

## ABSTRACT

GRAHAM, SYDNEY ELIZABETH. Improving Selection Methods for Development of Freeze-Tolerant St. Augustinegrass Cultivars (Under the direction of Dr. Susana Milla-Lewis).

St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze) is a warm-season turfgrass primarily used for home lawns in the Southern United States. St. Augustinegrass is known for its dense canopy, aggressive stolon growth, and superior shade tolerance among warm-season turfgrasses. However, levels of freeze tolerance in the species are generally low. Elite St. Augustinegrass cultivars for North Carolina and the transitional climatic zone of the United States require superior turf quality in combination with improved tolerance to freeze, drought, and fungal pathogens, which would increase the sustainability of their management. Germplasm with superior performance for individual traits has been identified, but little research has been conducted on the relationship among these traits, and limited progress has been made in stacking them via conventional breeding. For example, cultivar 'Raleigh' is the industry standard for freeze tolerance but lacks superior turf quality.

A multi-environment trial was conducted to assess the relationships among economically important traits and to select lines with stable performance for them across North Carolina. Sixty-one St. Augustinegrass breeding lines were established in replicated field trials at three locations. Entries were evaluated from 2017 to 2020 for establishment rate, turf quality, density, genetic color, texture, uniformity, winter survival, fall color, drought tolerance, and gray leaf spot (*Pyricularia grisea*) resistance. Mixed model analysis was used to estimate best linear unbiased predictions (BLUPs), which were used to calculate a selection index in order to identify elite genotypes across traits. Generalized heritability estimates for each trait ranged between 0.38 and 0.87. The ten traits were generally well correlated with one another and clustered into three groups: winterkill and fall color; genetic color, texture, and gray leaf spot resistance; and establishment,

turf quality, density, uniformity, and drought tolerance. Selection of the best 10 genotypes using the selection index resulted in a positive estimated genetic gain for all ten traits, indicating this is an effective method for simultaneous selection. XSA 14271 outperformed the commercial checks for several traits and was the top-ranked line. It will be advanced to on-farm trials to evaluate traits essential for sod production in order to assess its potential for commercial release.

Additionally, in order to further elucidate the genetic control of freeze tolerance in St. Augustinegrass, quantitative trait loci analysis was conducted. Previous research identified QTL in a freeze-tolerant 'Raleigh' by freeze-susceptible 'Seville' (biparental) mapping population. The current study was performed in order to improve the resolution of the previously identified freeze tolerance QTL using a high-density SNP-based linkage map, and to validate these QTL in a 'Raleigh' selfing population. Utilization of the high-density biparental linkage map resulted in the identification of 39 QTL, including 17 not previously detected. Additionally, for the selfing population, a linkage map containing 119 SSR markers with a total genetic distance of 1641cM was developed and 16 putative QTL were identified using lab-based freeze tests. The linkage maps from both populations were aligned with a draft reference sequence of parent cultivar 'Raleigh' as means to compare freeze tolerance QTL identified in the different populations. Five genomic regions with significant overlap were found on chromosomes 1, 3, 4, 6, and 7. Gene annotation in these regions identified auxin response factors, transcription factors, and enzymes that affect freeze tolerance, including diacylglycerol kinases and cycloartenol synthase. These QTL and their associated markers may be used in future breeding efforts to develop a broader pool of freeze-tolerant St. Augustinegrass cultivars.

Overall, these findings can be utilized to implement an effective selection strategy for St. Augustinegrass, including marker-assisted selection and selection indexes for simultaneous selection on multiple traits including freeze tolerance.

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Improving Selection Methods for Development of Freeze-Tolerant St. Augustinegrass Cultivars

By  
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A thesis submitted to the Graduate Faculty of  
North Carolina State University  
in partial fulfillment of the  
requirements for the Degree of  
Master of Science

Crop Science

Raleigh, North Carolina  
2021

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## **BIOGRAPHY**

Sydney Graham, originally from Waukee, Iowa, received her bachelor's degree in genetics from the University of Wisconsin-Madison in 2017. Following graduation, she moved to Raleigh, NC, to pursue her master's degree in plant breeding at North Carolina State University under the direction of Susana Milla-Lewis. Sydney enjoys cooking, spending time with the other graduate students as well as hiking with her dog.

## ACKNOWLEDGEMENTS

Throughout my time at NC State University, I have received technical advice, support, friendship, and help in so many ways from so many people. First and foremost, I want to thank Susana Milla-Lewis for taking me on as her master's student and providing me with so many opportunities. Thank you not only for a great set of technical skills but for teaching me about working in plant breeding beyond that. I appreciate all the time you have taken to foster my interests and to tirelessly edit my conference submissions and, of course, thesis.

Thank you to all my committee members: Dr. Jeffrey Dunne, Dr. David Livingston, and Dr. Michelle DaCosta. Thank you, Jeff, for sharing your experiences and expertise about freezing tests with me; my experiments would not have been near as well-thought-out without your help. Thank you also for always making time to talk about data analysis with me, even when we have almost certainly gone over it before. Dr. Livingston – I appreciate all your physiology expertise and curiosity. Without the help of you and Tan Tuong, I would not have been able to work out all of the kinks of freezing tests. Thank you for letting me use space in your growth chambers and freezers, without which there would be no experiment. Thank you to Dr. DaCosta for your freezing expertise and experience and your thoughtful approach to experimental design.

Of course, my work was not done in isolation, so thank you to the past and current NC State Turfgrass Breeding and Genetics team: Jessica, Greta, Kirtus, Jenny, Xingwag, Rocio, Esdras, and Beatriz. Jessica, Greta, and Kirtus – thanks for being great friends and company no matter what we are working on and for always being willing to help talk through and troubleshoot projects. Dr. Jenny Kimball, thank you for getting this project off the ground and doing many of the freeze tests as well as the DNA extractions and genotyping. Xingwang, thank you for always helping me out with all the genomic data and letting me make my mistakes, and then showing a

better path forward when I come around. Beatriz, the advanced trial analysis would not be what it is without your help. Thank you so much for pushing it in that direction, I have lots left to learn, but with your help, I learned so much. And finally, Rocio and Esdras, you hold the lab together and always make sure things get done. Thank you for accommodating my ever-expanding number of benches in the greenhouse and making sure I had all the materials I needed.

Thank you to all my friends and family, near and far, for all the support, laughs, distractions, and love. Thanks to Anna Grassy for birthday surprises and to Lais Bastos Martins for both providing literal shelter for me and a place to vent. I miss you all, thanks to covid-19, and I look forward to spending time with you from near instead of far.

## TABLE OF CONTENTS

LIST OF TABLES .....	vii
LIST OF FIGURES .....	ix
<b>Chapter 1: Literature review.....</b>	<b>1</b>
Turfgrass Overview .....	2
St. Augustinegrass Overview .....	3
Turfgrass Breeding.....	4
St. Augustinegrass Breeding .....	5
Freeze Tolerance in St. Augustinegrass.....	7
Quantitative Trait Loci Mapping Overview .....	10
QTL Mapping in St. Augustinegrass .....	13
References.....	16
<b>Chapter 2: Multi-environment evaluation of St. Augustinegrass [<i>Stenotaphrum secundatum</i> (Walt.) Kuntze] breeding lines for turf quality traits and stress tolerance in North Carolina .....</b>	<b>26</b>
ABSTRACT.....	28
INTRODUCTION .....	30
MATERIALS AND METHODS.....	33
Plant Material and Field Trial.....	33
Phenotypic Evaluations.....	34
Statistical Analysis.....	35
RESULTS .....	39
Variance-Covariance Structures .....	39
Correlations among Traits.....	39
Generalized Heritability & Selection.....	40
DISCUSSION.....	42
Acknowledgments.....	45
REFERENCES .....	46
<b>Chapter 3: Validation of QTL Associated with Freezing Tolerance across Two St. Augustinegrass [<i>Stenotaphrum secundatum</i> (Walt.) Kuntze] Mapping Populations.....</b>	<b>58</b>
ABSTRACT.....	60
INTRODUCTION .....	62
MATERIALS AND METHODS.....	65
Plant Material.....	65
Freezing Evaluations.....	66

Genotyping and Linkage Mapping .....	70
QTL Analysis.....	71
QTL Validation & Sequence Annotation .....	72
<b>RESULTS .....</b>	<b>72</b>
Freezing Evaluations.....	72
Genotyping and Linkage Mapping .....	74
QTL Analysis.....	74
QTL Validation & Sequence Annotation .....	75
<b>DISCUSSION.....</b>	<b>78</b>
Acknowledgments.....	83
<b>REFERENCES .....</b>	<b>84</b>
<b>APPENDICES.....</b>	<b>112</b>
APPENDIX 1: Supplementary Tables for Chapter II.....	113
APPENDIX 2: Supplementary Figures for Chapter II .....	120
APPENDIX 3: Abbreviated ASReml-R Code for Chapter II.....	123
APPENDIX 4: Supplementary Tables for Chapter III .....	124
APPENDIX 5: Abbreviated SAS Code for Chapter III.....	136

