeze. The length of post-freeze evaluation for these different rating systems was also assessed and determined to vary depending upon the rating system. Additionally, we provide evidence that digital imaging techniques commonly utilized in turfgrass field studies are also useful in estimating surviving green tissue and regrowth in lab-based freeze tests. Percent green cover calculated using digital images was highly correlated with visual ratings for all ratings taken 2, 4, and 6 weeks post-freeze. This study provides unique information regarding freezing temperatures, genotype responses, and data collection methods in St. Augustinegrass, which should aid breeders in the improvement of freezing survival in this species.

INTRODUCTION

Winter survivability is a major limiting-factor in the production and widespread use of St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze) as turf in the transitional climatic region of the United States. Therefore, improved winter survival could increase St. Augustinegrass' competitiveness in the marketplace. Field and lab-based studies have reported significant variability in winter survival and freezing tolerance among present-day cultivars (Moseley et al., 2010, Reynolds et al., 2009, Milla-Lewis et al., 2013) despite the fact that St. Augustinegrass is native to subtropical and tropical climates (Judd, 1975). A recent study evaluating response to winter stress in Arkansas, measured as percent winterkill, revealed a range of zero to 100 percent survival among cultivars (Moseley et al. 2010).

While field evaluations can provide plant breeders with the most accurate assessment of winter survivability, environmental conditions are often unpredictable and difficult to reproduce (Anderson and Taliaferro, 2002). To circumvent these issues and to expand testing year-round, laboratory-based experiments can be a reliable and reproducible method to evaluate freeze tolerance. Such a system could facilitate selection and aid in the improvement of winter survivability within a species in an efficient, inexpensive manner.

Two main laboratory-based methods, electrolyte leakage (EL) and tissue regrowth measurements are commonly used to assess freeze tolerance in turfgrass species. However, studies implementing the EL procedure have reported inconsistencies, including underestimation (Cardona et al., 1997; Fry et al., 1993) and overestimation (Maier et al., 1994a) of freeze tolerance in several warm-season turfgrass species. This suggests the evaluation of regrowth may potentially be a better option to evaluate freeze tolerance in grasses (Patton and Reicher, 2007).

Post-freeze survival of rhizomes, single stolon nodes, whole-stolons, and whole-plants has been used to estimate freeze tolerance in turf species (Bush et al., 2000; Dione et al., 2001; Qian et al., 2001; Anderson et al., 2003; Sahba et al., 2003; Patton et al., 2007). In St. Augustinegrass, single-node (Philley, 1995; Li et al., 2010), four-node (Maier et al., 1994b), and whole stolons (Milla-Lewis et al., 2012) have been utilized. Differences between these experiments resulted in a 50% survival rate when freezing cultivar 'Raleigh' at -4^{o}C (Li et al, 2010) and 60% survival at -6.0^{o}C (Maier et al, 1994a). Moreover, Milla-Lewis et al. (2012) reported differences between survival of the basal, mid, and apical regions of St. Augustinegrass stolons indicating node age is an important factor in freeze tests. Therefore, based on previous research, differences in tissue-type and freezing methodologies are important factors to consider when comparing results across studies.

Ratings based on regrowth have been used as a measure of survival (Anderson et al., 2002; Anderson et al., 2003; Hinton et al., 2012) as well as a combination of surviving green tissue and regrowth (Maier and Lang, 1994; Cardona et al., 1997; Dunn et al., 1999; Espevig et al., 2011). Independent scores have been created for surviving green tissue and regrowth, yet ultimately both were included in an overall survival rating (Li et al., 2010; Milla-Lewis et al., 2013). Therefore, the relationship between surviving green tissue and recovery and whether one rating is more useful or predictive than the other in freezing tests is unclear.

Digital imaging techniques are commonly used in turf field studies to measure parameters such as establishment rates, percent green cover, and recovery from injury or dormancy (Richardson et al., 2001; Karcher and Richardson, 2003; Karcher and Richardson, 2005). While visual ratings can be subjective, digital imaging can provide consistent, accurate measurements regardless of time, evaluator, or environment (Karcher and

Richardson, 2005). Although digital imaging techniques have not been utilized in lab-based freeze tests, their success in field studies estimating percent green cover suggests they could be used to estimate survival and regrowth in lab-based freeze tests.

While several methods are available to estimate freeze tolerance in turfgrass, few are practical for measuring relative performance between large numbers of experimental lines. We were interested in developing a freezing protocol capable of distinguishing the variability in freeze tolerance found in St. Augustinegrass so that germplasm collections and breeding populations could be more accurately evaluated. Therefore, the objectives of this study were to evaluate four freezing temperatures for protocol development in St. Augustinegrass, and to identify a reliable method to evaluate the survivability and recovery of St. Augustinegrass after freezing.

MATERIALS AND METHODS

Plant Materials. Nine St. Augustinegrass genotypes varying in winter survivability based on Moseley et al. (2010) were evaluated in this study (Table 3.1). Cultivars 'Raleigh' and 'Floratam' were used as tolerant and sensitive standards, respectively. For each genotype, nine single node stolons were planted in Fafard 4P potting mix (Conrad Fafard Inc, Agawam, MA) and propagated in 7.62 x 7.62 x 7.62 cm plastic pots (Hummert International, Earth City, MO) and allowed to completely fill the container. Each pot represented one replication of a genotype. All plants were dethatched before freezing so only green tissue was present at testing. Before freezing, genotypes were cold-acclimated according to Milla-Lewis et al (2013). Three replications of each genotype were placed in a 13°C growth chamber with a 12 h photoperiod at 300 µmol m^{-2} s^{-1} for one week and then moved to a 3°C growth chamber with a 12 h photoperiod at 300 µmol m^{-2} s^{-1} for one more week before freezing.

Freezing Tests. For each experiment, plants were arranged in a randomized complete block design with three replications. Plants were put into two modified commercial freezers at -1°C. Ice shavings were placed on the soil of each pot to promote freezing and avoid supercooling. Each replication set of the nine genotypes was placed in a large plastic bag to minimize desiccation during freezing. Five thermocouples were placed both within random stolons as well as in the soil to monitor plant and soil temperatures during freezing tests to ensure plants reached target temperatures. Plants remained at -1°C for 15 h to allow for latent heat to dissipate from the soil. The temperature was then decreased at a rate of 1°C h^{-1} until reaching the target freezing temperature of -3°C, -4°C, -5°C, or -6°C, which was maintained for 10 h. The temperature was then increased at a rate of 2°C h^{-1} to 3°C. Plants were removed

from the freeze and allowed to thaw on at 20°C overnight before being placed in a 20°C temperature controlled greenhouse for evaluation. Two individual runs for each target temperature were conducted during the winter of 2014-2015.

Data Collection. Recovering genotypes were evaluated using visual ratings and digital imaging analysis. To account for time, space, labor costs, and difficulty, four rating methods were conducted. Pearson correlation coefficients were calculated to compare both rating systems and validate the accuracy of our visual ratings system.

For visual evaluation, surviving green tissue (SGT) and regrowth (RG) ratings were collected the day after freezing and then once a week for six weeks post-freeze. Surviving green tissue, based on percent green leaves and nodes, was rated on a scale of 0-5 with 0=all tissue and nodes were brown, 1=0-25%, 2=25-50%, 3=50-75%, 4=75-99%, and 5=100% surviving green tissue. It should be noted that surviving green tissue is representative of the severity of freezing injury a plant sustains during freezing tests. Regrowth was also rated on the same 0-5 scale with a recovery rating of 0=no evidence of regrowth and 5=complete recovery of the turf stand. Surviving green tissue and regrowth ratings were added together to create an overall survival (OS) rating on a 0-10 scale similar to Hinton et al. (2012) in order to compare to the digital %GC rating, which captures both SGT and RG. All ratings were converted from the 1-5 or 1-10 scales used to percentages on a 0-100 scale for data interpretation.

For digital imaging analysis, individual pictures of the plants were taken at five different time points: prior to freezing (after cold acclimation) and 1 d, 2 wks, 4 wks, and 6 wks post-freeze. The percent green cover for each individual container was calculated using Sigmascan Pro digital imaging software (SPSS, 2002) and the macro ‘Turf Analysis’

developed by Karcher and Richardson (2005).

Data Analysis. Initial analyses included all four freezing temperatures in the model. For each freezing temperature, the experimental design was a randomized complete block design with block representing two runs of each temperature. In the model, the main effects of freezing temperature and genotype, and their interactions were considered to be fixed. Experimental run and its interaction with fixed effects were considered to be random, as well as replications nested within each test. An analysis of variance (ANOVA) and adjusted means for all visual and digital ratings were generated using the mixed model procedure (PROC MIXED) in SAS statistical software version 9.3 (SAS Institute, 2011). Type 3 test of hypothesis for the fixed effects was used to determine significance of main effects and interactions (Table 2), and Fisher's protected LSD was used to calculate mean separation of genotypes across each individual temperature (Table 3).

Initial analyses revealed little to no variation between genotypes at -5°C or -6°C as the majority of plants died (Table 3). Therefore, subsequent analyses were performed for -3°C and -4°C tests only. A repeated measures ANOVA for each temperature was generated using the general linear models procedure (PROC GLM) in SAS statistical software version 9.3 and used to evaluate the effect of time on genotype response for all visual and digital ratings. For each genotype, the lethal temperature where 50 percent of plants died (LT_{50}) was calculated using SGT and RG data collected at six weeks post-freeze using the probit analysis procedure (PROC PROBIT) in SAS.

RESULTS AND DISCUSSION

Freeze Tests. Temperature, genotype, and their interaction were all statistically

significant for post-freeze SGT, RG, OS, and digital GC ratings according to the ANOVA type III tests (Table 3.2). In general, the degree of freeze injury (represented by SGT ratings) and recovery (represented by RG ratings) responses were variable among genotypes at -3°C and -4°C, while the large majority of genotypes showed 0% SGT and 0% RG at -5°C and -6°C (Figure 3.1). At -3ºC, SGT among genotypes ranged from 3.3% to 73.2% and their RG response ranged from 0% to 56.6% at six weeks post-freeze (Table 3.3). At -4ºC, SGT among genotypes ranged from 0 to 40% and their RG response ranged from 4% to 36% at six weeks post-freeze (Table 3.3). Therefore using this freezing method, -3 and -4ºC appear to be more suitable temperatures than -5 and -6°C for evaluating freezing survival in St. Augustinegrass. Also, freezing at -4ºC applies greater selection pressure than freezing at -3°C, which is important for future applications of this methodology in screening nurseries and large breeding populations.

While some rankings of genotypes did change significantly between -3°C and -4°C, they appeared to fall within three distinct groupings (Figure 2). Raleigh, 106T3, and GF2 were the top performing genotypes and represented the most freeze tolerant group across temperatures and traits (Figure 3.2). Floratam, our cold-sensitive standard, generally was the most freeze sensitive genotype with the worst performance across the different rating systems and temperatures. Cultivars Captiva, Palmetto, Sapphire, Seville, and Sunclipse ranked between the most cold-tolerant and cold-sensitive genotypes and represent the intermediate freeze sensitive group. When accounting for these different groupings or levels of freeze tolerance, the differences in genotype rank across temperatures for the different rating systems are primarily in magnitude, not direction.

For the freeze tolerant group, Raleigh, GF2, 106T3 were in the top three most freeze

tolerant performers, regardless of the rating system or the temperature (Figure 3.2). While at -3°C, Raleigh, GF2, and 106T3 ranked first, second, and third, respectively, for all ratings, 106T3 performed better than Raleigh in RG and GC ratings at -4°C, though the difference was not statistically significant (Figure 3.2d and h). Likewise, 3% of Raleigh and 2% of both 106T3 and GF2 survived -5°C, while 12% of Raleigh and 3% of GF2 plants survived at -6°C (Table 3.3). The improvement in Raleigh's response between -5°C and -6°C was caused by one plant's complete survival for one run at -6°C and was most likely an escape.

Raleigh's superior cold tolerance has been well documented in both field studies and laboratory-based freeze tests (Maier et al., 1994; Duble, 1996; Philley, 1998; Li et al., 2010; Moseley et al., 2010), however, 106T3 and GF2 performed similarly to Raleigh in this study. Both lines were selected for their high levels of cold-tolerance and semi-dwarf growth habit, and were reported to have similar winter survival to Raleigh in field studies (Reynolds et al., 2009). 106T3 is a somaclonal variant of Raleigh and GF2 is a mutant of Raleigh produced from gamma ray irradiation. Therefore, Raleigh, 106T3, and GF2 are most likely highly genetically similar. However, Moseley et al (2010) did not find similar field winter survival of 106T3 in comparison to Raleigh and GF2 in Arkansas. At -6°C in this study, Raleigh and GF2 survived while 106T3 did not. It is possible that at very low freezing temperatures or during severe winters that 106T3 survivability declines.

For the intermediate freeze sensitive group, genotype rankings tended to change across rating systems and temperatures. In Zoysiagrass (*Zoysia* spp. Willd.), Patton and Reicher (2007) found genotypes classified with intermediate winter injury varied in their response over years, while genotypes exhibiting low or high winter injury were stable across years. However, it should be noted that in this study, differences in freeze response between

the intermediate genotypes were not statistically significant with the exception of Seville. Seville's SGT, RG, OS, and %GC responses were significantly higher at -3°C than -4°C (Table 3.3). Likewise, Seville ranked fourth across all rating systems at -3°C and ranked seventh across rating systems at -4°C (Figure 3.2). This is the largest consistent change in genotype rank between the two temperatures and contributes a large amount of the variability found in the temperature-by-genotype interaction. Seville was released in 1980 by the O.M. Scott and Sons Company, and while the cultivar's exact pedigree is unknown, the characteristics listed in its patent (USPP 4097) and registration (Riordan et al., 1980) suggests that Seville has some level of cold tolerance. Even though Seville has low winter survival in field trials (Philley et al., 1996; Moseley et al., 2010), it is possible that the cultivar harbors genes associated with cold tolerance. Only cold-acclimated plants were used in this study, and therefore, it would be of interest to further evaluate Seville and determine if acclimation impacts Seville's survival.

In previous studies, different freezing temperatures, typically ranging from -2°C to -6°C, have been suggested for St. Augustinegrass using different methodologies. For example, Milla-Lewis et al (2012) suggested -2°C was most suitable for differentiating genotypes using a whole stolon rolled method (WSRM), which was conducted with whole stolons placed in plastic containers. Since this study used a whole plant method, which meant nodes were rooted in soil, it seems appropriate that our methodology calls for lower freezing temperatures. Also, while -2°C was not tested in this study, we hypothesize that this freezing temperature could provide better separation of genotypes in the moderate freeze sensitive group.

Lethal Temperature. The lethal temperature where 50% of plants die (LT_{50}) was

calculated for all genotypes (Table 3.3). GF2 had the lowest LT_{50} at -4.9°C followed by Raleigh at -4.6°C. Previous studies have reported 50% survival of Raleigh at -6.0°C (Maier et al., 1994a) and -4°C (Li et al., 2010). In this study, the estimated LT_{50} for Floratam was -1.7°C, while LT_{50}'s as low as -6.1°C (Fry et al., 1991) and -4.5°C (Maier et al., 1994) have been reported. Both of these studies utilized an EL methodology and may have resulted in the overestimation of Floratam's LT_{50}. Additionally, Floratam consistently exhibited 0% SGT and 0% RG across all temperatures in this study and has performed poorly in both freeze (Li et al., 2010) and field studies (Murdoch et al., 1990; Moseley et al., 2010). Additionally, our whole container method provides greater separation of Raleigh and Floratam (2.9°C difference) than Maier et al., 1994a (1.5°C difference) which corresponds well with field studies (Wilson et al., 1977; Busey et al., 2003; Moseley et al. 2010) providing further evidence that tissue regrowth experiments are a better option for evaluating freeze tolerance in St. Augustinegrass.

Post-Freeze Evaluation. A repeated measures ANOVA was used to evaluate the effect of evaluation time and its interaction among genotypes (Table 3.4). For all ratings, evaluation time had a significant effect at -3°C and -4°C. The significant interaction of time by genotype at -3°C indicated that the response of genotypes changed over the course of the six week evaluation period when evaluated visually. In general, across ratings, this interaction was in magnitude when taking the three previously described freeze tolerance groupings into account. For example, the majority of freezing injury occurred by three weeks post-freeze as SGT had stabilized across all temperatures by that time (Figure 3.2a and b). Likewise, a steady increase in RG was recorded throughout the six week post-freeze evaluation period (Figure 3.2c and d).