## LIST OF TABLES

Table 2.1: Traits collected on advanced breeding lines of St. Augustinegrass evaluated 2017 through 2020 in replicated field trials at three locations in North Carolina. ....	51
Table 2.2: Significant factors from Mixed Model Analysis. All significance values were calculated by likelihood ratio test or Wald test of the best model selected for that trait. Heritability estimates as generalized heritability. Values that were not calculated are indicated by "--." Complete details of likelihood ratio tests and Wald tests are available in the appendix. ....	52
Table 2.3: Modified Base Index Values for top and bottom-ranked St. Augustinegrass lines as well as commercial checks evaluated in multi-environment trials in North Carolina 2017-2020. High positive values indicate superiority, and negative values indicate inferiority. A complete list of values is available in Supplementary Table 4.3.....	53
Table 2.4: Estimated genetic gain for each trait under direct and indirect selection with a 15% selection intensity (10 genotypes). Maximum genetic gains for each trait under direct selection are indicated in bold along the diagonal. Estimated genetic gains for each trait under indirect selection using the modified base index as the selection criteria are presented in the bottom row. ....	53
Table 3.1: Type 3 Analysis of Variance for freeze tolerance traits for the selfing population. DF = degrees of freedom, Green Cover Day 5 = green cover at day 5 post-freeze relative to the pre-freeze green cover from digital imaging, Green Cover Day 26 = green cover at day 26 post-freeze relative to the pre-freeze green cover from digital imaging, Days Survival = sum of the days where green tissue was present post-freeze, SS = sum of squares, VWC = volumetric water content. ....	94
Table 3.2: Adjusted means for number of days of survival, post-freeze green cover on day 5 and post-freeze green cover on day 26 measured in lab based freeze tests of a cultivar ‘Raleigh’ selfing population. Number of days survival can range from 0 to 26 where 26 indicates the plant had green tissue every day after freezing. Post-freeze green cover values are a proportion of pre-freeze values where 0 indicates no change in the amount of green tissue, 1 indicates the same amount of green tissue, and negative values indicate loss of green tissue after freezing. F2 = freezer 2, F3 = freezer 3, CA = cold acclimated, NA = non-cold acclimated. ....	95
Table 3.3: Summary of the distribution of markers on the Raleigh selfing population linkage map. The map was developed using the package r/Onemap and the genetic distances estimated using the Kosambi function. cM = centimorgan, LG = linkage group. ....	96
Table 3.4: Quantitative Trait Loci associated with Freeze tolerance traits for the Raleigh x Seville biparental population based on a high-density SNP linkage map and field and lab-based freeze test data. QTL mapping was conducted using r/QTL using the stepwise model function and QTL identified as significant ( $p > 0.05$ ) are presented here. Physical positions were identified using the sequences of the flanking markers mapped to the ‘Raleigh’ reference genome. When these	

sequences could not be aligned to the reference the region was expanded to the next marker to identify a physical position, these are denoted with \*. When the flanking markers could not be used for alignment the markers in the expand to next marker were used. CHR = chromosome, LOD = logarithm of odds, RG = regrowth, SGT = surviving green tissue, SGU = spring green-up, WK = winterkill. ....97

Table 4.1: Bayesian information criterion (BIC) for multi-environment analysis models of eight St. Augustinegrass traits using compound symmetry (CS) for the genetic (G) variance-covariance matrix and different structures in the residual (R) variance-covariance matrix. The lowest value for each trait is indicated in bold. ....113

Table 4.2: Significance tests and variance component estimates for each trait. The significance of each factor was evaluated by likelihood ratio test (LRT) for random effects (entry, entry\*location, entry\*year, entry\*year\*location, rep(location)) and by Wald tests for fixed effects (location, year, location\*year). SS = sum of squares, LR = likelihood ratio, EST = establishment, TQ = turf quality, DEN = density, GC = genetic color, TX = texture, UN = uniformity, FC = fall color, WK = winterkill, DRY = drought, GLS = gray leaf spot. ....114

Table 4.3: Normalized BLUPs and Modified Base Index Values. Normalized best linear unbiased prediction values for each trait used in the modified base index calculation (Eq. 7). All genotypes in the study listed in order of rank using the selection index. ....117

Table 4.4: SSR primer sequences for ‘Raleigh’ x ‘Raleigh’ population. Tm = melting temperature. ....124

## LIST OF FIGURES

Figure 2.1: Pairwise Spearman rank correlation between traits. The lower triangle displays the correlation coefficients. In the upper triangle, the size of the circle corresponds to the absolute value of the correlation coefficient, and the intensity of color indicates a positive or negative correlation. Dark blue indicates a correlation coefficient, of 1 and dark red of -1. Black stars indicate significance as ‘\*\*\*\*’ < 0.001 ‘\*\*\*’ < 0.01 ‘\*’ < 0.05 correlations. EST = establishment, TQ = turf quality, DEN = density, GC = genetic color, TX = texture, UN = uniformity, WK = winterkill, FC = fall color, DRY = drought. .... 54

Figure 2.2: Type-B genetic correlations for St. Augustinegrass breeding lines evaluated in multi-environment trials in North Carolina. EST = establishment, TQ = turf quality, DEN = density, GC = genetic color, TX = texture, UN = uniformity, WK = winterkill, FC = fall color. .... 55

Figure 2.3: Genotype by Trait Biplot. Principal components analysis generated using the normalized predicted values for each trait. Traits are listed in green and genotypes are listed in blue. Principal component 1 is on the x-axis and represents 39.58% of the overall variation, and principal component 2 is on the y-axis and represents 16.24% of total variation. EST = establishment, TQ = turf quality, DEN = density, GC = genetic color, TX = texture, UN = uniformity, WK = winterkill, FC = fall color, DRY = drought, GLS = gray leaf spot. .... 56

Figure 2.4: Studentized-BLUPs of selected St. Augustinegrass breeding lines and commercial checks evaluated in multi-environment trials in North Carolina 2017-2020 for EST = establishment, TQ = turf quality, FC = fall color, WK = winterkill, DRY = drought, l, GLS = Gray leaf spot. \* denotes significance at 5% probability by t-Student test. .... 57

Figure 3.1: Schematic of the experimental design for the ‘Raleigh’ selfing population controlled freezing tests. The experimental design featured a completely nested design with trays within freezers within runs within replications. Each tray contained 45 pots including 3 acclimated ‘Raleigh’ and 3 not-acclimated ‘Raleigh.Raleigh’ as controls. Freezers can be considered analogous to the environment. Five replications were conducted in total. Trays 1-4 contain all 78 genotypes by treatment combinations as do trays 5-8. .... 105

Figure 3.2: Identified freeze tolerance QTL aligned to the physical map of 'Raleigh'. The flanking markers of each QTL were used to identify the physical position of each. If a flanking marker did not align, the region was expanded based on the next marker on the respective genetic map (refer to tables 3-6). QTL presented for all St. Augustinegrass chromosomes (1-9). CA = cold acclimated; GC5 = green cover at day five post-freeze; DS = number of days of survival; F2 = freezer 2; F3 = freezer 3; NA= Not-acclimated; RG= regrowth; SGT = surviving green tissue; SGU = spring green-up; WK = winterkill. .... 106

Figure 4.1: Monthly Temperature and Rainfall at the Upper Mountain Research Station in Laurel Springs, NC. Temperature and precipitation data from the North Carolina State Climate Office from April 2017 to April 2020. Minimum, average, and

maximum temperature are presented in °F along the primary axis (left), and accumulated precipitation is measured in inches and follows the secondary axis (right). ..... 120

Figure 4.2: Monthly Temperature and Rainfall at the Turfgrass Field Laboratory in Raleigh, NC. Temperature and precipitation data from the North Carolina State Climate Office from April 2017 to April 2020. Minimum, average, and maximum temperature are presented in °F along the primary axis (left), and accumulated precipitation is measured in inches and follows the secondary axis (right). ..... 121

Figure 4.3: Monthly Temperature and Rainfall at the Sandhills Research Station in Jackson Springs, NC. Temperature and precipitation data from the North Carolina State Climate Office from April 2017 to April 2020. Minimum, average, and maximum temperature are presented in °F along the primary axis (left), and accumulated precipitation is measured in inches and follows the secondary axis (right). No data was collected in April 2020. .... 122

## **Chapter 1**

### **Literature review**

## **Turfgrass Overview**

Turfgrasses are narrow-leaved grass species that form living ground cover and are generally maintained at a height of a few inches. They are grown in all fifty of the United States and offer functional, recreational, and aesthetic benefits (Beard and Green, 1994). Turfgrasses can reduce runoff and increase water infiltration, thereby reducing soil erosion, and thus are widely used on roadsides (Beard and Green, 1994; Easton and Petrovic, 2004). In urban settings, turfgrasses can reduce temperature extremes (Wang et al., 2016), minimize noise pollution (Onuu, 2007), and reduce urban glare (Sliney et al., 1991). Turfgrasses are used for many sports surfaces, including golf, tennis, football, soccer, and baseball (Monteiro, 2017). While some of these surfaces have been replaced by artificial turf, living turfgrasses provide cooler surfaces (Serensits et al., 2011), have a lower incidence of injury (Loughran et al., 2019), and are preferred by athletes (Owen et al., 2017). Turfgrass lawns are perceived as improving quality of life and having strong community benefits (Khachatryan et al., 2014), as well as increase the resale value of homes (Behe et al., 2005). In 2002, the economic impact of turfgrasses in the United States was valued at nearly \$60 billion and the industry employed over 800,000 people. The industry includes sod production, retail, golf courses, and equipment manufacturing (Haydu et al., 2006).

Turfgrasses are divided into cool-season and warm-season grasses, where generally, cool-season turfgrasses are grown in the Northern US, and warm-season turfgrasses are grown in southern states. While cool-season turfgrasses are characterized by their winter hardiness but can be susceptible to high temperatures, warm-season turfgrasses thrive in the summer heat but lack freezing tolerance. There are no turfgrass species ideally suited for the transition zone – a region with hot, humid summers but cold winters – which includes North Carolina, Virginia, Tennessee, and parts of Arkansas and Texas. Furthermore, turfgrasses are available to consumers either as

seed or as sod, and while some species – such as fescue and bermudagrass – are available in both forms, others can only be produced as one or the other, such as St. Augustinegrass, which is only available as sod.

### **St. Augustinegrass Overview**

St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntz) is a warm-season turfgrass typically grown in home lawns in the Southeastern United States. St. Augustinegrass is characterized by coarse, round-tipped leaf blades and aggressive stolons or runners. It lacks below ground rhizomes, but due to its rapid and aggressive stoloniferous growth, it forms a dense, spongy canopy (Busey, 2003b). St. Augustinegrass can outcompete weeds due to its aggressiveness, has good salinity and shade tolerances, and can be grown in a wide range of soil pH (Busey, 2003a; Miller, 2018; Segars and Bowling, 2020). However, its lack of winter hardiness has limited its use into, and north of, the transition zone, including widespread use in North Carolina.

Despite some of its limitations, the 2020 North Carolina sod producers report shows an increasing demand for St. Augustinegrass in the state (Miller, 2020). In 1999, the turfgrass industry in North Carolina was estimated to have an economic impact of \$4.6 billion dollars, and it likely has increased since then (Murphy and Hayes, 1999). As turfgrasses are grown in all 100 counties in North Carolina covering over two million acres, development of ideally suited St. Augustinegrass cultivars has high demand and could have a significant market impact (Brandenburg et al., 2004).

St. Augustinegrass is a member of the Poaceae family and has a base chromosome number of  $x = 9$ . The center of origin for the genus is believed to be tropical regions of Africa and southeastern Asia, but it can be found wildy in tropical and subtropical regions in the Caribbean,

North America, South America, and Australia as well (Busey, P., 1995; Sauer, J.D., 1972). Of the seven species identified within the genus only *secundatum* is widely cultivated. While many cultivars are diploid ( $2x = 2n = 18$ ), sterile triploid ( $3x = 3n = 27$ ), and tetraploid ( $4x = 4n = 36$ ) varieties exist and are on the market (Long and Bashaw, 1961). Aneuploids are also not uncommon, and ploidy levels are challenging to assess using only flow cytometry (Milla-Lewis et al., 2013a). While diploid germplasm is commonly used in breeding programs for its finer texture and superior turf quality, genotypes with higher ploidy levels often have improved resistance to pests and pathogens (Genovesi et al., 2009). Other polyploid *Stenotaphrum* genotypes can be used as a pool of genetic diversity for St. Augustinegrass breeding, but the success of developing interploid hybrids has been limited (Genovesi et al., 2009; Carbajal et al., 2019).

### **Turfgrass Breeding**

Turfgrass breeding programs aim to develop regionally adapted cultivars with improved traits such as turfgrass quality in combination with tolerance to abiotic and biotic stresses. Each breeding program is tailored to the species, geographic stresses, and consumer demand; for example, a sodded variety will need good tensile strength, and seed yield will not be important, but for a seeded variety, high seed yield is necessary for production. Furthermore, for sodded species, cultivars can be more easily preserved vegetatively and are “immortal” once developed, whereas seeded species need to be produced through either apomictic reproduction or controlled pollination.

A key feature of breeding programs are multi-environment trials (MET), in which germplasm are screened across locations and years. A classic evaluation of MET uses analysis of variance (ANOVA) to partition variance into different sources including genotype, location, year,

their interactions, and error variation (Smith et al., 2005). Additionally, in order to determine the effect of genetics relative to the environmental influence for a given trait, breeders often estimate heritability. Heritability can be measured in the broad sense, which includes additive, dominance, and epistatic effects, or in the narrow sense, including only additive effects. While narrow sense heritability estimates provide more specific information on the genetic contribution of a given trait, it requires specialized experimental designs for its calculation whereas broad sense heritability does not.

Linear mixed models can also be used to analyze MET. These models can reduce the impact of incomplete data, include some sources of variation as random rather than fixed, and handle realistic within-trial covariance structures (Smith et al., 2005). Covariance structures describe the relationship between measurements in a trial and can capture spatial, time-series, compound symmetry, diagonal, or unstructured relationships (Wolfinger, 1993). Complex covariance models have rarely been used in turfgrass breeding but are widely used in other crops.

### **St. Augustinegrass Breeding**

Consumer demand for St. Augustinegrass in the transition zone requires breeders to develop cultivars combining high turf quality, improved winter survival, and resistance to pests and pathogens. Turf quality is an assessment of the aesthetic value of turfgrass and includes color, texture, density, and uniformity (Morris and Shearman, 1998). These traits are evaluated individually, but an aggregate assessment of overall turf quality is also taken. Turf quality is often assessed on a 1-9 scale, which can be easily used by breeders, sod producers, and golf course superintendents alike. Because visual ratings can be subjective, other methods, including the use of handheld sensors (Bell et al., 2009; Rodriguez and Miller, 2000) or unmanned aerial vehicles

(UAV), have been evaluated for effectiveness and efficiency (Zhang, J. et al., 2019). Significant variation among genotypes have been identified for overall turf quality (Kimball et al., 2018; Philley et al., 1996; Reynolds et al., 2009) and genetic color, as well as leaf texture, although the range for texture is narrower within diploid materials (Kimball et al., 2016). In a diallel study, Kimball et al. found additive gene effects for turf quality and texture were higher than non-additive effects, but the opposite was true for genetic color. These data indicate that both selection of improved parents and crosses with high specific combining ability are important for improving St. Augustinegrass performance (2016). Mississippi State University released ‘MSA 2-3-98’, which has similar freeze tolerance to ‘Raleigh’ but improved turf quality (Reasor and Philley, 2019).

Gray leaf spot (GLS) is a fungal disease (caused by *Pyricularia grisea*), which causes gray/brown lesions to develop on the leaves. If untreated and severe, turf can appear scorched, and ultimately large patches may die. Gray leaf spot is currently controlled through the use of fungicides, which can be costly and difficult for homeowners. Carbajal et al. (2020) identified significant variation for gray leaf spot resistance among 58 genotypes and identified both polyploid and diploid genotypes with high levels of resistance. Multiple breeding programs are working to improve gray leaf spot resistance, including the University of Florida, which released ‘NUF-76’ in 2015 with resistance to some races of GLS (Lu et al., 2015), and Texas A&M University, which released ‘DALSA 0605’ with improved GLS tolerance (Chandra et al., 2015).

Heritability has been estimated for many traits in St. Augustinegrass. Estimates of narrow-sense heritability for winter survival ranged from 0.70 to 0.98 (Philley et al., 1998). Generalized heritability estimates for overall turf quality in a multi-state trial yielded estimates of 0.16-0.90, but values were typically over 0.5 (Gouveia et al., 2020). A broad sense heritability estimate of susceptibility to gray leaf spot has been estimated to be 0.69 (Atilano and Busey, 1983).

Additionally, a diallel with reciprocals indicated that general combining ability was the most significant source of variation for winter survival and lethal temperature (Philly et al., 1998). Moreover, the large proportion of additive variance identified indicates that freeze tolerance should respond to selection. Another diallel where the hybridity of crosses was confirmed using SSR markers found that both specific combining ability (SCA) and general combining ability (GCA) were significant sources of variation, but that SCA accounted for three times as much genetic variance than GCA (Kimball et al., 2016). These moderate to high heritability estimates across the board indicate the potential for rapid genetic gain for each trait.

### **Freeze Tolerance in St. Augustinegrass**

St. Augustinegrass is susceptible to winter injury which can result in the loss of the turfgrass stand over winter, known as winterkill. Plant response to freezing stress is a complex quantitative trait as winterkill can be caused by damage from low temperatures resulting in desiccation and death of tissue. This complexity confounds the ability to determine the genetic control of freezing tolerance (Fowler et al., 1993).

Although variation among genotypes of St. Augustinegrass for winter survival in field studies is well reported, cultivar ‘Raleigh,’ released in 1980, remains the industry standard for freezing tolerance (Bateman, 1980). A large-scale trial in Arkansas reported 0-10.8% winter survival for 30 genotypes, with cultivar ‘Raleigh’ being the most winter hardy and ‘Seville’ among the least (Moseley et al., 2012). A lab-based freeze study by Milla-Lewis et al. (2013b) reported that ‘Raleigh’ is significantly more freeze tolerant than ‘Seville,’ but that the latter benefited more from cold acclimation than ‘Raleigh’. A multi-year field trial in northern Mississippi showed cultivars varied significantly in winter survival and that diploid varieties had greater winter

survival than polyploid ones (Phillee et al., 1996). Additionally, F<sub>1</sub> hybrid lines were found to show significant variation for winterkill in a North Carolina trial (Kimball et al., 2018). Another set of field evaluations in North Carolina identified several genotypes with comparable freeze tolerance to 'Raleigh' but also found levels of freeze tolerance varied by location (Reynolds et al., 2009). Despite ample identification of freeze-tolerant germplasm, limited progress has been made in breeding for freeze tolerance in St. Augustinegrass, perhaps in part due to the limited understanding of genetic and environmental control of the trait.

While field winter survival is a key long-term breeding goal, field evaluations are often unpredictable, challenging to reproduce, and require significant land and labor resources (Anderson and Taliaferro, 2002). In addition, environmental effects have been found to significantly affect winter survival and spring green-up responses in St. Augustinegrass. Meanwhile, lab-based freeze tests have been shown to correlate well with field winter survival evaluations (Kimball et al., 2017a). Different plant materials have been used to assess freeze tolerance in St. Augustinegrass in lab-based tests, including freezing of single stolon nodes (Li et al., 2010; Phillee et al., 1995), 4-node stolon cuttings (Maier et al., 1994a), whole stolons (Milla-Lewis et al., 2013b), and whole plants (Kimball et al., 2017a; Kimball et al., 2017b). Milla-Lewis et al. (2013b) observed differences in freeze survival of basal, mid, and apical regions of the stolon, indicating plant age affects freeze tolerance. Since node age confounds freeze tolerance and whole plant evaluations correlate well with field winter survival -which is the ultimate goal in breeding for freeze tolerance- the latter constitute arguably the best materials for lab-based freeze assessments. Additionally, freeze tolerance responses have been assessed by electrolyte leakage (EL), visual ratings, and digital image analysis, all of which detect significant differences for genotype. Evaluation of EL indicates that the method might produce inconsistent values and result

in underestimation (Cardona et al., 1997; Fry et al., 1993) or overestimation (Maier et al., 1994b) of freeze tolerance. Visual ratings, which may include regrowth (RG), surviving green tissue (SGT), or a combined score of overall survival, are consistent with field evaluations (Kimball et al., 2017a). Visual ratings are also quick to conduct and do not require any technology or extensive data storage, but they can be subjective. Digital imaging of plants in lab freeze tests measures the percentage of green coverage for each pot over time. These measurements are quantitative and objective but can take more time if individual pots need to be photographed and photos processed with image analysis software. Kimball et al. (2017a) found that while visual ratings of overall survival were highly correlated with digital percent green cover measurements, visual ratings were more cost and labor efficient.