While differences between genotypes were statistically significant across all weeks for SGT, they were only significant at week six for RG ratings at -3°C (Table 3.5). These results indicate that at -3°C, collecting SGT and RG data two to three weeks and six weeks post-freeze, respectively, are adequate evaluation times for each of the traits. For OS and %GC, significant differences between genotypes could be distinguished from two to six weeks post-freeze (Table 3.5). A six week evaluation time for RG is consistent with previously reported tissue regrowth studies in St. Augustinegrass (Maier and Lang, 1994; Li et al., 2010; Milla-Lewis et al., 2013). For freeze tests in St. Augustinegrass, it appears that a final evaluation measurement of both SGT and OS at three weeks post-freeze would save post-freeze evaluation time, labor, and space.

Visual Ratings. Previous studies have commonly evaluated freeze tolerance using a simple yes or no rating system for survival, while some included surviving green tissue as a measure of survival (Maier and Lang, 1994; Cardona et al., 1997; Philley et al., 2001; Anderson et al., 2002; Li et al., 2010; Espevig et al., 2011; Milla-Lewis et al., 2013). In this study, both SGT and RG measurements were measured to establish whether genotypes responded differently to freezing injury vs recovery. An OS rating was also included to estimate survival ratings based on a combination of SGT and RG. Significantly high Pearson's correlation coefficients (0.79 to 0.93) were calculated between SGT and RG ratings for each of the six weeks of evaluation at -4°C (Table 3.6). Over time, correlations between SGT and RG tended to increase at -3°C, while correlations were higher at early weeks and lower at the final weeks at -4°C (Table 3.6). Lower, non-significant correlations between SGT and RG found in the two and three weeks post-freeze at -3°C could be a result of the increased variability seen at this temperature in comparison to -4°C (Table 3.6). For

example at -3ºC, Palmetto was able to retain green color (SGT) similarly to the most freeze-tolerant genotypes but was not able to recover as well. Additionally, the decrease in correlation between SGT and RG after 4 weeks at -4ºC was most likely attributed to high rates of injury, where SGT ratings stabilized between 0-25% green tissue, while recovery rates continued to increase over time. These results appear to indicate that RG is a more stable measure of freeze tolerance among St. Augustinegrass genotypes. If SGT is taken into account in analyses, an OS rating may be a more appropriate measure of survival as it reflects both SGT and RG, especially when evaluation time was considered (Table 3.5). It should also be noted that measuring SGT and RG independently from one another, and then combining the two as in previous St. Augustinegrass studies (Li et al., 2010; Milla-Lewis et al., 2012) could reduce the chance of errors in scoring, especially for genotypes with intermediate survival.

Digital Imaging Analysis. Digital images were taken in order to compare and evaluate percent GC as a rating system for freeze tests in St. Augustinegrass, and to gauge the accuracy of our visual ratings. The calculated percent green cover showed similar trends when compared with visual ratings in the statistical analyses performed including significant temperature, genotype, and temperature by genotype effects (Table 3.2, 3.3, and 3.4). The time of regrowth could also be pinpointed as the first two weeks percent green cover dropped and then started to rebound at week 4 for both -3°C and -4°C (Figure 3.2g and h).

To compare visual ratings vs digital ratings, percent SGT and RG were combined to create an overall survival (OS) rating as digital images were able to detect both traits. Pearson's correlation coefficients between OS and digital rating systems increased over the weeks for both temperatures (Table 3.6). High correlations in the later weeks post-freeze

indicate that both OS and digital ratings systems are comparable and measured the survivability of St. Augustinegrass genotypes in a similar manner. Therefore, it appears that digital imaging analysis techniques can be translated from the field to the lab and provide an alternative to visual ratings. However, while digital ratings are not subjective and are more stable over time, they do require more time, labor, and equipment than visual ratings.

CONCLUSIONS

This study provides a new methodology for freeze tests in St. Augustinegrass using a whole container approach. For screening St. Augustinegrass germplasm collections and breeding populations for adaptation to the transition zone, and thus high levels of freeze tolerance, we recommend using a -4^{o}C freezing temperature, visually rating SGT at three weeks post-freeze, and finally RG at six weeks post-freeze. Overall, this study provides unique information regarding freezing temperatures, genotype responses, and data collection methods for freezing survival evaluations in St. Augustinegrass, which should aid breeders in improving the selection efficiency for this trait.

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Table 3.1. Cultivars of St. Augustinegrass and experimental genotypes used in the U.S and their breeding information and release date.

Genotypes	Experimental notation	Source/Breeder	Year Released
106T3	106T3	North Carolina State Univ., Raleigh, NC	NA[†]
Captiva	NUF-76	Univ. of Florida	2007[‡]
Floratam	FA-110	Florida Agriculture Experiment Station and Texas Agriculture Experiment Station	1973[§]
GF2	GF2	North Carolina State Univ., Raleigh, NC	NA
Palmetto		Sod Solutions	1995
Raleigh		North Carolina State Univ., Raleigh, NC	1983
Sapphire		Sod Solutions	2003
Seville	S-6-68-516	Pursely, Inc.; T.P. Riordan; O.M. Scotts & Sons	1978[¶]
Sunclipse	S-6-72-130	Pacific Sod Co., T.P. Riordan, O.M. Scotts & Sons	1988

[†] NA, not applicable. Experimental germplasm under development.

[‡] Lu, H., R. Nagata, K. Kenworthy, K. Quesenberry, and P. Busey. 2015. Registration of 'NUF-76' St. Augustinegrass. J. Plant Registrations. doi:10.3198/jpr2014.10.0073crc.

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Table 3.2. Analysis of variance Type III tests of fixed effects across all four freezing temperatures after six weeks post-freeze for surviving green tissue (SGT), regrowth (RG), overall survival (OS), and percent green cover (%GC).

			SGT		RG		OS		%GC	
Effect	Num df	Den df	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F	F-value	Pr>F
Temperature	3	4	127.3	<.0001	101.05	<.0001	450.20	<.0001	43.27	<.0001
Genotype	8	32	43.68	<.0001	28.69	<.0001	139.29	<.0001	2.94	0.0044
Temperature*Genotype	24	32	38.34	0.0005	32.91	0.0007	133.22	<.0001	2.1	0.0038

† SGT, surviving green tissue; RG, regrowth; OS, overall survival; %GC, percent green cover

Table 3.3. St. Augustinegrass germplasm, LT50's based on this study's methodology, and mean separation using Fisher's protected LSD for surviving green tissue (SGT), regrowth (RG), overall survival (OS), and percent green cover (%GC) at six weeks post-freeze for -3°C, -4°C, -5°C, -6°C freezing temperatures.

			-3°C				-4°C			
No	Name	LT_{50}	SGT	RG	OS	%GC	SGT	RG	OS	%GC
1	Raleigh	-4.60	73.2a[†]	56.6a	65.0a	100.0a	40.0a	36.0ab	38.0a	29.2ab
2	GF2	-4.91	60.0ab	53.2a	56.6ab	92.9ab	32.0abc	28.0abc	30.0ab	19.2ab
3	106T3	-4.40	40.0bcd	50.0a	53.3ab	79.8abcd	36.0ab	40.0a	38.0a	32.8a
4	Palmetto	-3.89	40.0bcd	23.2c	31.6cd	74.8bcd	8.0bcd	12.0abc	10.0ab	8.4b
5	Sunclipse	-3.87	26.6de	26.6bc	26.6cd	19.3d	4.0cd	8.0bc	6.0b	17.0ab
6	Sapphire	-3.66	20.0de	20.0cd	20.0de	43.5d	0.0d	12.0abc	6.0b	16.6ab
7	Captiva	-3.89	33.2cd	26.6bc	30.0cd	59.8abcd	8.0bcd	12.0abc	10.0ab	6.0b
8	Seville	-3.98	56.6abc	46.6ab	43.3bc	79.5abc	4.0cd	8.0bc	3.0b	4.4b
9	Floratam	-1.70	3.32e	0.0d	1.6e	0d	0.0d	4.0c	4.0b	7.7b
			-5°C				-6°C			
No	Name	LT_{50}	SGT	RG	OS	%GC	SGT	RG	OS	%GC
1	Raleigh	-4.60	3.3a	3.3a	3.3a	3.3a	16.6a	0.07a	8.35a	16.7a
2	GF2	-4.91	3.3a	0.0b	1.7a	2.0a	0.03ab	0.03a	0.03ab	3.2ab
3	106T3	-4.40	3.3a	0.0b	1.7a	0.0a	0.0b	0.0a	0.0b	0.0a
4	Palmetto	-3.89	0.0a	0.0b	0.0a	0.0a	0.0b	0.0a	0.0b	0.0a
5	Sunclipse	-3.87	0.0a	0.0b	0.0a	0.0a	0.0b	0.0a	0.0b	0.0a
6	Sapphire	-3.66	0.0a	0.0b	0.0a	0.0a	0.0b	0.0a	0.0b	0.0a
7	Captiva	-3.89	0.0a	0.0b	0.0a	0.0a	0.0b	0.0a	0.0b	0.0a
8	Seville	-3.98	0.0a	0.0b	0.0a	0.0a	0.0b	0.0a	0.0b	0.0a
9	Floratam	-1.70	0.0a	0.0b	0.0a	0.0a	0.0b	0.0a	0.0b	0.0a

[†] SGT, surviving green tissue; RG, regrowth; OS, overall survival; %GC, percent green cover

[‡] Means within column followed by the same letter are not significantly different ($p<0.05$) from one another.

Table 3.4. Repeated measures analysis of variance testing for within time effects for visual and digital ratings at two different freezing temperatures.

	SGT	RG	OS	% GC
-3°C				
Time	<.0001	<.0001	<.0001	<0.0001
Time*Genotype	<.0001	0.0029	<.0001	0.3640
-4°C				
Time	<.0001	<.0001	0.0046	<.0001
Time*Genotype	0.9979	0.9411	0.6756	0.3259

† SGT, surviving green tissue; RG, regrowth; OS, overall survival; %GC, percent green cover

Table 3.5. Significance of genotype effects over time revealed by analysis of variance while testing at -3°C and -4°C individually for visual and digital ratings.

Post-Freeze	1day	Wk1	Wk2	Wk3	Wk4	Wk5	Wk6
-3°C							
SGT	0.1570	0.0279[†]	0.0093[‡]	0.0093[‡]	0.0058[‡]	0.0058[‡]	0.0058[‡]
RG	-	-	0.1650	0.1329	0.1035	0.1145	0.0094
OS	0.1570	0.0279[†]	0.0032	0.0085	0.0115	0.0127	0.0044
%GC	0.2105	-	0.0152	-	0.0058	-	0.0194
-4°C							
SGT	0.4051[†]	0.2005[†]	0.3349[‡]	0.3349[‡]	0.0638[‡]	0.0638[‡]	0.0638[‡]
RG	-	-	0.4144	0.3264	0.2810	0.1005	0.0896
OS	0.4051[†]	0.2005[†]	0.3756	0.3310	0.1245	0.0803	0.0756
%GC	0.4854	-	0.7253	-	0.6388	-	0.5510

[†] Weekly SGT and OS ratings with the same p-values are a result of no RG and therefore, only SGT is represented in OS ratings.

[‡] Weekly SGT ratings with the same p-values indicate that little to no change in ratings was detected between those weeks.

[§] SGT, surviving green tissue; RG, regrowth; OS, overall survival; %GC, percent green cover

Table 3.6. Pearson correlation coefficients comparing surviving green tissue (SGT) and regrowth (RG) as well as overall survival (OS) and percent green cover (%GC) over a six week evaluation period post-freeze at two freezing temperatures.

	Day 1	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
SGT vs RG †							
-3°C	-	-	0.17 NS	0.24 NS	0.37**	0.52***	0.62***
-4°C	-	-	0.88***	0.93***	0.79***	0.79***	0.80***
Visual vs Digital: OS vs %GC †							
-3°C	0.09 NS	-	0.76***	-	0.92***	-	0.87***
-4°C	0.31*	-	0.72***	-	0.79***	-	0.84***

† SGT, surviving green tissue; RG, regrowth; OS, overall survival; %GC, percent green cover
‡ * $p<0.05$; ** $p<0.01$; *** $p<0.0001$, NS=non-significant.

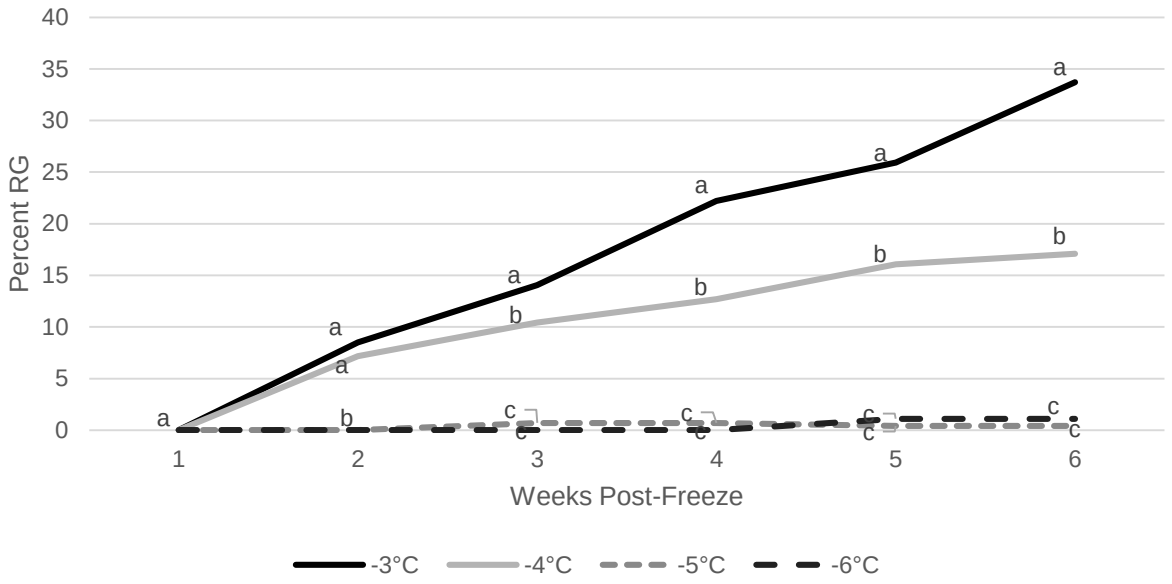

Figure 3.1. Average percent regrowth (RG) for nine St. Augustinegrass genotypes across four freezing temperatures (-3°C, -4°C, -5°C, and -6°C) over a six week evaluation period post-freeze.