Many factors influence freeze tolerance, including acclimation, de-acclimation, freeze injury, and desiccation. Cold acclimation is the increase in freezing tolerance as plants adapt to, non-freezing temperatures, and is characterized by the upregulation and downregulation of cellular components in response to cold temperatures (Fowler and Thomashow, 2002). Cool-season turfgrasses are well known for their ability to acclimate to the cold, while warm-season turfgrasses vary in that ability. In St. Augustinegrass, cold acclimation varies by genotype (Kimball et al., 2017b; Li et al., 2010; Maier et al., 1994a; Milla-Lewis et al., 2013b). Genotype ‘Floritam’ appears to not respond to cold acclimation (Busey, 2003b; Fry et al., 1991), while ‘Raleigh’ shows a significant acclimation effect (Kimball et al., 2017b; Li et al., 2010; Maier et al., 1994a). ‘Seville,’ known for its lack of freeze tolerance, was one of the top performers when cold-acclimated (Kimball et al., 2017b). Kimball et al. reported that genotypes with the highest field winter survival also had the highest acclimation ability indicating cold acclimation is crucial to freeze tolerance (2017b).

De-acclimation refers to the loss of cold acclimation or de-hardening of plants and plays a large role in winter survival. De-acclimation results from warming temperatures in late winter and early spring and can occur in one day (Gay and Eagles, 1991). If the warming event is followed by freezing temperatures, which can happen more frequently in the transitional climatic region of the US, plants could sustain serious injury. Studies on cool-season turfgrasses suggest both temperature and duration of a de-acclimation event can affect the plant's ability to remain freeze tolerant. Warmer de-acclimation events with longer durations result in less freeze tolerance retention (Gay and Eagles, 1991; Hoffman et al., 2010; Jørgensen et al., 2010). The effects of de-acclimation on warm-season turfgrasses have been less well characterized, but in bermudagrass, de-acclimation causes rapid, detrimental loss of freeze tolerance (Chalmers and Schmidt, 1979; Zhang, X., Wang, et al., 2011; Zhang, X. et al., 2011). In St. Augustinegrass, Kimball et al. found that de-acclimation affects genotypes differently, with some being more susceptible than others (2017b). Differences between non-acclimated and de-acclimated plants for a given genotype were rarely observed, indicating de-acclimation may negate the increase in hardiness from cold acclimation (Kimball et al., 2017b). The impact on temperature and duration of de-acclimation events on freeze tolerance, as well as the variation in response to de-acclimation by genotype, need to be further investigated to better understand overall freeze tolerance in St. Augustinegrass.

### **Quantitative Trait Loci Mapping Overview**

In most crop species, many important traits, such as yield, disease resistance, and stress response, are controlled by several genes, which results in a continuous distribution of values. Quantitative trait locus (QTL) analysis aims to identify regions of a genome that contribute to the

phenotype of the trait of interest. The location of a QTL can be identified using linkage maps and phenotypic data from a population (Collard et al., 2005).

The population used for QTL analysis is important as it must be segregating for the trait of interest. Moreover, the type of population that is used to construct the linkage map affects the statistical assumptions and the QTL resolution (Keurentjes et al., 2011). For many self-pollinating species, inbred lines can be used to develop a complex population structure, but when species do not tolerate inbreeding, other populations can be developed (Collard et al., 2005). In general, populations that are the result of many recombination events have the potential for improved precision because recombination blocks are broken down. In St. Augustinegrass and other turf and forage grasses, which are outcrossing species,  $F_1$  (often called “pseudo-testcross” or “pseudo-  $F_2$ ”) populations are typically used for linkage mapping (Brown et al., 2020; Hatier et al., 2014; Khanal et al., 2019; Takahashi et al., 2014; Yu et al., 2018). These populations are developed by crossing two heterozygous parents. If one parent is heterozygous at a given locus and the other is homozygous or null, the segregation ratio at that locus in the  $F_1$  progeny is 1:1, the same as the segregation ratio for a testcross (Grattapaglia and Sederoff, 1994). Likewise, if both parents are heterozygous at a given locus, there will be a 1:2:1 genetic ratio in the progeny, which is the classic genotypic ratio for  $F_2$  populations.

Methods for identifying QTL include single marker analysis (SMA), simple interval mapping (SIM), and composite interval mapping (CIM), where SMA is the simplest and CIM is the most complex. In SMA, a t-test, analysis of variance (ANOVA), or linear regression can be used to test if individuals in one genotypic class are statistically different in phenotype from those in other genotypic classes. While SMA does not require a complete linkage map, the effect of the QTL may be underestimated, and the further the marker is from the QTL, the less likely it will be

detected (Collard et al., 2005). Both SIM and CIM use adjacent markers to test the likelihood of a true QTL within the interval. However, CIM uses a multiple QTL model that takes into account there might be QTL elsewhere in the genome, which can interfere with detection (Akond et al., 2019). While SIM is the most widely used QTL detection method, CIM is more effective at identifying QTL (Akond et al., 2019).

As the complexity and accuracy of QTL detection methods increase, so does the need for specialized statistical software and analysis. There are many software options available: MapQTL, a paid program with a graphical user interface (GUI) making it simple to use (Van Ooijen, 2009); R/qtl, a widely used free R package that requires the use of the R coding language (Broman, 2012); QTL Cartographer, a free command-line based software with a GUI implementation called Windows QTL Cartographer (Wang et al., 2012); and MVQTL-CIM, a free command-line based software developed for F<sub>1</sub> hybrid mapping (Liu et al., 2017). QTL Cartographer is limited to analysis of inbred populations while MapQTL, r/qtl, and MVQTL-CIM are capable of --or built for-- analyzing hybrid F<sub>1</sub> populations.

After primary identification of QTL, it is important to validate that the QTL can be detected under other conditions and in different genetic backgrounds to rule out statistical errors or artifacts. Such population-specific QTL are common, and in some cases, most QTL may be dependent on the background or location (Chen et al., 2020; Han et al., 2012). In order to confirm QTL are independent of the genetic background or environment, they should be able to be detected in multiple populations (Gutiérrez et al., 2013). Once the validity of QTL is established, they can be utilized for marker-assisted breeding.

Marker-assisted selection (MAS) is a form of indirect selection wherein DNA markers are used to make selections rather than the trait itself. Therefore, one objective of QTL analysis is to

identify genetic markers that are tightly linked to the QTL of interest so they can be used for MAS. Markers can be used to help pyramid together several QTL to improve the trait of interest more rapidly than traditional breeding. Advantages of MAS over conventional selection include: there is no confounding effects from the environment, it can be carried out on young plants and the undesirable plants can be discarded (saving resources), and single plants can be selected, which may be unreliable with traditional evaluations (Collard and Mackill, 2008). MAS can be a cost-effective tool, particularly in turfgrass breeding, because it can reduce the need for multi-year field evaluations, thus improving the efficiency of the breeding pipeline (McClure et al., 2014).

Freeze tolerance is important in all turf- and forage-grass species. Therefore, multiple studies have been conducted to identify genomic regions controlling this trait. In switchgrass, a biparental mapping population was used to identify six QTL for freeze tolerance, including one associated with median lethal temperature (LT50) on chromosome 5K, which corresponds to the upland parent (Poudel et al., 2019). In *Zoysia japonica*, nine QTL were identified for surviving green tissue, eight for regrowth following freezing, and 22 for both traits. All these QTL each accounted for 6.4-12.2% of the phenotypic variance (Brown et al., 2020). In bermudagrass, a diverse panel of seeded varieties was used in an association mapping study, which identified 34 markers associated with turf quality and cold tolerance traits (Fan et al., 2020). These studies underscore the complexity of plant response to freezing and the challenges plant breeders face in incorporating freeze tolerance into elite lines.

### **QTL Mapping in St. Augustinegrass**

While genomic tools for St. Augustinegrass still lag behind many other crops, significant advances have been made during the past decade. Mulkey et al. (2014) used a set of 190

polymorphic simple sequence repeat (SSR) markers to construct the first partial linkage map for the species. The first complete linkage map for *St. Augustinegrass* was developed using 160 SSR markers for a pseudo-F<sub>2</sub> ‘Raleigh’ x ‘Seville’ population (Kimball et al., 2018). The map included all nine linkage groups, covered a total distance of 1176.24cM, and had an average marker distance of about 7.5cM (Kimball et al., 2018). Shortly afterward, a high-density linkage map was developed from the same population using genotyping by sequencing (GBS). This map contained 2,871 single nucleotide polymorphisms (SNPs) spanning 1241.7cM with an average marker distance of 0.4cM (Yu et al., 2018). Most recently, the first reference genome for the species is being developed for cultivar ‘Raleigh’ (Schoonmaker, unpublished). Additionally, transcriptome analysis is underway via RNA sequencing of two *St. Augustinegrass* genotypes in response to drought stress (Yu, unpublished).

QTL have been identified in *St. Augustinegrass* for several traits, including drought, freezing tolerance, and turf quality. Kimball et al. (2018) identified six QTL for field winter survival (winterkill, spring green-up), twenty QTL for freeze tolerance (regrowth and surviving green tissue measured under controlled environment freeze tests), and fourteen QTL for turf quality traits evaluated in the field (turf quality, turf density, leaf texture, and genetic color). Notably, QTL for both field and lab freeze traits that co-localized on linkage group 3 were identified (Kimball et al., 2018). Yu et al., using the same population as Kimball et al. (2018), developed a high-density SNP-based linkage map and used comparative genomics to number the linkage groups according to the foxtail millet genome. The study then identified 48 putative turf quality QTL, including overlapping regions for leaf texture, turf density, genetic color, and overall turf quality on linkage groups 3 and 8, which corresponded to linkage groups 3 and 5 presented in Kimball et al. (Yu et al., 2018). Furthermore, 70 QTL were later identified in the population for

relative water content, chlorophyll content, leaf firing, leaf wilting, green cover, and normalized difference vegetative index under drought conditions (Yu et al., 2019). Several regions of co-localization were identified, including one on linkage group R6 (where R represents cultivar Raleigh), where three traits overlapped (corresponding to linkage group 4 in Kimball et al.) (Yu et al., 2019). In an F<sub>1</sub> population derived from Raleigh x PI 410353, two major effect QTL were identified for gray leaf spot resistance (GLS) on linkage groups P2 and P5 (where P indicates resistant parent PI 410353) (Yu et al., 2020). Sequence analysis in these regions identified two  $\beta$ -1,3-glucanases in these regions, which might represent candidate genes for GLS resistance.

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## **Chapter 2**

**Multi-environment evaluation of St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] breeding lines for turf quality traits and stress tolerance in North Carolina**

**Multi-environment evaluation of St. Augustinegrass [*Stenotaphrum secundatum* (Walt.)  
Kuntze] breeding lines for turf quality traits and stress tolerance in North Carolina**

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## ABSTRACT

St. Augustinegrass (*Stenotaphrum secundatum* [Walt.] Kuntze) is a warm-season turfgrass primarily used for home lawns and commercial landscapes in the Southern United States. New cultivars that possess desirable turf quality in combination with improved tolerance to freeze, drought, and fungal pathogens are needed to increase the sustainability of St. Augustinegrass maintenance. This study's objectives were to 1) evaluate St. Augustinegrass breeding lines in multi-environment trials to select genotypes with stable performance across North Carolina, and 2) assess relationships among economically important traits. Sixty-one St. Augustinegrass breeding lines were established in replicated field trials at three locations. Entries were evaluated for establishment rate, turf quality, turf density, genetic color, leaf texture, uniformity, winterkill, fall color, drought tolerance, and gray leaf spot resistance from 2017 to 2020. Mixed model analysis was used to estimate best linear unbiased predictions (BLUPs), which were used to calculate a selection index to identify elite genotypes across traits. Generalized heritability estimates for each trait ranged between 0.38 and 0.87. The ten traits were generally well correlated with one another and clustered into three groups: winterkill and fall color; genetic color, texture, and gray leaf spot resistance; and establishment, turf quality, density, uniformity, and drought tolerance. Selection of the top 10 genotypes using the selection index resulted in positive estimated genetic gains for all ten traits, indicating it is an effective method for simultaneous selection. XSA 14271 outperformed the commercial checks for several traits and was the top-ranked line. It will be advanced to on-farm trials to evaluate sod production in order to assess its potential for commercial release.

**KEYWORDS:** St. Augustinegrass, turfgrass breeding, heritability, multi-environment trials

**Abbreviations:** AR1, first-order autoregressive matrix; AR1H, first order autoregressive heterogeneous matrix; BIC, Bayesian information criterion; BLUP, best unbiased linear predictor; CS, compound symmetry matrix; CSH, compound symmetry heterogeneous matrix; DEN, turf density; EST, establishment rate; FA1, first-order factor analytic matrix; FC, fall color; G, genetic covariance matrix; GC, genetic color; GE, genotype-by-environment; GLS, gray leaf spot;  $h^2$ , narrow-sense heritability;  $H^2$ , board-sense heritability; LRT, Likelihood Ratio Test; MET, Multi-environment trial; MVN, Multivariate Normal; NCSU, North Carolina State University; NTEP, National Turfgrass Evaluation Program; R, residual covariance matrix; REML, restricted maximum likelihood; t-BLUP, studentized best linear unbiased predictors; TEX, leaf texture; TQ, turfgrass quality; UN, uniformity; US, unstructured matrix; WK, winterkill.

## INTRODUCTION

St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] is a warm-season turfgrass typically grown in landscapes and home lawns in the Southeastern United States. The species is characterized by its course leaf blades, aggressive growth, salinity tolerance, and superior shade tolerance. However, a lack of winter hardiness has limited its use into the transition zone of the United States, including North Carolina. Despite this, data from the 2020 sod producers report shows the demand for St. Augustinegrass in the state is increasing (Miller, 2020).

To meet the growing demand, St. Augustinegrass breeders focus on developing cultivars with regional adaptation and superior performance for multiple important traits, including turf quality, speed of establishment, which influences sod production, and biotic and abiotic stress tolerances such as drought stress, winter injury, and gray leaf spot (caused by *Pyricularia grisea*). The assessment of turf quality, defined as the aesthetic value of turfgrass, includes genetic color, leaf texture, turf density, and uniformity. These traits are evaluated individually, but an aggregate assessment of turf quality is also taken (Morris and Shearman, 1998). Reducing the need for irrigation is a key challenge for the turfgrass industry; therefore, retaining turf quality under drought stress is an important breeding objective. St. Augustinegrass is susceptible to winter injury, which can result in the loss of the turfgrass stand over winter, known as winterkill (Beard, 1963). Winterkill can be caused by either damage from low temperatures or desiccation of tissue (Beard, 1963; Fowler et al., 1993). Gray leaf spot is a fungal disease, which causes gray/brown lesions to develop on the leaves (Harmon, 2018). If untreated and severe, turf can appear scorched, and ultimately large patches may die. Gray leaf spot is typically controlled through the use of fungicides, which can be costly and difficult for homeowners (Harmon, 2018).

Multi-environment trials are used by breeders to evaluate the performance of breeding lines across different environments and estimate the level of genotype-by-environment interaction (GEI) present. Linear mixed models can be used to analyze multi-environment trials in order to reduce the impact of incomplete data, include some sources of variation as random rather than fixed, and handle realistic within-trial covariance structures (Smith et al., 2005). Covariance structures describe the relationship between measurements in a trial and can capture spatial tendencies and consider the non-independence of the plots in time-series data and the heterogeneity of variance of the residual and genetics effects (Wolfinger, 1993). Mixed model analysis of multi-environment trials can be used to predict genotype performance and conduct index selection, which allows multiple traits to be selected simultaneously rather than sequentially (Lin, 1978; Smith et al., 2005). Furthermore, this analysis can be used to calculate type-B genetic correlations, an estimate of genotype-by-environment interaction, as well as heritability (Yamada, 1962).

Heritability is defined as the proportion of the phenotypic variance among individuals in a population that is due to genetic effects and is helpful for estimating expected gains from selection (Holland et al., 2002). Heritability can be measured in the broad sense ( $H^2$ ), which includes additive, dominance, and epistatic effects, or in the narrow sense ( $h^2$ ), including only additive effects (Schmidt et al., 2019). Additionally, a generalized heritability can be estimated. In a simple case with balanced data, this will be equal to the broad-sense heritability, but in more complex scenarios, the generalized heritability is a better estimator of response to selection (Cullis et al., 2006). Heritability estimates vary for traits within a population but can be used alongside genetic correlations among traits to identify the most effective indirect selection schemes (Holland et al., 2002).

While St. Augustinegrass has been previously evaluated for several traits via multi-environment trials, these studies often focused on only one or a few traits. Significant variation among genotypes has been identified for overall turf quality (Kimball et al., 2018; Philley et al., 1996; Reynolds et al., 2009; Gouveia et al., 2020), genetic color, texture (Kimball et al., 2016), winter survival (Moseley et al., 2012; Milla-Lewis, 2013; Philley et al., 1996; Kimball et al., 2018; Reynolds et al., 2009), and gray leaf spot resistance (Carbajal et al., 2020). Additionally, heritability has been estimated for several of these traits. Generalized heritability estimates for overall turf quality in a multi-state trial yielded estimates of 0.16-0.90, but values were typically over 0.5 (Gouveia et al., 2020). Heritability in the broad sense for susceptibility to gray leaf spot was estimated to be 0.69 (Atilano and Busey, 1983). Estimates of narrow-sense heritability for winter survival ranged from 0.70 to 0.98 (Philley et al., 1998). Despite moderate to high heritability estimates and identification of sources of variation for this trait, limited improvements have been made and cultivar 'Raleigh,' released in the 1980s, remains the industry standard for freeze tolerance (Bateman, 1980).

While research has been conducted on evaluating several St. Augustinegrass traits of agronomic and economic importance, little is yet known about the relationship among these traits or the best selection scheme for improving this species. As the market demand for St. Augustinegrass grows in North Carolina, there is a need for cultivars that combine superior turf quality with tolerance to local biotic and abiotic stresses. To this aim, there is a need to understand the relationships among important traits in order to further advance the use of St. Augustinegrass in the transition zone. Thus, the objectives of this study were to i) to evaluate St. Augustinegrass lines in multi-environment trials in order to select lines with stable performance across North Carolina, and ii) report on the relationships among important phenotypic traits to improve selection

efficiency, and 3) assess the efficiency of using a selection index for selection of superior breeding lines.

## **MATERIALS AND METHODS**

### **Plant Material and Field Trial**

Sixty-one advanced breeding lines from the North Carolina State University (NCSU) turfgrass breeding program and five commercial checks, cultivars ‘Palmetto’, ‘Raleigh’, ‘Captiva’, ‘Seville’ and ‘Tamstar’, were evaluated from 2017 to 2019 in a field study. The trial was planted in a randomized complete block design with three replications at three locations in North Carolina: the Lake Wheeler Turfgrass Field Laboratory in Raleigh (LW, Lat. 35.72816° Long. 78.67981°, 116.434m elevation, 11.34°C to 22.58°C average temperature range, average rainfall = 4.06 in monthly), the Upper Mountain Research Station in Laurel Springs (LS, Lat. 36.40232°, Long. 81.29711°, 917m elevation, 6.36°C to 16.82°C average, average rainfall = 5.77 in monthly), and the Sandhills Research Station in Jackson Springs (SH, Lat. 35.18782°, Long. 79.68437°, 190.5m elevation, 11.57°C to 22.61°C average temperature range, average rainfall = 4.47 in monthly). Monthly temperature and precipitation data for the duration of the study is available in Supplementary Figures 4.1 – 4.3. In the early summer of 2017, eight 10.16 x 10.16 cm plugs were planted in 0.91x 0.91 m plots with 0.46 m alleys between plots. Plots were mowed weekly at a height of 7.62cm and irrigated as needed until established. Nitrogen (N) fertilizer was applied at 48.8 kg ha<sup>-1</sup> monthly over the growing season for a total accumulation of 244 kg N/ha per year. Chemical and manual weed control were used within and between plots as needed and according to recommended St. Augustinegrass management practices for NC (Miller et al., 2016).

## Phenotypic Evaluations

The ten response variables evaluated included rate of establishment (EST); turf quality traits: overall turf quality (TQ), turf density (DEN), genetic color (GC), leaf texture (TX), and uniformity (UN); fall color (FC); winterkill (WK); drought resistance (DRY); and gray leaf spot incidence (GLS). In total, the trial was evaluated over a four-year period from 2017 to 2020. However, only EST and TQ were evaluated in the first year of the trial, and since WK is rated at the very end of the season, following winter, this trait was evaluated 2018-2020. Additionally, DRY and GLS were evaluated only in years and locations where natural incidence occurred.