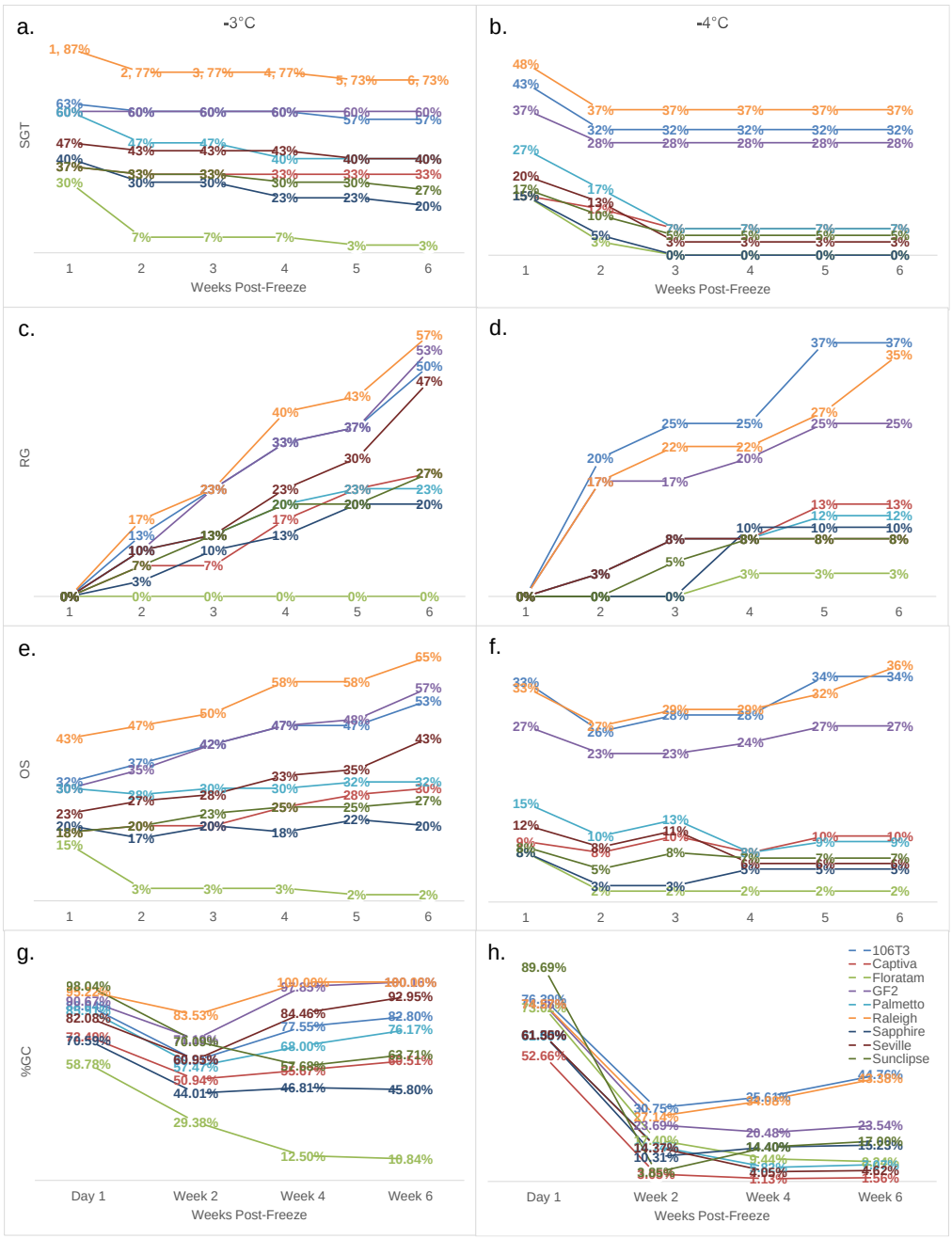

Figure 3.2. LSmeans of nine St. Augustinegrass genotypes for a. surviving green tissue (SGT) at -3°C, b. SGT at -4°C, c. regrowth (RG) at -3°C, d. RG at -4°C, e. overall survival (OS) -3°C, f. OS at -4°C, g. percent green cover (%GC) -3°C, b. %GC at -4°C evaluated over a six week period post-freeze. Mean separation using Fisher's LSD values are included across temperatures for each trait.

-CHAPTER IV-

Freeze-Testing in St. Augustinegrass II: Evaluation of Acclimation Effects

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ABSTRACT

The adaptation of St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze) in the transitional climatic zone is marginal due to a lack of sufficient winter survival. Lab-based freeze tolerance testing method not only afford plant breeders a reliable method for evaluating freeze tolerance, they also provide the ability to investigate different underlying mechanisms. The objective of this research was to evaluate the effects of cold acclimation and deacclimation across two freezing temperatures on nine St. Augustinegrass genotypes. Results indicate that recovery measurements provide the best mean separation for genotype response and more specifically, recovery at -4°C identifies the best separation of freeze-tolerant genotypes from the intermediate and least tolerant genotypes. Accounting for all levels of acclimation provided excellent genotype separation at both freezing temperatures (-3 and -4 °C) and supports the hypothesis that the inclusion of different acclimation response traits offers the best overall assessment of freeze tolerance in St. Augustinegrass. This research indicates that cold acclimation plays a crucial role in the improvement of winter survivability of St. Augustinegrass. A significant loss of freeze tolerance was also identified when plants were subjected to deacclimation events suggesting that St. Augustinegrass can be negatively affected by rapid temperature changes in the transitional climatic zone leading to increased sensitivity to winterkill. Overall, this study provides unique information regarding the effects of cold acclimation and deacclimation and the complex relationships within and between mechanisms underlying freeze tolerance in St. Augustinegrass.

INTRODUCTION

The production and widespread use of St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze) in the transitional climatic zone is largely hindered by a lack of sufficient cold tolerance in this grass species which is native to subtropical and tropical climates (Judd, 1975). As an alternative to field testing, lab-based methods can provide breeders with an expedited, accurate, and reliable method for evaluating freeze tolerance, which has been positively correlated with winter survival (Humphreys and Eagles, 1988). These lab-based tests can ultimately aid in the efficiency of breeding for cold tolerance. Moreover, lab-based freezing methods afford breeders the power to explore different physiological mechanisms affecting freeze tolerance, such as cold acclimation and deacclimation.

Cold acclimation is a natural process whereby plant cells upregulate and downregulate production of various cellular components when exposed to low, non-freezing temperatures (Fowler and Thomashow, 2002). As a result, cold acclimation improves a plant's freezing tolerance. Previous studies have indicated warm-season grass species, including St. Augustinegrass, respond well to cold acclimation (Anderson et al, 1988; Anderson et al, 1993; Fry et al., 1993; Qian et al., 2001; Anderson et al, 2003; Sahba et al., 2003; Patton and Reicher, 2007; Li et al., 2010). The St. Augustinegrass cultivar 'Raleigh' readily acclimates to cold temperatures (Maier et al., 1994b; Li et al., 2010) and based on the cultivar's freeze response, Li et al. (2010) developed an acclimation protocol for St. Augustinegrass. Milla-Lewis et al. (2012) also found differences between Raleigh and Seville's cold acclimation response. 'Floratam', however, appears not to respond to cold acclimation (Fry et al., 1991; Busey, 2003).

Deacclimation, referring to the de-hardening of cold acclimated plants, occurs

naturally during late winter or early spring when plants undergo physiological and metabolic changes to prepare for plant growth (Sasaki et al., 2001; Arora et al., 2004). Previous studies in cool-season turf species have reported both temperature and duration of the deacclimation event can affect a plant's ability to retain freeze tolerance gained from cold acclimation (Gay and Eagles, 1991; Jørgensen et al., 2010; Hoffman et al., 2013). Specifically, plants exposed to the highest deacclimation temperatures for the longest periods of time experience the greatest decreases in freezing tolerance (Gay and Eagles, 1991; Hoffman et al., 2013). Although relatively few studies have investigated the effects of deacclimation in warm-season turf species, available research in bermudagrass suggests that deacclimation adversely affects freezing tolerance and can rapidly cause significant physiological and metabolic changes (Chalmers and Schmidt, 1979; Zhang et al., 2011a; Zhang et al., 2011b). In the transitional climatic zone, drastic temperature shifts during the winter are common and can trigger the deacclimation process leading to a loss of freezing tolerance and ultimately, low temperature kill. Deacclimation of St. Augustinegrass could potentially play a significant role in winterkill levels.

Aside from assessing the freeze tolerance of St. Augustinegrass genotypes (Kimball et al., 2015), we were also interested in exploring the effects of acclimation and deacclimation on freeze tolerance. As studies have reported varying effects of acclimation treatments on genotype response, further evaluation of cold-acclimation and deacclimation could provide insight into the freeze tolerance of St. Augustinegrass genotypes. Likewise, incorporating specific acclimation treatments into our freeze protocols could improve our ability to identify higher levels of freeze tolerance during the selection process when screening breeding populations. Therefore, the objective of this research were twofold (1) to

evaluate the effects of cold-acclimation and deacclimation on St. Augustinegrass' freeze tolerance and (2) to explore the relationships between individual genotypes known winter survivability and their acclimation and de-acclimation response.

MATERIALS AND METHODS

Plant Materials. Nine St. Augustinegrass genotypes varying in winter survivability used in Moseley et al. (2010) were evaluated in this study (Table 4.1). Cultivars Raleigh and 'Floratam' were used as tolerant and sensitive checks, respectively. Genotypes were planted and propagated in 7.62 x 7.62 x 7.62 cm plastic pots (Hummert International, Earth City, MO) filled with Fafard 4P potting mix (Conrad Fafard Inc, Agawam, MA) soil. The St. Augustinegrass genotypes completely filled the container prior to the initiation of acclimation treatments.

Acclimation Treatments. Four acclimation treatments were used to evaluate the St. Augustinegrass genotypes as follows: (1) Cold-acclimation (CA) treatment according to Milla-Lewis et al. (2013) where genotypes were placed in a 13°C growth chamber with a 12 h photoperiod at 300 µmol m^{-2} s^{-1} for one week and then moved to a 3°C growth chamber with a 12 h photoperiod at 300 µmol m^{-2} s^{-1} for one more week before freezing; (2) non-acclimation (NCA) treatment, where genotypes were placed in a greenhouse held at a 21°C for two weeks prior to freezing; (3) deacclimation at 13°C (DCA13) treatment, where following the CA treatment, genotypes were placed into a 13°C growth chamber for three days prior to freezing; and (4) deacclimation at 21°C (DCA21) treatment, where following CA treatment, genotypes were placed into a 21°C temperature-stable greenhouse for three days prior to freezing.

Freezing Tests. As -5°C and -6°C tests provided little information using this methodology (Kimball et al., 2015), independent experiments were conducted using -3°C and -4°C, with two individual experimental runs per temperature, and each run included three replications of NCA, CA, DCA13, and DCA21 treatments per genotype. Freezing protocols were the same as described in Kimball et al. (2015a) with the exception that modified refrigerated-chamber with vertical air flow as well as three vertical rotating fans were used to provide constant air circulation.

Data Collection. For this study, plants were evaluated visually for surviving green tissue (SGT) and regrowth (RG) according to Kimball et al. (2015) in January and February of 2015 in a 21°C temperature-stable greenhouse. Ratings were taken once a week for five weeks starting two weeks post-freeze. Surviving green tissue, which is reflective of the amount of freezing injury a plant sustains during freeze tests, was rated on a scale of 0-5 with 0=all tissue and nodes were brown, and 1=0-25%, 2=25-50%, 3=50-75%, 4=75-99%, and 5=100% surviving green tissue. Regrowth was also rated on the same 0-5 scale with a recovery rating of 0=no evidence of regrowth and 5=complete recovery of the turf stand.

Data Analysis. The experimental design was a randomized complete block design and the experiment was conducted twice. A repeated measures ANOVA procedure was used to evaluate the effect of time on genotype response for all visual ratings. An analysis of variance (ANOVA) and least square (LS) means were generated using the GLM procedure in SAS statistical software version 9.3 (SAS Institute, 2011). Standard *F*-tests in all analyses were used to determine significance of main effects and interactions. In the model, tests and its interaction with fixed effects were considered to be random, as well as replications nested within each test. The main effects and their interactions were considered to be fixed. Fisher's

protected LSD method (α=0.05) was used to separate means. The SLICE option within PROC GLM was also used to test for differences between the simple main effects in regards to their interaction.

RESULTS AND DISCUSSION

Evaluation Time. Surviving green tissue and regrowth were evaluated over a five week period starting two weeks after freeze tests. A repeated measures analysis of variance was performed to test for the effect of time on both SGT and RG in order to determine an appropriate evaluation time for each trait (Table 4.2). For SGT, time's interaction with temperature, acclimation, genotype, and temperature-by-acclimation-by-genotype were all significant. However, these effects were no longer significantly different between weekly evaluations after week three (*data not shown*) indicating the differences identified in Table 1 were due to variability seen between two and three weeks post-freeze. These results indicate SGT stabilized with little change after two weeks post-freeze and that an appropriate evaluation time for SGT should be three weeks post-freeze. Therefore, SGT data collected at three weeks post-freeze are presented.

For RG, time's interaction with temperature, acclimation, genotype, replication, temperature-by-genotype, acclimation-by-cultivar were all significant (Table 4.2). Comparison of evaluation times indicated that significant differences between time and treatments decreased over time except for genotype response, which remained constant ($P<0.001$). Only genotype response was significant between week 5 vs week 6 ($P=0.0013$, *data not shown*) and the best mean separation for genotype was detected at week 6 post-freeze. These results indicate that unlike SGT, RG does not stabilize and continues to change

over time, which is expected as plants continue to recover from freeze injury. Therefore, six weeks post-freeze recovery was the most appropriate evaluation timing for RG, which is consistent with previous studies (Maier and Lang, 1994; Li et al., 2010; and Milla-Lewis et al., 2013). Therefore, RG data collected at six weeks post-freeze are presented.

Freeze Temperature. An overall model for the Type III test of fixed effects for SGT and RG across temperature, acclimation, genotype, and their interactions is presented in Table 4.3. For both SGT and RG, temperature-by-genotype, and acclimation-by-genotype were significant, while temperature-by-acclimation and temperature-by-acclimation-by-genotype were not. Genotype ranks across freezing temperatures were stable with the main exception being Seville, which shows a large improvement in survivability at -3°C in comparison to -4°C (Table 4.4) and largely contributes to the temperature* genotype interaction. GF2 also performs better than Raleigh at -3°C, though not statistically different, in comparison to -4°C.

Three groupings of genotypes based on varying levels of freeze tolerance were identified in Kimball et al. (2015) as follows: Raleigh, GF2, and 106T3 represent the most freeze tolerant group, Floratam represents the least tolerant, and Captiva, Palmetto, Sapphire, Seville, and Sunclipse generally represent intermediate response. These groupings are clearly identifiable at -3°C in Table 3 as well as Figure 1. Moreover, SGT and RG mean separation indicated that Raleigh, GF2, and 106T3 performed significantly better than all other genotypes at -3°C when evaluated across acclimation regime (Table 4). Although at -4°C, separation of genotypes was not as marked, these three genotypes were still the top three performers. These results are consistent with other studies reporting the highest levels of freeze and/or cold tolerance identified in St. Augustinegrass can be found among these three

genotypes (Reynolds et al., 2009; Li et al., 2010; Moseley et al., 2010).

Interestingly, averaging genotype responses over acclimation treatments (Table 4.4) provides very good separation of genotypes at both temperatures in accordance with their reported varying levels of freeze tolerance. After unexpected LT_{50} estimates of cold-acclimated annual bluegrass (*Poa annua* L.) plants, Dionne et al. (2001) recommended that additional traits be evaluated to fully assess the winter survivability of the species. Similarly, Hoffman et al. (2013) suggested that cold acclimation and deacclimation response are separate traits that should both be evaluated to understand mechanisms underlying cold tolerance in a species. Results of this study support this hypothesis and suggest that the inclusion of different traits offers the best overall assessment of freeze tolerance in St. Augustinegrass.

Genotype Response. In freeze tests, Johnson (1989) suggested that if significant interactions are caused by large rank changes, then different freeze temperatures should be analyzed separately. Therefore, in this study the effect of acclimation on genotype response was estimated individually for each temperature. Significant interaction between acclimation treatment and genotype was identified (Figure 4.1). For acclimation-by-genotype sliced by acclimation, significant differences were observed among genotypes within each acclimation treatment for all trait by temperature combinations with the exception of NCA for SGT at -4°C (Table 4.5). This is expected as very few non-acclimated individuals survived at -4°C. As for acclimation-by-genotype interaction sliced by genotype, results varied. Raleigh was the only genotype for which significant differences were detected among acclimation treatments for all trait by temperature combinations indicating that it might be the genotype most responsive to acclimation. Conversely, Floratam, Captiva and Sunclipse showed no

significant differences among acclimation treatments for any of the traits. Since these genotypes are in the groups with intermediate or no tolerance to freezing, their lack of response to acclimation treatments likely explains the reason for their poor freezing tolerance and survival in the field.

A number of rank shifts can be seen between the nine genotypes across the four acclimation treatments at -3°C, while changes at -4°C were less drastic (Figure 4.1). This indicated that as most genotypes experienced higher levels of freeze injury and death at -4°C, the potential for specific acclimation treatments to improve survivability was diminished and therefore, the threshold for acclimation benefits for most genotypes may have been surpassed. For example, evaluating SGT of CA, DCA13, and DCA21 treated plants, Raleigh, GF2, and 106T3 responses are fairly similar to one other at -3°C (Figure 4.1a), while Raleigh significantly outperforms both GF2 and 106T3 at -4°C (Figure 4.1b). Therefore, Raleigh appears to be able to continue to resist deacclimation and lower temperatures than GF2 and 106T3. Likewise, CA Seville experiences a 50% RG response and is among the top performers at -3°C (Figure 4.1c), while only experiencing 12% RG response and is among the lowest performers at -4°C (Figure 4.1d). A previous study in perennial ryegrass (*Lolium perenne* L.) identified a large cold acclimation effect in Pennfine, a low performing variety (Ebdon et al., 2002). These results indicated that this variety did share some freeze-stress characteristics in common with high performing varieties and reflected the importance of screening for cold acclimation ability in perennial ryegrass (Ebdon et al., 2002). Additionally, changes in genotype rank in field studies are common when measurements are taken throughout the fall, winter, and early spring, which may reflect exposure to different temperatures as well as acclimation regimes (Dunn and Nelson, 1974; Qian et al., 2001;

Ebdon et al., 2002). Therefore, for freeze tests in St. Augustinegrass, the choice of freeze temperatures and acclimation treatments is crucial in identifying the nuances between genotypes as well as reflecting the different environmental conditions experienced in the field.