Evaluations of EST (percent turfgrass cover at the end of planting year) were performed visually (0-100%) at SH, while at LW and LS, EST was evaluated using digital image analysis (Richardson et al., 2001) taken inside a 61 L x 52 W x 56 H cm light box fitted with four 9-watt compact fluorescent light bulbs (TCP 5800965K, TruStart) and then, images were batch analyzed (Karcher and Richardson, 2005). However, unfortunately, due to a data storage error, digital images of plot coverage at LW were lost.

Turf quality traits were evaluated visually on a 1 to 9 whole number scale according to the National Turfgrass Evaluation Program guidelines (Morris and Shearman, 1998) as follows: TQ, 1 = poorest or dead turf, 9 = outstanding or ideal turf, and 6 = minimum acceptable quality; DEN, 1 = sparse turf to 9 = densest turf per unit area; GC, 1 = light green to 9 = dark green; TX, 1 = coarse leaf blades to 9 = fine leaf blades; and UN, 1 = highly variable to 9 = very consistent turf surface. Additionally, FC, WK and DRY were also evaluated visually as follows: FC, 1 = straw brown to 9 = dark green; WK, 1 = 100% injury and 9 = no injury, and DRY, 1 = complete wilting to 9 = no wilting, no leaf firing.

When incidence of GLS occurred (2017 at LW and SH, 2018 at LS, and 2019 at LW), it was evaluated either on a 1-9 scale where 1 = no damage and 9 = 100% damage or as a percentage of the plot covered by lesions (0-100%). For the combined analysis, so that higher values would indicate superiority for all ten traits, GLS values were converted to percentages and flipped to indicate resistance rather than GLS incidence.

## Statistical Analysis

Data were analyzed using mixed-models via *ASReml-R* within the RStudio environment (Butler et al., 2018; RStudio Team, 2020). The significance of each factor was evaluated by likelihood ratio test (LRT) for random effects and by Wald tests for fixed effects. The variance components were estimated with restricted maximum likelihood (REML).

For each multi-year trait, repeated measure multi-location analysis was performed using the model:

$$y = \mu 1 + X_1 l + X_2 t + X_3 lt + Z_1 r(l) + Z_2 g + Z_3 gl + Z_4 gt + Z_5 glt + e \quad (\text{Eq. 1})$$

Where  $y$  is the vector of phenotypic values,  $\mu$  is the overall mean,  $X$ 's and  $Z$ 's represent the incidence matrices for fixed and random effects, respectively;  $l$  is the fixed vector of location effects;  $t$  is the fixed vector of year effects;  $lt$  is the fixed vector of the effects of the interaction between locations and years;  $r(l)$  is the random vector of replication effect nested within location where  $r(l) \sim \text{MVN}(0, \sigma_{r(l)}^2 \mathbf{I})$  where  $\sigma_{r(l)}^2$  is the variance component of the replication effect nested within location;  $g$  is the random vector of genotype effects with  $g \sim \text{MVN}(0, \sigma_g^2 \mathbf{I})$  where  $\sigma_g^2$  is the variance component of the genotype effects;  $gl$  is the random vector of the effects of the interaction between genotype and location with  $gl \sim \text{MVN}(0, \sigma_{gl}^2 \mathbf{I})$  where  $\sigma_{gl}^2$  is the variance component of the effects of the interaction between genotype and location;  $gt$  is the random vector of the effects of the interaction between genotype and year with  $gt \sim \text{MVN}(0, \sigma_{gt}^2 \mathbf{I})$  where  $\sigma_{gt}^2$  is

the variance component of the effects of the interaction between genotype and year;  $glt$  is the random vector of the effects of the interaction between genotype, location, and year with  $glt \sim \text{MVN}(0, \sigma_{glt}^2 \mathbf{I})$  where  $\sigma_{glt}^2$  is the variance component of the effects of the interaction between genotype, location and year; and  $e$  is the random vectors of errors with  $e \sim \text{MVN}(0, \mathbf{R} \otimes \mathbf{I})$ .  $\mathbf{R}$  is the covariance matrix of residuals,  $\mathbf{I}$  is an identity matrix of proper size, and  $\mathbf{1}$  is a vector of ones. The Kronecker product is denoted by  $\otimes$ .

For EST, which was a single year trait, the multi-location analysis followed the model:

$$y = \mu \mathbf{1} + X_1 l + Z_1 r(l) + Z_2 g + Z_3 gl + e \quad (\text{Eq. 2})$$

Where  $y$  is the vector of phenotypic values,  $\mu$  is the overall mean,  $X$ 's and  $Z$ 's represent the incidence matrices for fixed and random effects, respectively;  $l$  is the fixed vector of location effects;  $r(l)$  is the random vector of replication effect nested within location where  $r(l) \sim \text{MVN}(0, \sigma_{r(l)}^2 \mathbf{I})$  where  $\sigma_{r(l)}^2$  is the variance component of the replication effect nested within location;  $g$  is the random vector of genotype effects with  $g \sim \text{MVN}(0, \sigma_g^2 \mathbf{I})$  where  $\sigma_g^2$  is the variance component of the genotype effects;  $gl$  is the random vector of the effects of the interaction between genotype and location with  $gl \sim \text{MVN}(0, \sigma_{gl}^2 \mathbf{I})$  where  $\sigma_{gl}^2$  is the variance component of the effects of the interaction between genotype and location; and  $e$  is the random vectors of errors with  $e \sim \text{MVN}(0, \mathbf{R} \otimes \mathbf{I})$ .  $\mathbf{R}$  is the covariance matrix of residuals,  $\mathbf{I}$  is an identity matrix of proper size, and  $\mathbf{1}$  is a vector of ones. The Kronecker product is denoted by  $\otimes$ .

Drought only occurred at SH in a single year. Thus, analysis was performed using the model:

$$y = \mu \mathbf{1} + Z_1 r + Z_2 g + e \quad (\text{Eq. 3})$$

Where  $y$  is the vector of phenotypic values,  $\mu$  is the overall mean,  $X$ 's and  $Z$ 's represent the incidence matrices for fixed and random effects, respectively;  $r$  is the random vector of replication effect with  $r \sim \text{MVN}(0, \sigma_r^2 \mathbf{I})$  where  $\sigma_r^2$  is the variance component of the replication effect;  $g$  is

the random vector of genotype effects with  $g \sim \text{MVN}(0, \sigma_g^2 \mathbf{I})$  where  $\sigma_g^2$  is the variance component of the genotype effects; and  $e$  is the random vectors of errors with  $e \sim \text{MVN}(0, \sigma_e^2 \mathbf{I})$  where  $\sigma_e^2$  is the variance component of the errors.  $\mathbf{I}$  is an identity matrix of proper size, and  $\mathbf{1}$  is a vector of ones.

For Eq. 1, as suggested by Smith et al. (2007), the residual effects were structured considering locations and years, and thus  $\mathbf{R} = R_l \otimes R_t$ , being  $R_l$  the covariance matrix for locations, and  $R_t$  the covariance matrix for years. The residual covariance structures tested are in Supplementary Table 4.1. The R matrix was selected based on convergence, Bayesian information criterion (BIC), and likelihood ratio test (LRT). For each trait, the R matrix structure with the lowest BIC value was chosen for the model, and the selected model was compared to the CS model by LRT.

Type-B genetic correlations were calculated from all traits except drought using:

$$r_g = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_{gl}^2} \quad (\text{Eq. 4})$$

where  $\sigma_g^2$  is the variance component of the entries and  $\sigma_{gl}^2$  is the variance component of the entry by location interaction. Best linear unbiased predictions (BLUPs) were generated for each trait by entry using equations 1-3 and entry\*location using equations 1 and 2. A studentized BLUP (t-BLUP) was estimated for EST, TQ, FC, WK, DRY, and GLS by:

$$tBLUP = \frac{BLUP}{SE} \quad (\text{Eq. 5})$$

where BLUP is the prediction for each genotype and SE is the standard error of the respective BLUP (Yan et al. 2002). Due to space restraints, t-BLUPs for turf quality traits was limited to overall turf quality (TQ). Figures for type-B correlations and studentized BLUPs were created with the ggplot2 package (Wickham, 2016). Generalized heritability was estimated as follows:

$$H^2 = 1 - \frac{\bar{v}_{\Delta}^{\text{BLUP}}}{2 \sigma_g^2} \quad (\text{Eq. 6})$$

where  $\bar{v}_{\Delta}^{\text{BLUP}}$  is the mean variance of the difference of two BLUPs for the genotypic effect (Cullis et al., 2006). Spearman correlations between traits were calculated with a significance level of 0.05 and plotted using the function *corrplot*.

Principle component analysis was done using predicted values of entries for each trait in equations 1-3 using function *prcomp*. These values were used to construct a genotype by trait biplot which can identify elite lines across traits, genetic correlations between lines, and redundant traits using the *ggfortify* package (Kendal, 2019; (Tang et al., 2016)).

A modified base index was calculated in order to identify the top 15% of entries (corresponding to 10 genotypes). The index was calculated for each entry using the formula:

$$I_j = a_i H_i^2 P_i + \dots + a_n H_n^2 P_n \quad (\text{Eq. 7})$$

where  $I_j$  is the index value for  $j$ th entry,  $a_i$  is the weight for the  $i$ th trait,  $H_i^2$  is the generalized heritability for the  $i$ th trait, and  $P_i$  is the normalized phenotypic value (BLUP) for the  $i$ th trait.

The economic impact of each trait is not available, so the weights were assigned by the breeder based on the perceived importance of each trait as follows: 1.5 EST, 1.5 TQ, 1 WK, 1 DRY, 0.75 FC, 0.75 GLS. Since TQ encompasses all of the other aesthetic traits, only TQ was used in the index. Selection gain for each trait was calculated by:

$$SG(\%) = \frac{(\bar{X}_S - \bar{X}_O) * H^2}{\bar{X}_O} * 100 \quad (\text{Eq. 8})$$

where  $\bar{X}_S$  is the mean of the selected genotypes,  $\bar{X}_O$  is the mean of the whole population and  $H^2$  is the heritability as defined by equation 6.