Cold acclimation. Acclimation at low non-freezing temperatures typically increased the freezing tolerance of St. Augustinegrass genotypes as has been observed in both cool-season (Dionne et al., 2001; Hoffman et al., 2013) and warm-season grasses (Maier et al., 1994b; Cardona et al., 1997; Stair et al., 1998; Patton and Reicher, 2007; Li et al., 2010). Cold acclimation, which had the largest effect on genotype response, significantly increased freeze tolerance for Raleigh, GF2, 106T3, Palmetto, and Seville in comparison to NCA treated plants (Figure 4.1). Stolons of field-grown Raleigh have also been reported to exhibit a 75% increase in survival when cold-acclimated (Maier et al., 1994b). Therefore, in both lab and field-based testing of St. Augustinegrass, cold acclimation response by genotype as triggered by genetic variability appears to be the most important and influential mechanisms of improving cold tolerance in the species.

Conversely, Captiva, Floratam, Sapphire, and Sunclipse appeared unresponsive to cold acclimation, which most likely drives their poor freeze tolerance. Conversely, Sapphire and Floratam appeared to be negatively affected by cold acclimation, which most likely drives their poor freeze tolerance. These results are consistent with previous studies evaluating Floratam's poor cold acclimation ability (Fry et al., 1991; Maier et al., 1994b). As genotypes such as Captiva, Floratam, Sapphire, and Sunclipse are exposed to low non-freezing temperatures, it is most likely that they are not undergoing the physiological and metabolic changes associated with cold acclimation in plants and instead, are incurring

chilling injury making them as sensitive to freezing temperatures as non-acclimated plants (Lyons, 1973). Chilling injury is a marked physiological dysfunction that occurring in tropical and subtropical plants in response to nonfreezing temperatures (Lyons, 1973).

In warm-season turf species with greater tolerance to low temperature, higher levels of proline (Cai et al., 2004; Munshaw et al., 2006; Patton et al., 2007b; Zhang et al., 2011) as well as N metabolites and certain dehydrin proteins (Patton et al., 2007a; Zhang et al., 2008) have been identified in cold-tolerant varieties relative to cold sensitive ones. Differences in soluble sugar concentrations associated with cold acclimation have been identified in warm-season turf species as well, however results have varied (White and Schmidt, 1990; Fry et al. 1991; Bush et al., 2000; Cai et al., 2004; Patton et al. 2007b). In St. Augustinegrass, starch and sucrose levels do not appear to be correlated with freezing tolerance (Fry et al., 1991; Maier et al., 1994b). Research comparing Raleigh (freeze tolerant) with Floratam (no freeze tolerance) and Sapphire (intermediate freeze tolerance) for different amino acids and proteins known to be associated with cold acclimation and freeze tolerance is needed to better understand the different physiological and metabolic mechanisms underlying cold acclimation in St. Augustinegrass.

Deacclimation. Significant differences between CA and DCA treated plants can be seen for RG for specific genotypes (Figure 4.1), which indicates that St. Augustinegrass can be affected by deacclimation and thus a loss of freeze tolerance. General rankings between the most freeze tolerant and freeze sensitive genotypes did not change when deacclimation occurred (Figure 4.1). However, this does not necessarily indicate that a genotype's ability to cold-acclimate is positively correlated with its ability to withstand deacclimation events. For example, Seville's unexpected ability to cold-acclimate drastically increases the genotype's

ability to recover as well as resist freezing injury and indicates that the genotype is equipped with cold acclimation genes. However, Seville's increased freeze tolerance is quickly lost after a deacclimation event (Figure 4.1). This indicates that deacclimation plays an important role in the low winter survivability of Seville in field studies and is representative of the complex relationships among mechanisms underlying freeze tolerance in St. Augustinegrass.

While survival was highest when plants were CA treated, DCA13 treated plants were generally the next top performers followed by DCA21 (Figure 4.1). However, significant differences between DCA13 and DCA21 were rarely identified as were differences between NCA and DCA treatments. The detrimental effects of DCA treatments may not be different from those of NCA and indicates that as St. Augustinegrass loses the improvement of freeze tolerance gained from cold acclimation, as it returns to a pre-acclimated state. This suggests that future screening of populations and nurseries could use NCA plants to gain an idea of a line's ability to resist deacclimation, especially using -4°C (Figure 4.1) and thus, minimize the loss of freeze tolerance in St. Augustinegrass.

During deacclimation events, temperature and deacclimation duration are two important factors affecting a plant's deacclimation sensitivity. To capture variation within St. Augustinegrass genotypes, it is possible that deacclimation temperatures should be increased. While genotypes appeared to be more sensitive to freeze injury and death when deacclimated at 21°C than 13°C, their responses were not significantly different. In bermudagrass, Zhang et al. (2011a) found significant deacclimation occurred as temperature changed from 21 to 23°C. However, in cool-season turf species annual bluegrass (*Poa annua* L.) and creeping bentgrass (*Agrostris stolonifera* L.), the largest differences in deacclimation response between the two species were detected at moderate temperatures (4 and 8°C), while

deacclimation sensitivity was more difficult to distinguish at higher temperatures (Hoffman et al., 2013). Since our deacclimation treatments (13°C and 21°C) were similar in this study, it may indicate that deacclimation in St. Augustinegrass is more strongly affected by duration of deacclimation rather than temperature. Regardless, further research is needed to fully understand important factors affecting deacclimation in St. Augustinegrass and its effects on winter survival.

CONCLUSIONS

This research demonstrated the positive effect of cold acclimation on freeze tolerance in St. Augustinegrass indicating that cold acclimation plays a crucial role in the winter survivability of the species. However, two genotypes were unresponsive to cold acclimation, which provides an excellent opportunity for future exploration into the physiological and metabolic changes that occur during cold acclimation in the species. A significant loss of freeze tolerance was also identified when plants were subjected to deacclimation events and suggests that St. Augustinegrass can be negatively affected by rapid temperature fluctuations. Further research is needed to explore the influence of temperature during deacclimation and the duration of deacclimation on St. Augustinegrass response.

Furthermore, these varied acclimation treatments will help to identify the highest levels of freeze tolerance during the selection process when screening genotypes as well as quantitative trait loci (QTL) associated with cold acclimation and deacclimation that can be stacked with freeze tolerance QTL for further improvement of cold tolerance in St. Augustinegrass. Overall, this study provides unique information regarding the complex relationships within and between mechanisms affecting freeze tolerance in St.

Augustinegrass.

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Table 4.1. Cultivars of St. Augustinegrass and experimental genotypes used in the U.S and their breeding information and release date.

Genotype	Experimental notation	Source/Breeder	Year Released
106T3	106T3	North Carolina State Univ., Raleigh, NC	NA[†]
Captiva	NUF-76	Univ. of Florida	2007[‡]
Floratam	FA-110	Florida Agriculture Experiment Station and Texas Agriculture Experiment Station	1973[§]
GF2	GF2	North Carolina State Univ., Raleigh, NC	NA
Palmetto		Sod Solutions	1995
Raleigh		North Carolina State Univ., Raleigh, NC	1983
Sapphire		Sod Solutions	2003
Seville	S-6-68-516	Pursely, Inc.; T.P. Riordan; O.M. Scotts & Sons	1978[¶]
Sunclipse	S-6-72-130	Pacific Sod Co., T.P. Riordan, O.M. Scotts & Sons	1988

[†] NA, not applicable. Experimental germplasm under development.

[‡] Lu, H., R. Nagata, K. Kenworthy, K. Quesenberry, and P. Busey. 2015. Registration of 'NUF-76' St. Augustinegrass. J. Plant Registrations. doi:10.3198/jpr2014.10.0073crc.

[§] Horn, G.C., A.E. Dudeck, and R.W. Toler. 1973. Floratam St. Augustinegrass: A fast growing new variety for ornamental turf resistant to St. Augustine decline and chinch bugs. Florida Agric. Exp. Stn. Circ. S-224.

[¶] Riordan, T.P., V.D. Meier, J.A. Long, and J.T. Gruis. 1980. Registration of Seville St. Augustinegrass. Crop Sci. 20:824-825.

Table 4.2. Repeated measures analysis of variance testing for the effect of time for surviving green tissue (SGT) and regrowth (RG) across temperatures, acclimation treatments, nine St. Augustinegrass genotypes, and experimental run.

		SGT		RG	
Source	DF	F-value	Pr > F	F-value	Pr > F
Time	4	52.44	<.0001	363.18	<.0001
Time*Temperature	4	7.13	<.0001	52.65	<.0001
Time*Acclimation	12	0.72	0.7288	8.14	<.0001
Time*Genotype	32	3.76	<.0001	13.99	<.0001
Time*Replication(Temperature*Acclimation*Run)	128	1.08	0.0951	1.27	0.0308
Time*Run(Temperature)	8	1.54	0.1404	0.77	0.6295
Time*Temperature*Acclimation	12	0.95	0.4949	1.35	0.1829
Time*Temperature*Genotype	32	0.98	0.4926	1.88	0.0024
Time*Acclimation*Genotype	96	1.01	0.4519	1.31	0.0297
Time*Acclimation*Run(Temperature)	24	1.24	0.5672	0.72	0.8371
Time*Genotype*Run(Temperature)	64	1.25	0.0922	0.93	0.6438
Time*Temperature*Acclimation*Genotype	96	1.53	0.0013	1.02	0.4307
Time*Acclimation*Genotype*Run(Temperature)	192	0.87	0.8933	0.72	0.9975

Table 4.3. Type III tests of fixed effects for surviving green tissue (SGT) and regrowth (RG) at two and six weeks post-freeze, respectively, for nine St. Augustinegrass genotypes across two freezing temperatures and four acclimation treatments.

		SGT Week 3		RG Week 6	
Effect	DF	F-value	Pr > F	F-value	Pr>F
Temperature	1	82.74	0.0119	221.19	0.0045
Acclimation	3	18.54	0.0019	33.41	0.0004
Genotype	8	24.18	<.0001	35.91	<.0001
Replication(Temperature*Acclimation*Run)	32	18.66	0.0822	17.26	0.1658
Run(Temperature)	1	1.11	0.264	0.24	0.7507
Temperature*Acclimation	3	3.24	0.1029	3.31	0.0987
Temperature*Genotype	8	3.75	0.0117	3.16	0.0238
Acclimation*Genotype	24	3.74	<.0001	3.15	0.0004
Acclimation*Run(Temperature)	6	0.09	0.9998	0.88	0.9125
Genotype*Run(Temperature)	16	7.34	0.3538	2.96	0.9727
Acclimation*Genotype *Run(Temperature)	48	8.29	0.9998	9.07	0.9995
Temperature*Acclimation*Genotype	24	12.56	0.1941	11.63	0.3715

Table 4.4. Mean separation of nine St. Augustinegrass genotypes response for surviving green tissue (SGT) and regrowth (RG) at three and six weeks post-freeze, respectively. Data are pooled over four different acclimation treatments.

	SGT† Week 3	RG† Week 6
-3°C		
GF2	0.44 a‡	0.49 a
Raleigh	0.43 a	0.47 a
106T3	0.42 a	0.43 a
Palmetto	0.21 b	0.28 b
Seville	0.20 b	0.27 b
Captiva	0.22 b	0.25 b
Sunclipse	0.08 c	0.19 b
Sapphire	0.20 b	0.18 b
Floratam	0.08 c	0.08 c
-4°C		
Raleigh	0.28 a	0.27 a
GF2	0.17 b	0.24 ab
106T3	0.15 bc	0.19 b
Palmetto	0.09 cd	0.11 c
Captiva	0.09 cd	0.10 cd
Sunclipse	0.05 de	0.08 cd
Sapphire	0.03 de	0.03 de
Seville	0.03 de	0.03 de
Floratam	0.01 e	0.00 e

†Surviving green tissue, which is reflective of the amount of freezing injury a plant sustains during freeze tests, was rated on a scale of 0-5 with 0=all tissue and nodes were brown, and 1=0-25%, 2=25-50%, 3=50-75%, 4=75-99%, and 5=100% surviving green tissue. Regrowth was also rated on the same 0-5 scale with a recovery rating of 0=no evidence of regrowth and 5=complete recovery of the turf stand.

‡Within columns and temperature, means followed by the same letter are not significantly different according to LSD (0.05).

Table 4.5. Testing for simple effects of genotype and acclimation for surviving green tissue (SGT) and regrowth (RG) three weeks and six weeks post-freeze, respectively, at -3°C and -4°C.

	SGT at -3°C		SGT at -4°C		RG at -3°C		RG at -4°C	
	F-value	Pr > F	F-value	Pr > F	F-value	Pr > F	F-value	Pr > F
Acclimation*Genotype Sliced by Acclimation								
CA	12.71	<.0001	9.27	<.0001	21.56	<.0001	8.02	<.0001
DCA13	5.8	<.0001	4.08	0.0002	6.82	<.0001	3.36	0.0016
DCA21	5.38	<.0001	3.75	0.0006	2.81	0.0066	2.28	0.026
NCA	6.65	<.0001	1.67	0.1122	3.81	0.0005	3.23	0.0022
Acclimation*Genotype Sliced by Genotype								
106T3	1.81	0.1491	3.75	0.0126	9.21	<.0001	3.58	0.0158
Captiva	1.81	0.1491	1.92	0.1293	0.35	0.7861	0.87	0.4599
Floratam	0.93	0.4282	0.13	0.9396	1.03	0.3803	0	1
GF2	2.21	0.0906	4.65	0.0041	5.28	0.0018	4.73	0.0036
Palmetto	6.46	0.0004	3.35	0.0211	3.87	0.0109	1.27	0.2892
Raleigh	3.83	0.0115	9.12	<.0001	8.74	<.0001	3	0.0331
Sapphire	1.49	0.2208	1.07	0.3632	3.19	0.026	0.87	0.4599
Seville	11.06	<.0001	1.21	0.3101	8.03	<.0001	1.73	0.1631
Sunclipse	1.17	0.3241	0.54	0.6582	1.74	0.1617	0.4	0.755

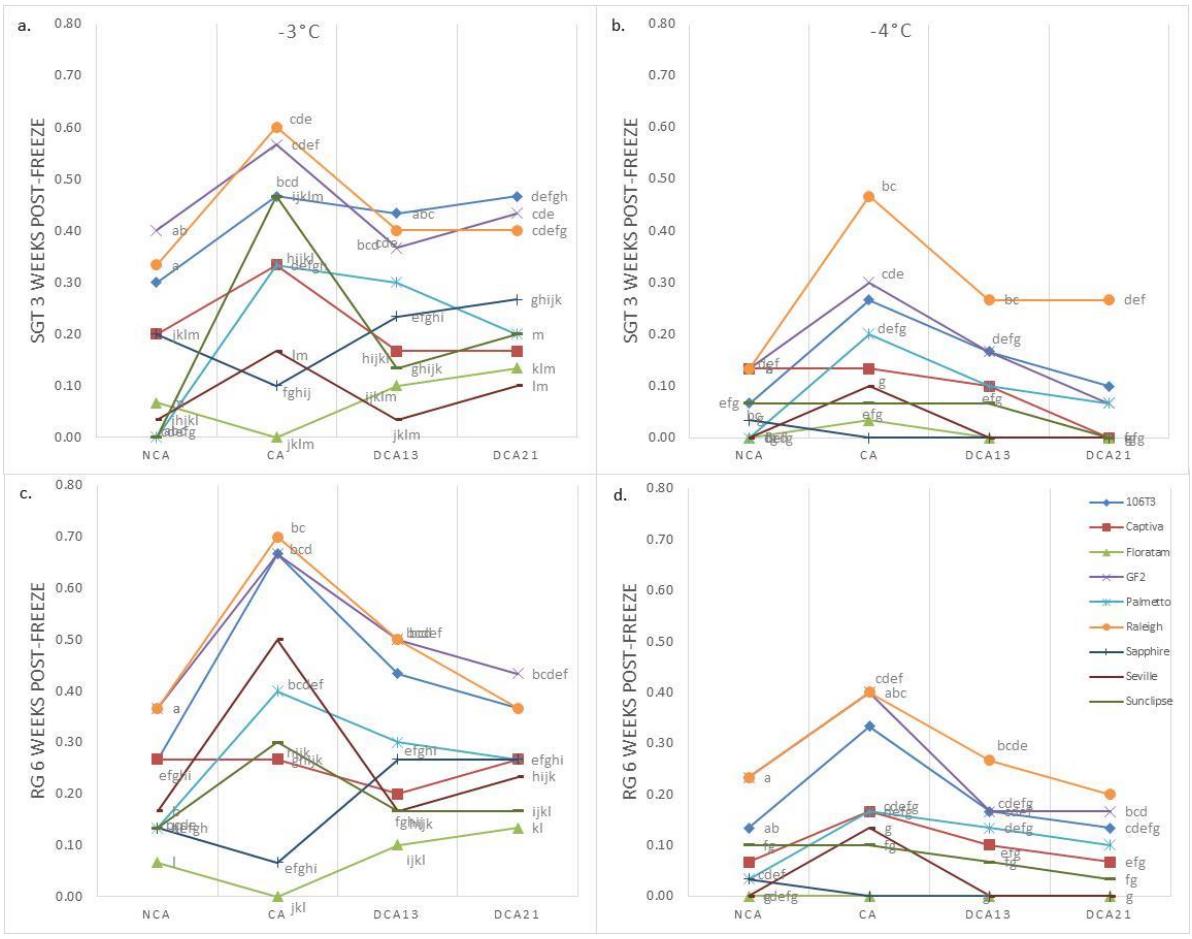

Figure 1: LSmeans and Fisher's LSD groupings for the acclimation by genotype interaction of nine St. Augustinegrass genotypes response for a. surviving green tissue at three weeks post-freeze at -3°C, b. surviving green tissue at three weeks post freeze at -4°C, c. regrowth at six weeks post-freeze at -3°C, d. regrowth at six weeks post-freeze at -4°C. Within subfigure, means followed by the same letter are not significantly different according to LSD (0.05).

-CHAPTER V-

Linkage Analysis and Identification of Quantitative Trait Loci Associated with Cold Tolerance and Turf Quality Traits in St. Augustinegrass

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ABSTRACT

St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] is a warm-season turfgrass commonly grown in the southern United States. Currently, no genetic linkage map exists to aid breeders in identifying loci controlling traits of interest in the species, such as winter survival and aesthetic characteristics. In this study, the first linkage map for the species was constructed for cultivar 'Raleigh' and cultivar 'Seville' using a pseudo-F_2 strategy. A total of 178 simple sequence repeat markers were mapped to nine linkage groups (LGs) covering a total distance of 1299.95 cM. To demonstrate the usefulness of the map, preliminary attempts were made to map quantitative trait loci (QTL) controlling field winter survival, freeze tolerance, and turf quality traits. Putative QTL were identified for all traits across multiple environments with the exception of winter survival, where QTL were only identified in single environments. Specific genetic regions were identified with overlapping QTL on LG4 for winterkill and spring green-up as well as on LGs 2, 3, 4, 6, and 9 between winter survival traits collected in the field, and survival and recovery traits collected in the laboratory post-freeze. These results provide strong support for cold tolerance related QTL in these regions. A large region on LG5 was also identified as a possible region for a large sweep of freeze tolerance and cold acclimation QTL due to the large number of QTL that were identified in survival and recovery traits across freeze temperatures and acclimation treatments. Similar overlapping QTL regions for turf quality traits were identified on LGs 3, 4, and 9. These results present the first complete linkage map produced for St. Augustinegrass, providing a template for further genetic mapping. Additionally, markers linked to the QTL identified may be useful to breeders for transferring these traits into new breeding lines and cultivars.

INTRODUCTION

St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze) is a warm-season, perennial turfgrass naturally adapted to tropical and subtropical climates. Commonly grown in home lawns and commercial landscapes in the southern United States, the species is utilized for its superior shade tolerance, stoloniferous growth habit, and moderately low input requirements when compared to other popular turf species (Busey, 2003). Conversely, St. Augustinegrass is one of the least cold tolerant, warm-season turfgrass species, which affects its marketability in the transitional climatic region of the United States. Studies have reported significant variability in winter survival and freeze tolerance among present-day St. Augustinegrass varieties (Moseley et al., 2010, Reynolds et al., 2009, Milla-Lewis et al., 2013, Kimball et al., 2016 b and c). 'Raleigh' is a popular, diploid St. Augustinegrass cultivar developed in the early 1980's (Bateman, 1980) and is still considered an industry standard for cold tolerance in the species. However, Raleigh's coarse leaf texture and long internodes when compared to other St. Augustinegrass cultivars as well as other warm-season turfgrasses are often undesirable (Reynolds et al., 2009). Conversely, cultivars with sought-after turf quality and related morphological characteristics often lack the necessary levels of cold tolerance to survive in the species' northern limits of adaptation. For example, 'Seville' is a diploid, semi-dwarf cultivar released in 1980 by O.M. Scott and Sons Company (Riordan et al., 1980) that has a finer leaf texture and lower growth habit than standard cultivars such as Raleigh (Busey, 1986). Like the majority of semi-dwarf cultivars though, Seville is cold sensitive and suffers significant winterkill (Philley et al, 1998).

While phenotypic assessment of these traits has been well documented (Reynold et al., 2009; Moseley et al., 2010; NTEP 2012; Kimball et al., 2016b; Kimball et al., 2016c),

their genetic components have not been studied. Genetic resources in warm-season turfgrasses, particularly St. Augustinegrass, have lagged behind crop species such as the cereals. Dense genetic maps, for example, have been developed in numerous crop species to study genome organization, dissect complex traits, and ultimately implement marker-assisted selection. Co-dominant molecular markers, particularly simple sequence repeats (SSRs), are very popular for genetic mapping due to their ease of assay, high polymorphic rates, and ability to detect heterozygosity at a locus. While SSR markers have been employed for linkage analysis in several warm-season turf species including bermudagrass (*Cynodon dactylon* (L.) Pers.) (Bethel et al., 2006; Harris-Schultz et al., 2010) and zoysiagrass (*Zoysia* spp.) (Cai et al., 2004; Li et al., 2009; Jessup et al., 2011), no such studies have been conducted in St. Augustinegrass. Recently, a large set of SSR markers have been developed for the species (Mulkey et al. 2013) making it possible to conduct linkage analyses using a co-dominant marker system possible.

Linkage maps are typically constructed using inbred populations derived from an F_1 of a cross between two homozygous inbred parents. In outcrossing plants, such as St. Augustinegrass, segregating F_2 or backcross populations are rarely available due to the difficulty of developing these crosses, and a significant genetic load. Therefore, a "pseudo-F_2" mapping strategy can be employed using F_1 crosses developed from heterozygous parents to construct individual linkage maps for each parent and then to create a consensus linkage map for the entire population (Grando et al., 2003). This strategy has been applied in many turfgrass species including bermudagrass (Bethel et al., 2006), creeping bentgrass (*Agrostis stolonifera* L.) (Chakraborty et al., 2005; Bonos et al., 2011; Zhang et al., 2012), ryegrass (*Lolium* spp.) (Jones et al., 2002; Warnke et al., 2004; Studer et al., 2010), and tall

fescue (Saha et al., 2005).

Like linkage mapping, quantitative trait locus (QTL) mapping for important agronomic traits has been extensive in major agricultural species for a range of phenotypic characteristics, and abiotic and biotic stresses (Forster et al., 2004). In turfgrass, the majority of QTL mapping studies have been in the cool-season grasses, particularly tall fescue (Xu et al., 1995; Saha et al., 2005; Fribourg et al., 2009) and perennial ryegrass (*Lolium perenne* L.) (Hayward et al., 1994; Bert et al., 1999; Jones et al., 2002; Warnke et al., 2004; Yamada and Forster, 2005; Xiong et al., 2007), and have mainly focused on disease resistance, abiotic stress tolerance, reproductive development (i.e. self-incompatibility), and morphological characteristics (Forster et al., 2004). For warm-season turfgrass species, QTL mapping has been limited. Salinity tolerance (Guo et al., 2014), fall army worm resistance (Jessup et al., 2011), and cold tolerance (Ding et al., 2010) have been mapped in zoysiagrass. In St. Augustinegrass, genetic linkage maps for QTL analysis of resistance to gray leaf spot have been constructed (Mulkey, 2012). However, only three consensus linkage groups were created from parental linkage maps. To date, a full haploid map of St. Augustinegrass (1n=9) has not been constructed.

There is a need within the breeding community for a better working knowledge of the underlying genetics, including causal polymorphisms, conferring cold tolerance as well as economically important morphological traits in St. Augustinegrass. Additionally, molecular markers linked to traits of interest would provide a stepping stone to implementing marker-assisted selection (MAS) within the species. For this purpose, a mapping population of a cross between cultivars Raleigh and Seville was created to study the genome's organization and to identify QTL conferring cold hardiness. The objectives of this study are to (1) to

develop an SSR linkage map using a Raleigh x Seville mapping population, and (2) to identify QTL associated with winter survival and turf quality-related traits using both field evaluations and laboratory-based freeze tests.

MATERIALS AND METHODS

Population Development. One hundred and twenty F_1 hybrids were developed from crosses between Raleigh and Seville following embryo rescue methods described by Genovesi et al. (2009). Plants were grown in the greenhouse in plastic containers containing Fafard potting mix (Conrad Fafard Inc., Agawam, MA) and fertilized at a rate of 0.45 kilograms of nitrogen per 1000 square feet every month using Scotts® Starter® Fertilizer (The Scotts Company LLC, Marysville, OH) prior to field and laboratory evaluations.

Genotypic Analysis. DNA was extracted according to Kimball et al (2013). Six hundred SSR markers were screened for polymorphism using parental DNA. Specific primer information for 215 of the SSRs is reported in Mulkey et al (2013); information for the remainder is in Supplementary Table 1. Progeny lines were genotyped with two hundred polymorphic SSRs following PCR conditions described in Mulkey et al (2013). SSR fragments were separated by polyacrylamide gel electrophoresis (PAGE) on a LI-COR 4300 DNA Analyzer Sequencer on 25-cm gels using 8% v/v denaturing polyacrylamide gels.

Linkage Mapping. For linkage analysis, a χ^2 test was performed for each marker to test for segregation distortion. Markers showing the appropriate Mendelian segregation ratios for individual markers were used to construct a framework map for each parent using Joinmap 4 (Van Ooigen, 2006). The population was treated as a 'cross pollinator' and a logarithm of odds ratio (LOD) of 4.0 was used to identify initial marker groupings. A 1:1

segregation ratio indicates that loci are in a heterozygous state in one parent and a homozygous state in the other parent. Markers showing a 1:1:1:1 segregation pattern, which indicates heterozygosity in both parents, were used to find homologous groups between the parental maps. The homologous group nodes from each parental map were selected and used to construct integrated linkage groups (LG). Map distances were calculated using the Kosambi mapping function (Kosambi, 1994).

Field Testing. Progeny and parental lines were planted in a randomized complete block design (RCBD) with three replications at two locations, the Lake Wheeler Turfgrass Field Lab (LW) in Raleigh, NC, and the Upper Mountain Research Station (LS) in Laurel Springs, NC. The LW environment (106m elevation, 32.0°C average summer high temperature, and 1.1°C average winter low temperature), located in the Piedmont region of North Carolina, has higher year-round temperatures and less snowfall than the LS environment (838m elevation, 26.6°C average summer high temperature, and -7.2°C average winter low temperature), which is located in the Blue Ridge/Appalachian mountain range of North Carolina. Plots were 0.91m x 091m in size and mowed weekly at a height of 6.35 cm, fertilized monthly at a rate of 0.23 kg ha^{-1} from May to October to accumulate 1.36 kg ha^{-1} for a year, and irrigated to avoid drought stress. Plots were evaluated over the 2012-2015 seasons.

Traits were grouped into two categories: cold hardiness-related traits, which included winterkill and spring green-up; turf quality-related traits, including overall turf quality, leaf texture, genetic color, and turf density. The remaining traits were evaluated visually on a 1 to 9 scale according to the National Turfgrass Evaluation Program's (NTEP) guidelines (NTEP, 2012) as follows: (1) spring green-up, 1=no green-up and 9=complete green-up; (2)

winterkill, 1=complete winterkill and 9=complete survival; (3) turf quality, 1=poor quality and 9=excellent quality where 5= minimum acceptable quality; (4) leaf texture, 1= coarsest texture and 9=finest texture; (5) genetic color, 1=light green/yellow and 9=dark green; (6) turf density, 1=sparsest density and 9=densest turf.

Laboratory-based Freeze Testing. Laboratory-based freeze tests were conducted to assess cold acclimation ability and freeze tolerance in progeny lines according to Kimball et al (2016b) freeze methods with the exception that a modified refrigerated chamber with vertical air flow as well as three vertical rotating fans were used to provide constant air circulation. Before freeze tests, progeny lines were cold-acclimated (CA) as well as non-acclimated (NCA) according to Kimball et al (2016c). Progeny lines were evaluated at two freezing temperatures, -3°C and -4°C to ensure a range of freeze tolerance variability. Due to the size of the population, one replication of CA and NCA-treated progeny lines were frozen at a time per temperature for a total of three runs per temperature.

Progeny lines were evaluated visually for surviving green tissue (SGT) and regrowth (RG) according to Kimball et al (2016b) with the exception that ratings were taken once every two weeks for six weeks post-freeze. These ratings assessed the whole turf stand in each pot. Surviving green tissue, based on percent green leaves and nodes, was rated on a scale of 0-5 with 0=all tissue and nodes brown, 1=0-25%, 2=25-50%, 3=50-75%, 4=75-99%, and 5=100% surviving green tissue. It should be noted that surviving green tissue is representative of the severity of freezing injury a plant sustains during freezing tests. Regrowth was also rated on the same 0-5 scale with a recovery rating of 0=no evidence of regrowth and 5=complete recovery of the turf stand.

Phenotypic Data Analysis. Due to limited variability mainly caused by high levels

of winterkill, data from locations at different years were dropped from the analysis. Because year by location interactions could not be adequately tested, these factors were pooled together as "environments", with each year by location combination composing a separate environment. An analysis of variance (ANOVA) and least square (LS) means were generated using the general linear model procedure (PROC GLM) in SAS statistical software version 9.4 (SAS Institute, 2015) for both field and freeze testing. Standard *F*-tests in all analyses were used to determine significance of main effects and interactions. In the model, tests were considered random, while the main effects were considered fixed. Individual hypothesis testing of effects and interactions was designated using an appropriate error term in the GLM procedure.

QTL Mapping. Using WinQTL Cartographer 2.5.009 (Wang et al., 2011), composite interval mapping (CIM) was used to identify QTL, estimate QTL LOD scores, and measure the proportion of phenotypic variation explained by individual QTL. Because pseudo-F_2 mapping populations can have up to four segregating alleles per locus and WinQTL Cartographer requires genotypic data to be in bi-allelic form, SSRs were rescored as dominant for each allele when more than two alleles were present. Single marker analysis was first performed to quickly identify regions strongly correlated with winter survival, freeze tolerance, and turf quality traits. Following this, interval mapping (IM) and 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 500 times at a significance level of $P<0.05$.

RESULTS

Genotypic Analysis and Linkage Mapping. Of the 600 SSR markers screened, 295 were initially identified as polymorphic between the parents. Due to difficulty of scoring, repeated amplification failure, or skewed segregation ratios, 117 markers were excluded from analysis. Using a LOD of 4.0, a total of 178 SSRs were mapped to 18 initial LGs (nine for each parent) consistent with the 2x=2n=18 genetic makeup of St. Augustinegrass. The individual parental LGs were successfully merged with one another to create a consensus linkage map with nine LGs. The final map, composed of 178 SSRs on nine merged LGs, covered a total distance of 1299.95 cM, with an average marker distance of 7.6 cM (Figure 5.1). Total distance of individual LG ranged from 109.0 to 169.4 cM with an average LG size of 144.44 cM. The number of SSRs in individual LGs ranged from 13 to 28 with an average of approximately 20 SSRs per LG. The gaps between markers ranged from 0 cM to 34.6 cM.