## RESULTS

### Variance-Covariance Structures

Many different residual (R) variance-covariance structures were tested, but several did not converge due to the number of parameters. For each trait, the best R matrix was chosen based on the lowest BIC estimate (Supplementary Table 4.1) and LRT. The best R matrix for FC was compound symmetry (CS) across locations, while for GC, TX, and GLS, the best R matrix was also CS but within locations. The compound symmetry heterogeneous (CSH) R matrix across locations was selected for DEN. For TQ, UN, and WK, the first-order autoregressive heterogeneous (AR1H) R matrices that were homogeneous across locations presented the lowest BIC estimates.

### Correlations among Traits

Many of the ten traits evaluated were positively correlated with one another, but few correlations were significant (Figure 2.1). TQ was significantly correlated with DEN and UN with Spearman coefficients of 0.80 and 0.77, respectively. Meanwhile, GC was also significantly correlated with both DEN (0.54) and UN (0.78). Density and uniformity were highly correlated with a coefficient of 0.78. In general, the turf-quality traits were well correlated with one another. Other significant correlations included DRY and EST (0.27), FC and WK (0.5), and GLS and GC (0.34). The only significant negative correlation was GLS and FC, which had a correlation coefficient of -0.14.

The type-B correlation was used to estimate the stability of traits across locations. Estimates ranged from 0.67 to 1.0 (Figure 2.2). GC, UN, FC, and GLS all had estimates of 1. Meanwhile, type-B correlation estimates for EST, TQ, DEN, TX, and WK were 0.67, 0.93, 0.80,

0.87, and 0.97, respectively. Since DRY was evaluated in a single location, no type-B genetic correlation was estimated.

A genotype-by-trait biplot was developed using the predicted values for each trait. The first two principal components of this biplot accounted for 55% of the total variance (Figure 2.3). The biplot shows three clusters of correlated traits: FC and WK; EST, TQ, DEN, UN, and DRY; and GC, TX, GLS. The biplot did not indicate any strong negative correlations (180 ° angle), but many traits were not correlated (90 ° angle). While there are no distinct clusters of genotypes, the biplot graph showed genotypes with great potential performances for some traits.

### **Generalized Heritability & Selection**

For all ten traits (EST, TQ, DEN, GC, TX, UN, WK, FC, DRY, and GLS), entry was found to be a significant factor (Table 2.2). Location was found to be significant for all traits except GC and FC while year was significant for all traits except TQ and FC. Replication was a significant effect except for DEN, UN, and DRY. The two-way interaction of entry\*year did not have a significant effect on any trait. The genotype\*location interaction was only significant in the case of EST, but the entry\*location\*year three-way interaction was found to be significant for all traits where it was estimated. Complete results for LRT and Wald tests are available in the appendix.

The heritability of all traits ranged from 0.38 to 0.87, representing moderately low to high values (Table 2.2). DRY had the lowest heritability of 0.38, while GC and FC had moderate estimates of 0.55 and 0.48, respectively. TQ, DEN, TX, UN, EST and GLS, all fell within a narrow range from 0.65-0.72, while WK had the highest heritability value estimated at 0.87.

Studentized BLUPS (t-BLUPS) were generated by entry for each trait for which the entry variance was significant. Due to space limitations, t-BLUPS in Figure 2.4 are presented only for

commercial checks, the top ten rated lines, and the bottom three rated lines. For EST, no genotype significantly outperformed the mean. For TQ, several genotypes outperformed the mean including cultivars 'Palmetto' and 'Captiva' as well as several experimental lines. Experimental line XSA 14271 performed as well as check 'Palmetto' for FC and both were significantly above the mean; 'Tamstar' had below-average performance for FC. For WK, 'Palmetto', 'Raleigh' and many experimental lines surpassed 'Palmetto' and 'Raleigh' for WK, but XSA 14271 and XA 14132 had similar performances, and many genotypes significantly outperformed the mean. No genotype significantly outperformed the mean for DRY, and XSA 14450 was the only entry that had significantly improved GLS.

Elite lines across important traits were identified using a modified base index that allowed for simultaneous selection on EST, TQ, FC, WK, DRY, and GLS. Index values ranged from 5.60 to -10.19, where higher numbers indicate better performance. The top-ranked genotypes based on the selection index were XSA 14271, 'Palmetto', XSA 14506, XSA 14450, XSA 14464, XSA 14146, XSA 14132, and XSA 14315 (Table 2.3). The commercial checks 'Tamstar', 'Raleigh', and 'Captiva,' were ranked 9<sup>th</sup>, 11<sup>th</sup>, and 14<sup>th</sup>, respectively, while 'Seville' was ranked 40<sup>th</sup> with a value of -0.56, indicating inferior performance. A complete list of modified base index values is available in Supplementary Table 4.3.

The genetic gain for each trait was estimated under direct selection for each trait as well as using the modified base index (Table 2.4). For each trait, the maximum predicted genetic gain ranged from 3.7% for GC to 39% for WK. Indirect selection using the modified base index and direct selection for TQ, DEN, UN resulted in positive genetic gains for all traits. Direct selection for TX resulted in the largest decrease in genetic gain, -4.2% for WK, and also resulted in negative estimated gains for TX and DRY.

## DISCUSSION

Multi-environment trials are never completely homogeneous but ignoring the heterogeneity can bias genomic predictions thus, fitting a model with a heterogeneous variance-covariance structure can improve the fit of the overall model by accounting for relationships between measurements (such as time measurements were taken and physical proximity) (Isik et al., 2017). Compound symmetry covariance structure is a simple structure where there is a relationship between measurements, but it is not impacted by distance between measurements. This was the case for FC, while for GC, TX, and GLS CS within location had the best fit. The difference being a single covariance parameter was estimated for FC and a covariance estimate for each location was used in the case of GC, TX, and GLS. The heterogeneous extension of compound symmetry (CSH) across locations provided the best fit for DEN. Univariate models utilizing heterogeneous first-order autoregressive R matrices had the best fit for TQ, UN and WK. The first-order autoregressive (AR1) covariance structure is robust in many plant breeding applications as it takes into account the distance between measurements and covariance decreases as distance increases; the heterogeneous variation of this (AR1H) includes heterogeneous variance estimates (Isik et al., 2017). These commonly used covariance structures improved the overall fit of the model for each trait.

In plant breeding, genotypes with superior performance, but low GEI are preferred as they can express their genetic potential in different environments (Ceccarelli, 1989). Genotype-by-environment interaction (GEI) poses a challenge as plant breeders must either select for genotypes that perform well across environments sacrificing potential genetic gain or develop separate populations for each environment where performance is significantly different (Kang, 1997). While some studies in St. Augustinegrass have found genotype-by-environment interaction to have

an effect on St. Augustinegrass performance (Reynolds et al., 2009), others found that environment plays a minor role in genotype variability (Kimball et al., 2016) and indicated it has better stability across locations than other turfgrass species (Gouveia et al., 2020). In this study, St. Augustinegrass performance was stable across locations as evidenced by entry\*location not being a significant factor with the exception of EST. Furthermore, type-B genetic correlations, which indicate rank changes among the same genotypes across different locations, revealed no genotype-by-location interaction for GC, UN, FC, and GLS and minimal interaction for DEN, TX, and WK. However, while entry\*location was only significant for EST and entry\*year was not significant for any of the traits for which it was evaluated, entry\*location\*year was a significant factor for all traits that had these three-way interactions. Therefore, GEI doesn't appear to have a large effect on St. Augustinegrass performance, and it may be possible to expand evaluations to a regional trial with fewer locations within North Carolina in order to advance breeding objectives.

Heritability values for the traits evaluated in this study ranged from moderately low to high (0.38 to 0.87). The estimate for TQ of 0.72 fell within the range of previously estimated values (Gouveia et al., 2020) as did heritability estimates for WK and GLS (Atilano and Busey, 1983, Philley et al., 1998). Heritability can be utilized as an indicator of response to selection, and therefore improving the accuracy of heritability estimates can improve selection efficiency (Cassell, 2009; Schmidt et al., 2019). As improving phenotyping accuracy can increase heritability, St. Augustinegrass breeders should consider the use of high-throughput phenotyping tools, such as digital image analysis or the use of unoccupied aerial systems (Madec et al., 2017) to improve traits such as drought tolerance for which heritability was moderately low.

A unique challenge for turfgrass breeders is improving multiple essential traits at the same time and breaking any negative associations among traits. In this study, most of the traits were

found to be positively correlated with one another. However, modest negative correlations were observed among TX and EST, FC, WK, and DRY, suggesting it may be difficult to improve TX without sacrificing the other traits, which corresponds with anecdotal accounts. Kimball et al. (2016) also identified mostly positive correlations among St. Augustinegrass traits with the exception of leaf texture and winter survival. Improvement in one trait might positively or negatively affect another, and thus, consideration of all traits of importance for a particular crop is an important consideration in plant breeding (Baker, 1986). While genetic gains can be increased for a single trait through the use of direct selection, this might occur at the expense of gain in other traits. The use of the selection index, first proposed by Smith (1936) to reflect the merits and demerits of several traits, has long been recognized as an appropriate method for improving simultaneously multiple traits in a breeding program (Baker, 1986). In our study, despite the presence of some negative correlations among traits, the use of a modified base index resulted in positive genetic gains for all ten traits. Furthermore, we were able to quantify the relationship among many economically important traits and identify an efficient selection strategy. While the use of selection indices has been investigated in several major crops, their use in turfgrass breeding has been limited, and to our knowledge, this is the first report for St. Augustinegrass. However, a few studies have been conducted recently on the economic value of different turfgrass traits (Chung et al., 2018; Ghimire et al., 2019). As more of such studies are conducted, better selection indices might be developed, but the one utilized in this study indicates its potential for improvement across all evaluated traits.