Field Testing. Significant differences were identified for genotype as well as environment for winterkill, spring green-up, turf quality, leaf texture, genetic color, and turf density (Table 5.1). The genotype by environment interaction was only significant for winterkill and spring green-up, and not for turf quality traits. Due to limited variability mainly caused by high levels of winterkill, several environments (year x location combination) had to be dropped from analysis. Three environments were utilized in the final analyses including LW 2013, LS 2013, and LW 2015. Due to limited variability for winterkill and spring green-up in LS 2013, this environment was dropped for these traits in the QTL analyses.

Among the parents, Raleigh had the highest tolerance to winterkill and the best spring green-up, while Seville exhibited the best turf quality, leaf texture, genetic color, and turf

density within each environment as well as averaged over the three (Table 5.2). Progeny averages fell in between parental means for all traits with the exception of turf quality and turf density, where progeny means were lower than either parent (Table 5.2). In different environments, several progeny performed as well or better than Raleigh in terms of winterkill (LW 2015) and spring green-up (LW2013). Additionally, some progeny performed as well or better than Seville in terms of turf quality (LS 2013 and LW 2015), leaf texture (LS 2013 and LW 2015), genetic color (LS 2013), and turf density (LW 2013). For spring green-up, progeny maximum values were similar to Raleigh's performance.

Laboratory Freeze Testing. An overall model for the Type III test of fixed effects for SGT and RG across temperature, acclimation, genotype, and their interactions is presented in Table 5.3. Temperature, acclimation treatment, and genotype were statistically significant for RG (Table 5.3). Replications were not significantly different for visual ratings of SGT and RG. Significant two-way and three-way interactions were only identified in SGT (Table 5.2). Among the parents, Raleigh performed the best across both freezing temperatures and acclimation treatments for all traits with the exception of cold acclimated Seville at -3°C for SGT (Table 5.4). Cold-acclimated Raleigh recovered better than NCA Raleigh and Seville as well. Progeny averages were lower than Raleigh and closer to Seville's mean for all traits (Table 5.4). However, several progeny outperformed Raleigh for all freezing survival and recovery traits.

QTL Identification. Windows QTL cartographer 2.5 was used to identify putative QTL for winter survival and turf quality traits evaluated in the field as well as for survival and recovery after freeze tests based on genotypic and phenotypic data from 121 progeny. A LOD threshold of 2.5 for the detection of significant QTL was determined through WinQTL

Cartographer's permutation testing function.

Field Testing. Due to significant variability found among environments and also to evaluate QTL stability, QTL analysis was performed with phenotypic data from individual environments and also with means across environments. The majority of traits with the exception of winterkill yielded significant LOD peaks at similar genetic locations across multiple environments through the use of composite interval mapping. A summary of each QTL, their estimated effects, and the closest flanking markers is provided in Table 5.5.

Severe winter stress at the LS field location in 2013 completely killed the majority of lines, resulting in very little variation among genotypes. Consequently, winterkill and spring green-up were only evaluated for the LW location in 2013 and 2015. A total of six QTL-containing regions were discovered for both winterkill and spring green-up when analyzing the traits within and across all environments (Table 5.5). Estimates of the variation explained by individual QTL ranged from 2.4-44.4 percent for winterkill, and 0.9-76.9 percent for spring green-up.

Two QTL were identified for winterkill on LG3 (27 cM and 48 cM) at LW in 2013. In LW 2015, two QTL were located to LG4 (3 cM and 96 cM), and one QTL to LG8 (76 cM). Interestingly, when performing QTL analysis using LS means derived from both locations, an additional QTL on LG2 (12 cM) was identified. Spring green-up showed much greater reproducibility across environments. Analysis of LW data in both 2013 and 2015 located QTL to LGs 2 (between positions 80-115 cM), 4 (between positions 38-59 cM and at 100 cM), and 6 (between position 74-90 cM). Almost all of the QTL identified had major effects (R^2 > 20%). One QTL on LG3 (between positions 69-99 cM) was detected when analyzing LW 2015 data individually as well as when analyzing all environments together.

Another QTL on LG9 (73 cM) was detected only at LW in 2015.

An analysis of turf quality traits within environments identified a total of three putative QTL for turf quality, and five putative QTL each for each of the leaf texture, genetic color, and turf density traits, respectively (Table 5). The proportion of variation (R^2) explained by individual QTL for these traits ranged from 7.4 to 28.2%, 1.1 to 69.8%, 6.0 to 27.1%, and 0.49 to 50.8 cM%, respectively. Analysis using LS means from across all environments identified two, three, four, and one QTL for turf quality, leaf texture, genetic color, and turf density, respectively. The putative QTL derived from an all-environment analysis were located at similar positions to those found for single-environment analyses in all cases except one (genetic color on LG9), and had similar R^2 estimates. Examples of the overlap in QTL across environments using turf quality and genetic color are depicted in Figures 5.2a and 5.2b, respectively.

For turf quality traits, many of the regions containing QTL were consistent across at least two of the three environments. Estimates of the proportion of variability explained by putative QTL in a particular region were generally consistent as well, though in some cases R^2 estimates varied by up to 20%, *e.g.*, turf quality at LG2. In the case of genetic color, a drastic difference in QTL estimates was observed (R^2 from 1.4-50.8). Despite this apparent lack of consistency, similar patterns of LOD peaks were observed across environments, even though the peaks did not surpass the minimum LOD threshold (Figure 5.2b). These patterns were also observed for turf quality, leaf texture, and turf density as well.

Laboratory Freeze Testing. Freeze tests conducted at -3°C and -4°C tests were analyzed separately due to significant differences identified between experiments (Table 5.3). Acclimation treatments within each freezing temperature were also analyzed separately

in an attempt to identify putative cold acclimation QTL. Analysis of SGT within each of the four temperature by acclimation treatment combinations identified a total of seven putative QTL for NCA at -3°C, five for CA at -3°C, and four QTL for each NCA and CA at -4°C (Table 5.6). The proportion of variation explained by these individual QTL ranged from 1.3 to 89.4%, 0.1 to 88.2%, 0.1 to 6.6%, and 11.4 to 83.6% for each treatment combination, respectively. Evaluation of RG within each treatment combination identified one putative QTL for NCA at -3°C, six for CA at -3°C, seven for NCA at -4°C, and six for CA at -4°C (Table 5.6). The R^2 estimates for these individual QTL ranged from 85.2%, 0.5 to 88.6%, 0.4 to 70.1%, and 2.6 to 54.8%, respectively.

Putative QTL for both survival and recovery were often found at the same loci. For example, analysis of RG and SGT at -3°C each identified QTL at ~39 cM on LG1. Regrowth and survival QTL also collocated on LG1 (~103 cM), LG4 (~102 cM), LG5 (~14 cM), LG6 (68 cM), and LG7 (~26 cM). Similarly, the regions spanning 90.41-144.81 cM on LG5, and 47.51-69.11 on LG9 harbored several QTL for each trait (Table 5.6, Figure 5.1). These overlapping QTL regions were generally valid at both levels of freezing temperature. An example of this phenomenon is depicted in Figure 5.2c.

Field vs. Controlled Freezing Experiments. A comparison of field data and laboratory freeze test data revealed several common genetic regions that contributed to survival and recovery. Winterkill and RG QTL collocated on LG2 (~14 cM), spring green-up and SGT collocated on LG3 (~105 cM), and spring green-up and SGT QTL collocated on LG9 (~70 cM) (Table 5.5 and 5.6, Figure 5.2d). Linkage group four showed clusters of field winter survival and freeze tolerance QTL in two regions (at ~55 cM: spring green-up, SGT, RG; at ~100 cM: winterkill, spring green-up, SGT, and RG). An example of this phenomenon on

LG4 is depicted in Figure 2d. Linkage group six also had overlapping QTL for spring green-up, RG, and SGT at approximately 70 cM.

DISCUSSION

Genotypic Analysis and Linkage Mapping. In this study, a pseudo-F_2 mapping approach was utilized to generate the first complete linkage map with nine linkage groups representative of the nine haploid chromosomes present in St. Augustinegrass (2x=2n=18). Building consensus linkage maps using the pseudo-F_2 mapping strategy such as the one reported here have been successful in a number of species, especially tree and shrub species including *Citrus grandis* (L.) (Weber et al., 2003), cassava (*Manihot esculenta* subsp. *esculenta* Crantz) (Okogbenin et al., 2008), and eucalyptus (*Eucalyptus grandis* Hill ex Maiden) (Grattapaglia and Sederoff, 1994). This approach has also been utilized for several turfgrass species including creeping bentgrass (*Agrostis stolonifera* L.) (Bonos et al., 2011; Honig et al., 2014), ryegrass (Studer et al., 2010), and zoysiagrass (Jessup et al., 2010). A total of 178 SSR markers were used to develop the map spanning a total distance of 1299.95 cM. While the average distance between SSRs was 7.6 cM, gaps of 20-35 cM were present within the linkage groups, indicating areas of low recombination, limited marker coverage, or weak linkages (Jairin et al., 2013). Additional molecular markers could improve uniform coverage in these areas, which has been a successful approach in perennial ryegrass (Warnke et al., 2004) and zoysiagrass (Li et al., 2009), as well as other species with limited genetic resources such as rose (Rosa sp.) (Yu et al., 2014). As St. Augustinegrass is a vegetatively propagated species, the mapping population used in this study is immortal and the addition of more molecular markers, such as newly developed SSRs or SNPs, is possible in the future.

A preliminary St. Augustinegrass linkage map developed using a pseudo-F_2 population from a cross between Raleigh and PI410353 for mapping disease resistance was developed in 2012 (Mulkey, 2012). Markers in common between that map and the one produced here show conserved marker orders. For example, two or more SSRs within LGs 2, 4, and 5 in this study are consistent on linkage groups R12, R10, and R9/P9 in Mulkey (2012). Additionally, linkage groups P6 and R13 correspond to the large LG 3 in this study indicating that P6 and R13, two previously unmerged LGs on the individual parental Raleigh and PI410353 maps, could possibly be merged to create a consensus LG between the two parents. This provides an example of the importance of an abundant set of highly polymorphic co-dominant markers for combining parental maps in a pseudo-F2 mapping population. Markers showing polymorphism within each parent are especially crucial in the context of this mapping approach.

QTL Identification in Field Environments. One of the largest problems in characterizing the genetic control of winter survival is the inconsistency of field testing results. Intensity, frequency, and duration of freeze events, as well as snowfall and snow-cover can drastically affect assessments of winter survival (Lewitt, 1980; Blum, 1988). Changing environmental conditions can alter complex physiological pathways in numerous ways, causing variability in a plant's potential to cold acclimate or resist deacclimation (Palta et al., 1997). In turfgrass species, studies have shown that cold acclimation (Fry et al., 1993; Dionne et al., 2001; Milla-Lewis et al., 2013), deacclimation (Webster et al., 2005; Zhang et al., 2011; Kimball et al., 2016c), low temperature tolerance (Dunn et al., 1999), and freeze tolerance (Patton et al., 2007) all play important roles in winter survival. All of these specific factors which are critical for winter survival can fluctuate from year to year depending on the

environment.

Previous mapping studies have attempted to identify cold tolerance QTL in several forage and turfgrass species including perennial ryegrass (*Lolium perenne* L.) (Yamada et al., 2004), meadow fescue (*Festuca pratensis* Huds.) (Rognli et al., 2002; Alm et al., 2011), and zoysiagrass (Guo et al., 2012). In perennial ryegrass, Yamada et al. (2004) utilized the laboratory-based electrolyte leakage methodology to evaluate freeze tolerance after detecting no significant QTL for field winter survival. In this study, QTL for winterkill were detected, however, only in individual environments. Similar findings have been reported in lentil (*Lens cularis* Medik.) (Eujayl et al., 1999; Kahraman et al., 2002), alfalfa (*Medicago sativa* L.) (Brouwer et al., 2000), and pea (*Pisum sativum* L.) (Jenaut et al., 2008), where single winter survival QTL were detected within individual environments. The low level of consistency for winterkill across environments is indicative of the difficulty faced when trying to dissect the complex underlying genetic architecture regulating this trait. It also highlights how inconsistent field environments can be (Fowler, 1979), and the importance of using controlled environment testing to get a better estimate of the genetic differences influencing this trait.

While multi-environment QTL analysis is more powerful than single-environment analysis, phenotypic plasticity in plants can confound QTL mapping as genotype-by-environment (GxE) interactions induce alterations in gene expression and plant physiology (El-Soda et al., 2014). Phenotypic plasticity has previously been identified in both parents used in this study in regards to their cold acclimation and deacclimation abilities as well as their response to different freezing temperatures (Milla-Lewis et al., 2013; Kimball et al., 2016b, 2016c). Unlike the parameters for cold tolerance, spring green-up appears to be more

consistent across different environments, indicating that the genetic factors controlling this trait have a more straightforward interaction with environmental effects. Given that QTL for spring green-up overlapped with winterkill at only one locus on LG4, it seems unlikely that these two traits are controlled by the same mechanisms.

In many cases, QTL regions for different phenotypic parameters overlapped. For example, QTL for both genetic color and turf quality were found at 30 cM on LG3. Overlapping regions for turf quality, leaf texture, and turf density were identified at roughly 80 cM on LG3 as well. Similar overlap was identified on LG4 for turf density and leaf texture, and on LG9 for leaf texture and genetic color. As turf quality is essentially a composite parameter based on the other three factors (leaf texture, genetic color, and turf density), it is not surprising to see QTL for this trait overlapping with the others. However, the fact that overlapping regions were also observed for leaf texture, genetic color, and turf density indicates that these regions may contain genes controlling important morphological characteristics. Clusters of putative QTL within the same genetic region are commonly found in QTL mapping studies, suggesting the existence of gene regions governing specific traits (Zhang et al., 2006), and are good candidate regions for fine mapping as well as use in marker-assisted selection. Colocation of QTL for similar types of traits have been reported in perennial ryegrass for morphological characteristics (Yamada et al., 2004) and also in tomato (*Solanum lycopersicum* L.) for a number of horticulturally important traits such as fruit size and shape, fruit quality, and plant architecture traits (Haggard et al., 2013).

QTL Identification in Freeze Testing. There is strong evidence supporting the hypothesis that freeze tolerance plays a key role in a plant's ability to adapt to winter environments (Volence et al., 2002). Due to inconsistencies in field evaluations of winter

survival, freeze testing in a controlled environment is a powerful tool to identify novel genes involved in freeze tolerance. Multiple QTL were detected for survival and recovery traits in this study, many of which were consistent across freeze temperatures as well as acclimation treatments. These results suggest these putative QTL regions are good candidate regions for genes of interest related to freeze tolerance in St. Augustinegrass. However, several unique QTL were identified within specific testing schemes, *i.e.,* temperature and acclimation treatments. Similar findings have been reported in *Citrus* where unique QTL were identified in both -9°C and -15°C freeze test conditions (Weber et al., 2003) and in the genus *Salix* where individual QTL were detected within specific temperature x acclimation schemes (Tsarouhas et al., 2004). In Salix, they identified more QTL at higher freezing temperatures (Tsarouhas et al., 2004), while QTL detection at different freezing temperatures was comparable in this study (18 at -3°C and 21 at -4°C). These findings are very similar to field evaluations of winter survival, which further speaks to the complexity of the genetic architecture underlying evaluation of traits relating to cold tolerance.

Cold acclimation is a natural process whereby plant cells upregulate and downregulate the production of various cellular components when exposed to low, non-freezing temperatures (Fowler and Thomashow, 2002). As a result, cold acclimation improves a plant's freeze tolerance. Previous studies have indicated warm-season grass species, including St. Augustinegrass, respond well to cold acclimation (Anderson et al, 1988; Anderson et al, 1993; Fry et al., 1993; Qian et al., 2001; Anderson et al, 2003; Sahba et al., 2003; Patton and Reicher, 2007; Li et al., 2010). 'Raleigh' St. Augustinegrass readily acclimates to cold temperatures (Maier et al., 1994b; Li et al., 2010). Additionally, Milla-Lewis et al. (2013) identified differences between Raleigh and Seville's cold acclimation

response. In this study, QTL detected in the cold acclimated versus the non-acclimated treatments were considered putative QTL for cold acclimation ability. Several regions fit this criteria within specific temperature treatments, including two regions located on LG5 (~143 cM) for RG at -4°C and LG9 (~48 cM) for SGT at -3°C. Two additional regions were identified across temperature treatments located on LG2 (~77 cM) (Table 6, Figure 2c) and LG5 (~30 cM as well as 98 cM) that were identified across either trait and/or temperature combinations. Using the same rationale, cold acclimation-related QTL have been identified in multiple plant species including pea (Dunne et al., 2009), *Salix* (Tsarouhas et al., 2004), and faba bean (*Vicia faba* L.) (Arbaoui et al., 2008). Identified regions in this study may play an important role in regulating the cold acclimation of St. Augustinegrass during fall months and contribute to survival of turf stands throughout the winter.

Comparison of QTL identified in Field and Freeze Testing. The genetic components of cold tolerance and related traits have been examined in numerous plant species, including *Triticeae* (Cattivelli et al. 2002), oat (*Avena sativa* L.) (Wooten et al. 2009), ryegrass (Xiong et al. 2007), and rapeseed (*Brassica napus* L.) (Kole et al. 2002; Asghari et al. 2008). In these studies QTL commonly clustered at a limited number of specific regions. Here, we report the colocation of several QTL identified through both field and freeze test experiments. These results are congruent with studies in alfalfa (Brouwer et al., 2000), *Arabidopsis* (Oakley et al., 2014), legume (*Medicago truncatula*) (Avia et al., 2013), pea (Lejeuene- He´naut et al., 2008; Dumont et al., 2009), ryegrass (Xiong et al., 2007), and *Salix* (Tsarouhas et al., 2004), which also reported the colocation of QTL from both field and laboratory-based experiments. This consistency across environmental conditions, experimental testing schemes, and genetic backgrounds increases the likelihood

that the observed QTL are estimating true gene locations. The QTL that colocated in this study show great promise for the identification of putative genes contributing to cold tolerance within St. Augustinegrass, and are excellent candidates for additional research towards fine mapping. Furthermore, markers linked to the QTL identified here may be implemented via a marker assisted selection scheme for use in germplasm improvement.

CONCLUSIONS

Compared to other turfgrass species, St. Augustinegrass has lagged behind in the development of genetic resources for application in breeding programs. This paper presents an additional 200 SSR markers characterized for St. Augustinegrass and the first linkage map of the species, providing a foundation for the description of the species' genomic architecture. Additionally, putative QTL for winter survival, freeze tolerance, and turf quality traits have been identified along with linked markers which will facilitate the incorporation of these traits into elite germplasm. The colocation of several cold/freeze tolerance-related QTL as well as turf quality-related QTL provides strong support that the regions identified are good candidates for true gene locations. These resources will enable further investigation within the germplasm and can act as a framework for identifying loci underlying other important traits, such as disease resistance.

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Table 5.1. Analysis of variance significance values for 117 progeny of a Raleigh x Seville cross evaluated in three field environments for cold tolerance and turf quality traits.

Source	DF	Winterkill	Spring Green-up	Turf Quality	Leaf Texture	Genetic Color	Turf Density
Genotype	116	0.0081	0.0158	<.0001	<.0001	<.0001	<.0001
Environment	2	<.0001	<.0001	<.0001	0.0855	<.0001	<.0001
Replication(Environment)	6	0.0887	0.0017	0.0065	0.3987	0.0003	0.3877
Genotype*Environment	221	<.0001	<.0001	0.2533	0.3675	0.6651	0.6398

Table 5.2. Lsmeans for parents, Raleigh and Seville, and progeny average, minimum, and maximum trait values for winterkill, spring green-up, turf quality, leaf texture, genetic color, and turf density calculated for three individual field environments as well as an across environments calculation.

	Winterkill				Spring Green-Up				Turf Quality			
Environment†	LW 13	LS13	LW15	Across	LW 13	LS13	LW15	Across	LW 13	LS13	LW15	Across
Raleigh	8	0.7	4	4.2	6.3	0.7	5.7	4.2	6	6.3	6	6.1
Seville	1	0	1.7	0.8	0	0	1.7	0.5	8	7.3	7.7	7.6
Progeny Min	1	0	0.3	0.4	0	0	0.3	0.1	4.3	3.3	4	4.2
Progeny Max	7.5	0	6	4.2	7.3	0	5.3	4	7.7	7.7	8	7.3
Progeny Avg	2.9	0	3.2	2	1.7	0	2.3	1.3	5.9	5.7	6.1	5.9
	Leaf Texture				Genetic Color				Turf Density			
Environment†	LW 13	LS13	LW15	Across	LW 13	LS13	LW15	Across	LW 13	LS13	LW15	Across
Raleigh	5.3	5.3	5.3	5.3	5.7	7.7	6.3	6.5	6.3	5.7	6.3	6.1
Seville	8.3	6	7.7	7.3	9	8	9	8.6	7.3	7.3	7.3	7.3
Progeny Min	4.3	3.3	4	4.2	3.7	5.8	3.7	4.7	2.7	2.3	3	2.8
Progeny Max	7.7	7.3	8	7.3	9	8.7	8.3	8.5	7.7	7.3	7	6.9
Progeny Avg	5.7	5.6	5.7	5.7	6.6	7.7	6.6	6.9	5.3	4.6	5.3	5

† LW13, Lake Wheeler 2013; LS13, Laurel Springs 2013; LW15, Lake Wheeler 2015.

Table 5.3. Analysis of variance significance values for 120 progeny of a Raleigh x Seville cross evaluated in laboratory freeze tests at -3°C and -4°C for surviving green tissue (SGT) and regrowth (RG).

Source	DF	SGT†	RG†
Temperature	1	0.0078	0.0579
Acclimation	1	0.1092	0.0002
Replication(Acclimation)	4	0.154	0.1688
Genotype	119	0.0098	<.0001
Temperature*Acclimation	1	0.7387	0.2083
Temperature*Replication(Acclimation)	4	0.009	0.1236
Temperature*Genotype	116	0.0301	0.9999
Acclimation*Genotype	118	0.0432	0.9982
Genotype*Replication(Acclimation)	467	0.0478	0.9999
Temperature*Acclimation*Genotype	116	0.025	0.9999
Temperature*Replication(Acclimation)*Genotype	427	0.0278	0.9989

†SGT, visually rated surviving green tissue; RG, visually rated regrowth

Table 5.4. Lsmeans for parents, Raleigh and Seville, and progeny average, minimum, and maximum trait values for surviving green tissue (SGT) and regrowth (RG) evaluated in laboratory freeze tests at -3°C and -4°C.

Temperature	-3°C				-4°C			
Trait[†]	SGT		RG		SGT		RG	
Acclimation[‡]	NCA	CA	NCA	CA	NCA	CA	NCA	CA
Raleigh	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.2
Seville	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0
Progeny Min	0.5	0.4	0.0	0.0	0.1	0.2	0.0	0.0
Progeny Max	0.0	0.0	0.1	0.3	0.0	0.0	0.1	0.2
Progeny Avg	0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.0

[†] SGT, visually rated surviving green tissue; RG, visually rated regrowth

[‡] NCA, no cold acclimation; CA, cold acclimation

Table 5.5. Characteristics of the QTL detected for winterkill, spring green-up, turf quality, turf density, leaf texture, genetic color, mid-season cover, and end-of-season cover.

QTL #	Parameter	Environment	LG	Location	Flanking markers†	R^2 (%)	LOD threshold††
1	Winterkill	All	2	12.31	SAG03749, SAG02877	44.4	2.5
2	Winterkill	LW2013	3	27.11	SAG12198, SAG04343	2.4	2.5
3	Winterkill	LW2013	3	48.81	SAG16064, SAG15446	9.2	2.5
4	Winterkill	LW2015	4	3.01	SAG30521, SAG23965	3.0	2.5
5	Winterkill	LW2015	4	96.61	SAG29525, SAG28482	6.4	2.5
6	Winterkill	LW2015	8	76.51	SAG12250, SAG17353	16.6	2.5
1	Spring Green-up	LW2013, LW2015, All	2	117.51	SAG28745, SAG03298	59.4, 71.2, 64.8	2.5
2	Spring Green-up	LW2015, All	3	69.51-99.21	SAG29118, SAG17356	9.4	2.5
3	Spring Green-up	LW2013, LW2015, All	4	38.51-59.61	SAG05258, SAG02442	27.3, 0.9, 12.3	2.5
4	Spring Green-up	LW2013, LW2015, All	4	98.61	SAG29525, SAG28482	76.9, 55.6, 56.5	2.5
5	Spring Green-up	LW2013, LW2015	6	74.81-90.51	SAG12250, SAG00102	26.2, 49.9	2.5
6	Spring Green-up	LW2015	9	73.01	SAG12250, SAG17353	23.4	2.5
1	Turf Quality	LS2013, LW2015, All	2	119.51	SAG17596, SAG03298	8.5, 28.2, 7.4	2.5
2	Turf Quality	LW2013, LW2015	3	31.01	SAG04343, SAG11035	13.9, 10.6	2.5
3	Turf Quality	LS2013, All	3	68.51-77.51	SAG29118, SAG21486	27.7	2.5
1	Leaf Texture	LW2015, All	3	69.51- 77.71	SAG29118, SAG21486	5.2	2.5
2	Leaf Texture	LW2013	4	77.51	SAG12104, SAG06099	69.8	2.5
3	Leaf Texture	LS2013, All	7	13.21	SAG14644, SAG17015	13.2, 29.5	2.5
4	Leaf Texture	LW2015, All	7	44.41	SAG10061, SAG14922	1.3, 1.1	2.5
5	Leaf Texture	LW2013	9	49.51	SAG22229, SAG17122	59.4	2.5
1	Genetic Color	LS2013, LW2015, All	1	49.71	SAG17849, SAG10212	50.8, 1.4	2.5
2	Genetic Color	LS2013, LW2015	2	39.61	SAG01356, SAG30021	0.49, 0.49	2.5
3	Genetic Color	LW2013, LS2013, All	3	31.01	SAG04343, SAG11035	3.8, 2.2, 1.4	2.5
4	Genetic Color	LW2013, All	7	26.21	SAG01952, SAG00325	4.3, 8.6	2.5
5	Genetic Color	All	9	69.11	SAG11435, SAG15851	2.7	2.5
1	Turf Density	LW2013	2	113.51	SAG10259, SAG03298	6.0	2.5
2	Turf Density	LS2013, LW2015	3	69.51-91.01	SAG29118, SAG17356	10.2	2.5

Table 5.5, Cont'd. Characteristics of the QTL detected winterkill, spring green-up, turf quality, turf density, leaf texture, genetic color, mid-season cover, and end-of-season cover.

QTL #	Parameter	Environment	LG	Location	Flanking markers†	R^2 (%)	LOD threshold ††
3	Turf Density	LW2013	4	59.81	SAG17002, SAG03140	10.9	2.5
4	Turf Density	LW2013, LW2015, All	5	111.91	SAG02460, SAG09943	10.8, 25.6, 27.1	2.5
5	Turf Density	LW2013, LS2013	9	105.71	SAG05455, SAG25882	7.7, 6.06	2.5

†Markers located closest on either side of detected QTL peaks. For QTL detected in multiple environments, listed markers flank all regions of detected QTL.

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

Table 5.6. Characteristics of the QTL detected surviving green tissue (SGT) and regrowth RG) across two acclimation treatments (NCA and CA) and two freezing temperatures (-3°C and -4°C).

QTL #	Parameter	Temperature	Acclimation	LG	Location	Closest marker†	R^2 (%)	LOD threshold††
1	SGT	-3°C	NCA	1	39.31	SAG23374, SAG17849	0.06	2.5
2	SGT	-3°C	NCA	1	91.51	SAG11067, SAG07483	0.89	2.5
3	SGT	-3°C	NCA	1	104.01	SAG03634, SAG01288	0.05	2.5
4	SGT	-3°C	NCA	4	45.81	SAG04527, SAG23320	0.10	2.5
5	SGT	-3°C	NCA	4	103.61	SAG29525, SAG28482	0.01	2.5
6	SGT	-3°C	NCA	5	14.01	SAG13043, SAG04205	0.01	2.5
7	SGT	-3°C	NCA	5	144.81	SAG11704, SAG00610	0.04	2.5
1	SGT	-3°C	CA	2	80.41	SAG28745, SAG19047	0.88	2.5
2	SGT	-3°C	CA	5	98.31	SAG00332, SAG02460	0.01	2.5
3	SGT	-3°C	CA	5	122.51	SAG04381, SAG11704	0.01	2.5
4	SGT	-3°C	CA	5	141.81	SAG11704, SAG00610	0.01	2.5
5	SGT	-3°C	CA	9	52.41	SAG17122, SAG03614	0.49	2.5
1	SGT	-4°C	NCA	6	68.41	SAG21772, SAG12250	0.07	2.5
2	SGT	-4°C	NCA	7	27.21	SAG01952, SAG00325	0.01	2.5
3	SGT	-4°C	NCA	9	52.41	SAG17122, SAG03614	0.01	2.5
4	SGT	-4°C	NCA	9	69.11	SAG11435, SAG15851	0.01	2.5
1	SGT	-4°C	CA	2	75.81	SAG10863, SAG28745	0.11	2.5
2	SGT	-4°C	CA	3	107.21	SAG11424, SAG11218	0.21	2.5
3	SGT	-4°C	CA	7	72.91	SAG02330, SAG11492	0.65	2.5
4	SGT	-4°C	CA	8	92.41	SAG06868, SAG0417	0.84	2.5
1	RG	-3°C	NCA	4	100.61	SAG29525, SAG28482	0.85	2.5
1	RG	-3°C	CA	1	38.31	SAG23374, SAG17849	0.05	2.5
2	RG	-3°C	CA	4	64.81	SAG02442, SAG03140	0.01	2.5
3	RG	-3°C	CA	5	30.51	SAG04205, SAG21266	0.01	2.5
4	RG	-3°C	CA	5	38.41	SAG21266, SAG23382	0.01	2.5

Table 5.6 cont'd. Characteristics of the QTL detected surviving green tissue (SGT) and regrowth RG) across two acclimation treatments (NCA and CA) and two freezing temperatures (-3°C and -4°C).

QTL #	Parameter	Temperature	Acclimation	LG	Location	Closest marker†	R^2 (%)	LOD threshold††
5	RG	-3°C	CA	5	98.31	SAG00332, SAG02460	0.89	2.5
6	RG	-3°C	CA	5	137.81	SAG11704, SAG00610	0.01	2.5
1	RG	-4°C	NCA	1	71.31	SAG24167, SAG02502	0.70	2.5
2	RG	-4°C	NCA	5	90.41	SAG11328, SAG00332	0.03	2.5
3	RG	-4°C	NCA	5	105.01	SAG02460, SAG04381	0.04	2.5
4	RG	-4°C	NCA	5	139.81	SAG11704, SAG00610	0.01	2.5
5	RG	-4°C	NCA	6	68.41	SAG21772, SAG12250	0.46	2.5
6	RG	-4°C	NCA	7	26.21	SAG01952, SAG00325	0.47	2.5
7	RG	-4°C	NCA	9	47.51	SAG14922, SAG04450	0.07	2.5
1	RG	-4°C	CA	1	102.01	SAG03634, SAG01288	0.05	2.5
2	RG	-4°C	CA	2	16.91	SAG02877, SAG25120	0.03	2.5
3	RG	-4°C	CA	4	30.41	SAG03957, SAG03956	0.04	2.5
4	RG	-4°C	CA	5	15.01	SAG13043, SAG04205	0.03	2.5
5	RG	-4°C	CA	5	27.51	SAG04205, SAG21266	0.04	2.5
6	RG	-4°C	CA	9	49.51	SAG22229, SAG17122	0.55	2.5

†Markers located closest on either side of detected QTL peaks. For QTL detected in multiple environments, listed markers flank all regions of detected QTL.

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

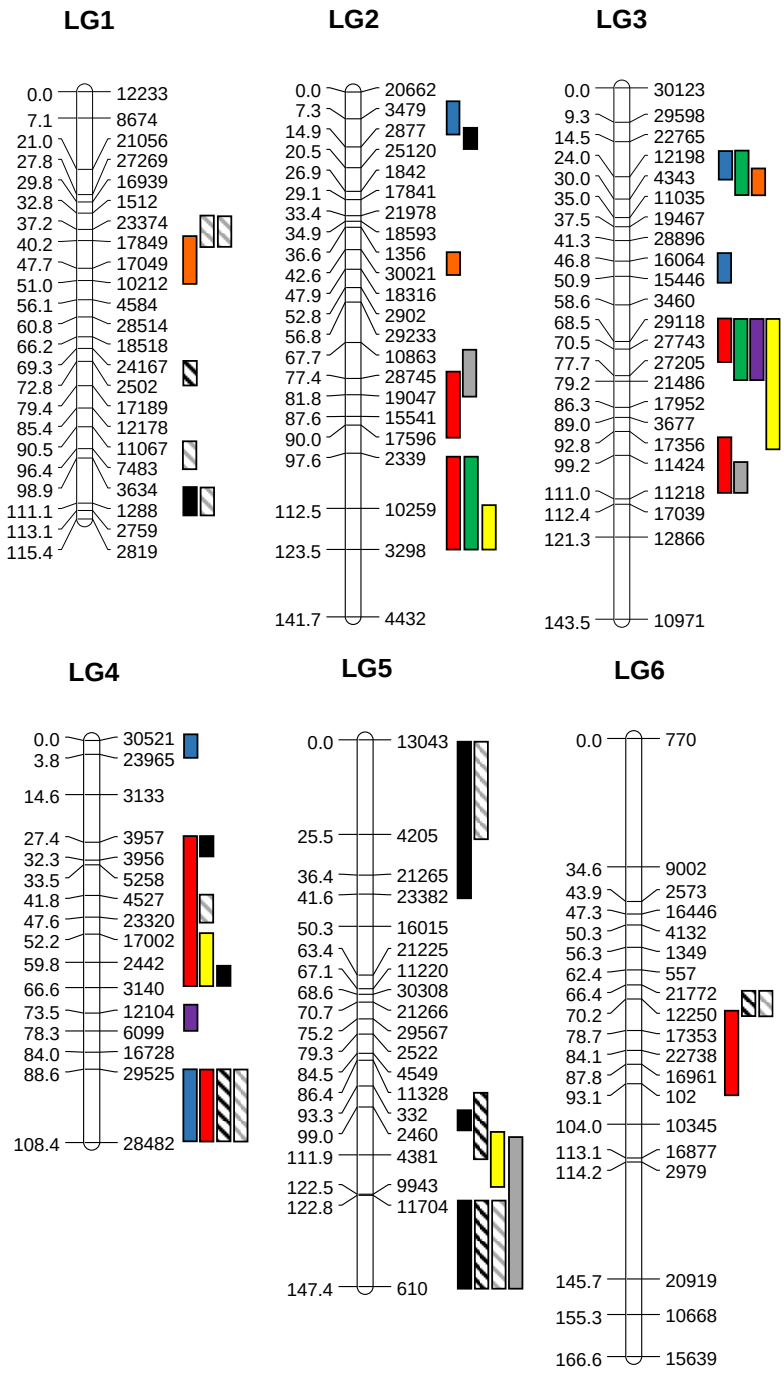

Figure 5.1. A genetic linkage map for St. Augustinegrass generated from 178 SSR markers mapped to 120 pseudo F_2 progeny from the cross of Raleigh and Seville. Genetic distances (centiMorgans: cM) were calculated using the Kosambi function and listed on the left of each linkage group (LG). Marker names are listed on the right. Quantitative Trait Loci are located on the far right of each LG. Individual field environments (LW2013, LS2013, LW2015, and across all environments) and freeze temperatures (-3°C and -4°C) were not distinguished on the map and can be found in Tables 5.5 and 5.6.

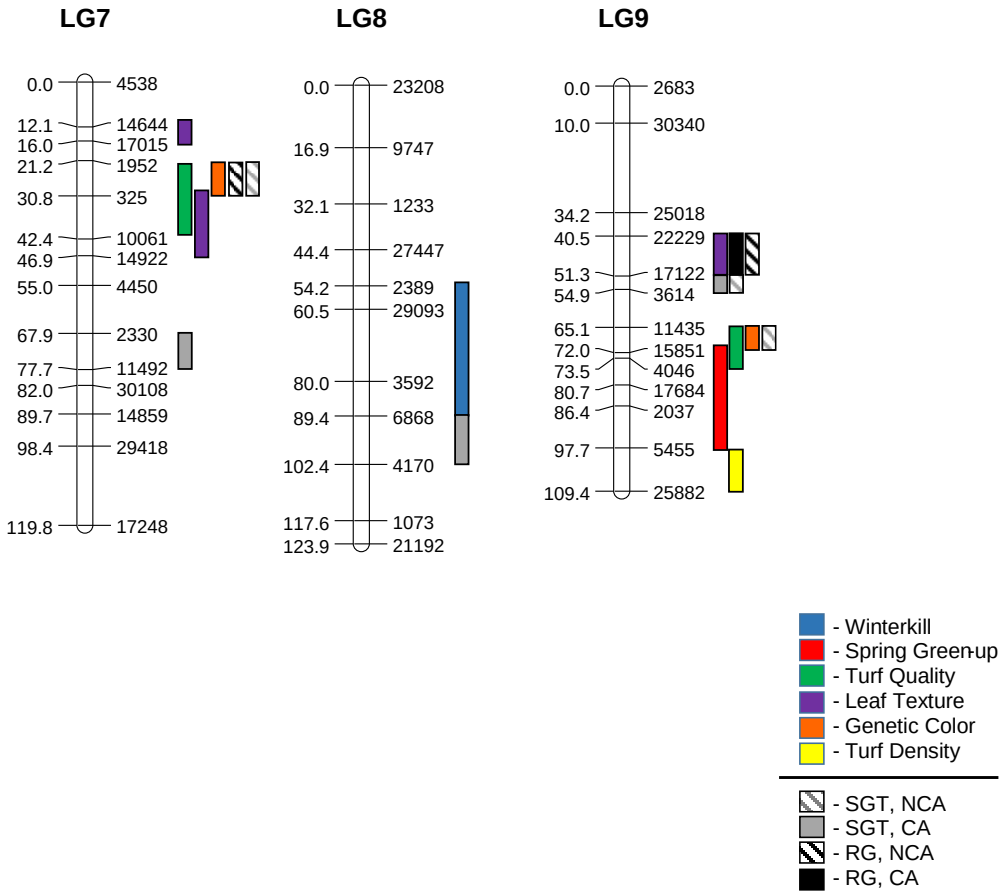

Figure 5.1 cont'd. A genetic linkage map for St. Augustinegrass generated from 178 SSR markers mapped to 120 pseudo F_2 progeny from the cross of Raleigh and Seville. Genetic distances (centiMorgans: cM) were calculated using the Kosambi function and listed on the left of each linkage group (LG). Marker names are listed on the right. Quantitative Trait Loci are located on the far right of each LG. Individual field environments (LW2013, LS2013, LW2015, and across all environments) and freeze temperatures (-3°C and -4°C) were not distinguished on the map and can be found in Tables 5.5 and 5.6.

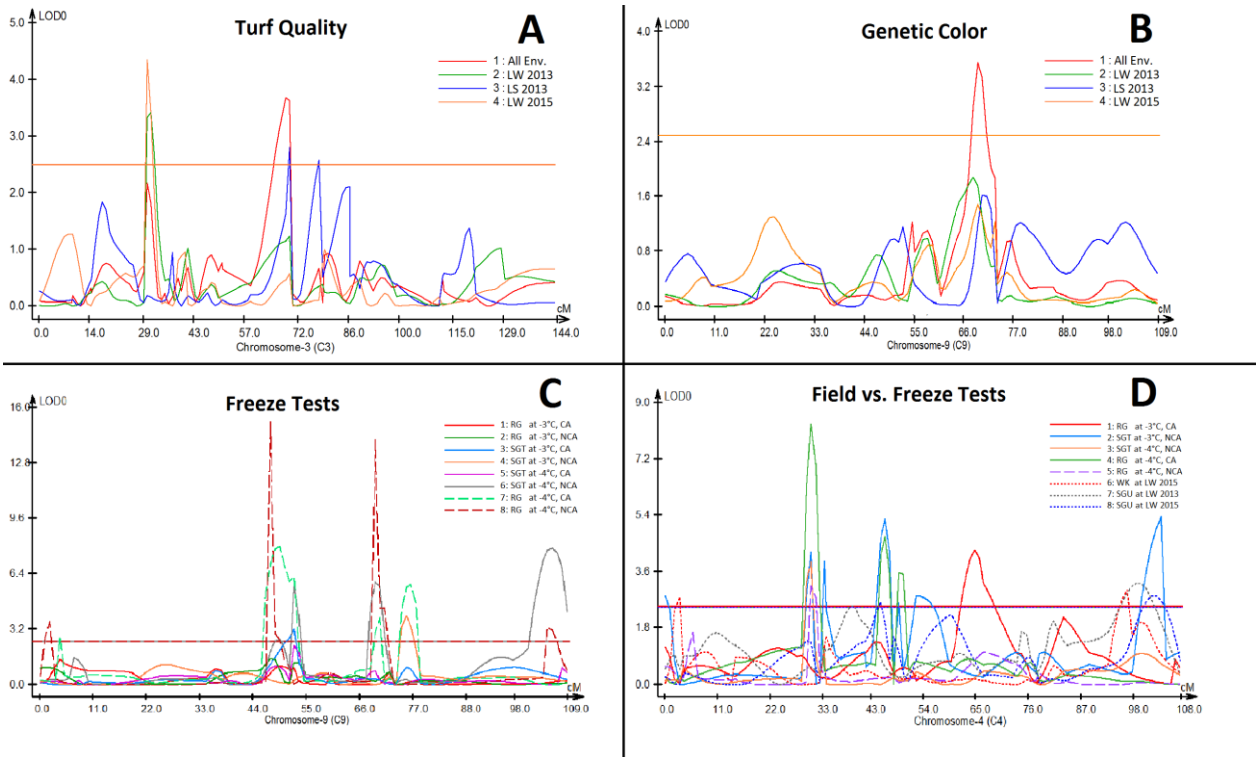

Figure 2. Examples of the Logarithm of odds (LOD) peaks for putative quantitative trait loci identified through composite interval mapping including a. an example of consistency across field environments using turf quality LOD peaks on LG3, b. an example of a significant LOD peak identified in an analysis using LSmeans from all environments where individual environments were not significant using genetic color LOD peaks on LG9, c. an example of co-localization of putative QTL identified under multiple freeze test conditions on LG9, and d. an example of co-localization of putative cold and freeze tolerant-related QTL identified under both field and freeze testing conditions on LG4.

-APPENDICES-

Appendix 1. SAS code for CHAPTER II. Combining Ability of Winterkill Tolerance and Turf Quality Traits in St. Augustinegrass

```
PROC GLM DATA=DIALLEL;
CLASS YEAR LOCATION REP ENTRY WK SGU TQ LT GC TD MS ES;
MODEL TRAIT = LOCATION YEAR REP AMG_PARENTS AMG_CROSSES CVP
LOCATION*YEAR LOCATION*REP LOCATION*AMG_PARENTS
LOCATION*AMG_CROSSES LOCATION*CVP
YEAR*REP YEAR*AMG_PARENTS YEAR*AMG_CROSSES YEAR*CVP
LOCATION*YEAR*REP LOCATION*YEAR*AMG_PARENTS
LOCATION*YEAR*AMG_CROSSES LOCATION*YEAR*CVPYEAR*LOCATION
P1 P2 P3 P4 P5 F1 F2 F3 F4 F5 G1 G2 G3 G4 G5 S12 S13 S14 S15 S23 S24 S25 S34 S35;
CONTRAST 'Crosses vs parents' cvp 1;
CONTRAST 'Selfs vs parents'   fvp 1 ;
CONTRAST 'Among parents'      p1 1,  p2 1,  p3 1,  p4 1,  p5 1 ;
CONTRAST 'Among selfs'        f1 1,  f2 1,  f3 1,  f4 1,  f5 1 ;
CONTRAST 'Among crosses'      g1 1,  g2 1,  g3 1,  g4 1,  g5 1,
                    s12 1, s13 1, s14 1, s15 1, s23 1, s24 1, s25 1, s34 1, s35 1 ;
CONTRAST 'GCA'              g1 1, g2 1, g3 1, g4 1, g5 1 ;
CONTRAST 'SCA'              s12 1, s13 1, s14 1, s15 1, s23 1, s24 1, s25 1, s34 1, s35 1 ;
ESTIMATE 'p6'   p1 -1  p2 -1  p3 -1  p4 -1  p5 -1 ;
ESTIMATE 'f6'   f1 -1  f2 -1  f3 -1  f4 -1  f5 -1 ;
ESTIMATE 'g6'   g1 -1  g2 -1  g3 -1  g4 -1  g5 -1 ;
ESTIMATE 's16' s12 -1 s13 -1 s14 -1 s15 -1 ;
ESTIMATE 's26' s12 -1 s23 -1 s24 -1 s25 -1 ;
ESTIMATE 's36' s12 -1 s23 -1 s24 -1 s25 -1 ;
ESTIMATE 's45' s12 -1 s13 -1 s14 -1 s15 -1 s23 -1 s24 -1 s25 -1 s34 -1 s35 -1 ;
ESTIMATE 's46' s12  1 s13  1 s15  1 s23  1 s25  1 s35  1 ;
ESTIMATE 's56' s12 -1 s13 -1 s15 -1 s23  1 s24  1 s34  1 ;
ESTIMATE 'p1'   p1  1 ;
ESTIMATE 'p2'   p2  1 ;
ESTIMATE 'p3'   p3  1 ;
ESTIMATE 'p4'   p4  1 ;
ESTIMATE 'p5'   p5  1 ;
ESTIMATE 'p6'   p1 -1  p2 -1  p3 -1  p4 -1  p5 -1 ;
ESTIMATE 'f1'   f1  1 ;
ESTIMATE 'f2'   f2  1 ;
ESTIMATE 'f3'   f3  1 ;
ESTIMATE 'f4'   f4  1 ;
ESTIMATE 'f5'   f5  1 ;
ESTIMATE 'f6'   f1 -1  f2 -1  f3 -1  f4 -1  f5 -1 ;
run;
```

Appendix 2. SAS code for CHAPTER III. Freeze Testing in St. Augustinegrass I: A Methodological Approach

```
PROC GLM DATA= FREEZE1 PLOTS=ALL;
CLASS BLOCK REP TEMP ENTRY ACCL;
MODEL TRAIT = TEMP BLOCK(TEMP)
            REP(TEMP*BLOCK)
        ENTRY ENTRY*TEMP ENTRY*BLOCK(TEMP);
RANDOM REP(TEMP*BLOCK) /TEST;
MANOVA H= ENTRY /PRINTE SUMMARY;
LSMEANS ENTRY*TEMP/LINES;
RUN;
```

Appendix 3. SAS code for CHAPTER IV. Freeze Testing in St. Augustinegrass II: Evaluation of Acclimation Effects

```
PROC MIXED DATA= FREEZE2;
CLASS BLOCK REP TEMP ENTRY ACCL;
MODEL  R6 =  TEMP
             ACCL        ACCL*TEMP
             ENTRY ENTRY*TEMP
             ENTRY*ACCL ENTRY*ACCL*TEMP;
RANDOM  BLOCK(TEMP) ACCL*BLOCK(TEMP)  ENTRY*BLOCK(TEMP)
ENTRY*ACCL*BLOCK(TEMP)  REP(TEMP*BLOCK*ACCL)  ;
REPEATED / SUBJECT =  REP *ENTRY(TEMP*BLOCK*ACCL)  GROUP = ENTRY;
LSMEANS ENTRY*ACCL/LINES;
RUN;
```

Appendix 4. SAS code for CHAPTER V. Linkage Analysis and Identification of Quantitative Trait Loci for Cold Tolerance and Turf Quality Traits in St. Augustinegrass.

```
PROC GLM DATA=FIELD;
CLASS ENTRY REP ENV WK SGU TQ LT GC TD;
MODEL TRAIT = ENTRY ENV REP(ENV)
                    ENTRY*ENV;
RANDOM REP(ENV);
TEST H=ENTRY E=ENTRY*ENV;
LSMEANS ENTRY*ENV;
RUN;

PROC GLM DATA=FREEZE;
CLASS TEMP REP ACCL ENTRY SGT RG;
MODEL TRAIT = TEMP REP(ACCL) ACCL ENTRY
              TEMP*REP(ACCL) TEMP*ACCL TEMP*ENTRY ACCL*ENTRY
              ENTRY*REP(ACCL)
              TEMP*ENTRY*REP(ACCL) TEMP*ACCL*ENTRY;
TEST H=ACCL E=TEMP*ACCL;
LSMEANS CULTIVAR*ACCL*TEMP/LINES;
RUN;
```