Significant genetic variation for all traits evaluated was identified in the present study. Experimental line XSA 14271 was the top-ranked line based on the selection index and performed as well as the checks for TQ, FC, and WK. Line XSA 14450 not only ranked highly (4<sup>th</sup>) on the

selection index but showed the highest level of GLS resistance indicating this line might be valuable for future breeding efforts to improve this trait. Although XSA 14271 will need to be advanced to on-farm trials to evaluate sod production traits in order to assess its potential for commercial release, its performance and stability appear promising. Furthermore, results presented here show that breeding efforts for St. Augustinegrass have been effective in achieving genetic gains for multiple traits of agronomic importance. As demand for this grass increases, planting cultivars with wider adaptation across North Carolina and improved stress tolerance will reduce reestablishment costs and ultimately increase sustainability.

### **Acknowledgments**

The authors wish to thank the personnel at the Lake Wheeler Turfgrass Field Lab, the Sandhills Research Station, and the Upper Mountain Research Station for assistance with maintenance of experimental plots. This research was supported by funding provided by the North Carolina Crop Improvement Association, the North Carolina State University Center for Turfgrass Environmental Research and Education, and Specialty Crop Research Initiative grant [2015-51181-24291] from the USDA National Institute for Food and Agriculture.

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**Table 2.1:** Traits collected on advanced breeding lines of St. Augustinegrass evaluated 2017 through 2020 in replicated field trials at three locations in North Carolina.

<b>Trait</b> <sup>a</sup>	<b>2017</b>	<b>2018</b>	<b>2019</b>	<b>2020</b>
EST	LW, SH, LS <sup>b</sup>	-	-	-
TQ	LW, LS	LW, SH, LS	LW, SH, LS	-
DEN	-	LW, SH, LS	LW, SH, LS	-
GC	-	LW, SH, LS	LW, SH, LS	-
TX	-	LW, SH, LS	LW, SH, LS	-
UN	-	LW, SH, LS	LW, SH, LS	-
FC	-	SH, LS	LW, SH, LS	-
WK	-	LW, SH, LS	LW, SH	LW, SH, LS
DRY	-		SH	-
GLS	LW, SH	LS	LW	-

<sup>a</sup> EST = establishment, TQ = turf quality, DEN = density, GC = genetic color, TX = texture, UN = uniformity, WK = winterkill, FC = fall color, DRY = turf quality under drought, GLS = gray leaf spot. DRY and GLS were only collected when the stress was present.

<sup>b</sup> LW = Lake Wheeler Turfgrass Field Laboratory (Raleigh, NC); SH = Sandhills Research Station (Jackson Springs, NC); LS = Upper Mountain Research Station (Laurel Springs, NC).

**Table 2.2:** Significant factors from Mixed Model Analysis. All significance values were calculated by likelihood ratio test or Wald test of the best model selected for that trait. Heritability estimates as generalized heritability. Values that were not calculated are indicated by "--." Complete details of likelihood ratio tests and Wald tests are available in the appendix.

	EST	TQ	DEN	GC	TX	UN	WK	FC	DRY	GLS
Entry	***	***	***	***	***	***	***	**	**	***
Location	***	***	***	.	***	***	*	N.S.	--	***
Year	--	.	*	***	***	***	***	N.S.	--	***
Entrv*Year	--	NS	NS	NS	NS	NS	NS	NS	--	N.S.
Entrv*Location	***	N.S.	.	NS	NS	NS	NS	NS	--	NS
Location*Year	--	***	***	***	***	***	NS	***	--	--
Entrv*Location*Yea	--	***	**	**	*	**	***	***	--	**
Rep(Location)	**	***	NS	*	*	NS	***	***	NS	***
Heritability	0.69	0.72	0.699	0.552	0.697	0.645	0.872	0.480	0.377	0.666

*EST = establishment, TQ = turf quality, DEN = density, GC = genetic color, TX = texture, UN = uniformity, WK = winterkill, FC = fall color, DRY = drought, GLS = gray leaf spot.*  
*Significance. codes: '\*\*\*' < 0.001 '\*\*' < 0.01 '\*' < 0.05 '.' < 0.10*

**Table 2.3:** Modified Base Index Values for top and bottom-ranked St. Augustinegrass lines as well as commercial checks evaluated in multi-environment trials in North Carolina 2017-2020. High positive values indicate superiority, and negative values indicate inferiority. A complete list of values is available in Supplementary Table 4.3.

Top Ranked Lines			Bottom Ranked Lines		
Entry	Selection Index	Entry	Selection	Entry	Selection
XSA 14271	5.60	XSA	5.60	XSA 14271	5.60
Palmetto	5.12	Palmetto	5.12	Palmetto	5.12
XSA 14506	3.97	XSA	3.97	XSA 14506	3.97
XSA 14450	3.96	XSA	3.96	XSA 14450	3.96
XSA 14464	3.90	XSA	3.90	XSA 14464	3.90
XSA 14146	3.55	XSA	3.55	XSA 14146	3.55
XSA 14132	3.41	XSA	3.41	XSA 14132	3.41
XSA 14315	3.14	XSA	3.14	XSA 14315	3.14

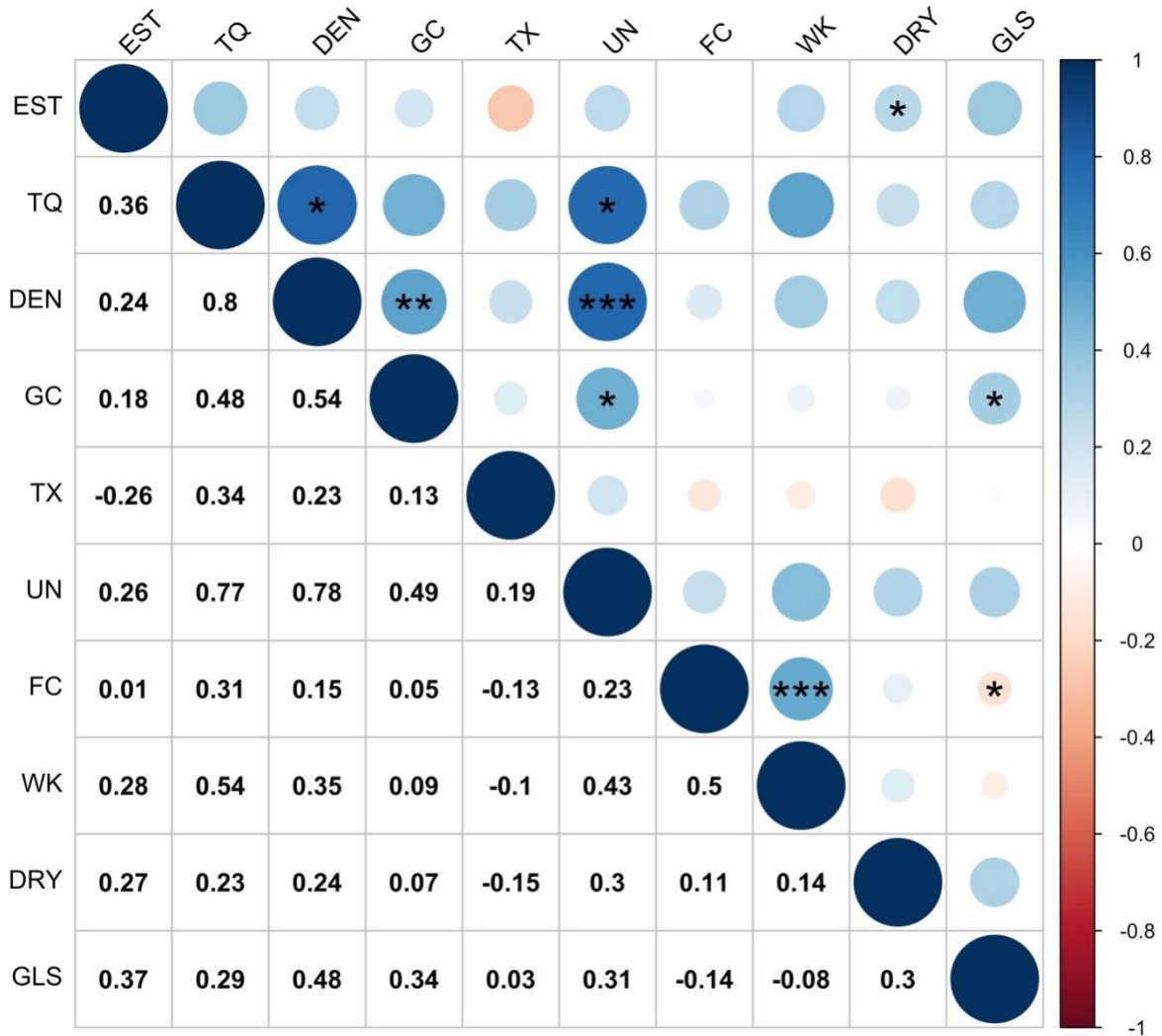
**Table 2.4:** Estimated genetic gain for each trait under direct and indirect selection with a 15% selection intensity (10 genotypes). Maximum genetic gains for each trait under direct selection are indicated in bold along the diagonal. Estimated genetic gains for each trait under indirect selection using the modified base index as the selection criteria are presented in the bottom row.

Selection <sup>a</sup>		Genetic Gain (%) <sup>b</sup>									
Type	Criteria	EST	TQ	DEN	GC	TX	UN	FC	WK	DRY	GLS
Direct	EST	<b>7.20</b>	4.32	7.30	0.00	-0.31	3.68	0.20	4.83	0.57	2.92
	TQ	3.58	<b>11.41</b>	23.79	2.08	2.92	11.27	3.20	20.06	1.32	2.66
	DEN	3.18	10.85	<b>25.88</b>	2.03	1.59	12.92	2.84	25.01	0.23	1.62
	GC	1.76	4.90	13.43	<b>3.66</b>	2.28	5.35	-1.41	0.13	-0.44	1.97
	TX	-2.95	4.42	8.65	0.66	<b>6.04</b>	1.11	0.81	-4.15	-0.25	0.26
	UN	1.68	9.99	23.81	1.70	1.53	<b>13.82</b>	4.04	32.03	0.81	0.29
	FC	1.24	4.14	3.98	-0.35	-0.61	3.34	<b>8.04</b>	29.43	-0.87	-1.94
	WK	1.58	5.80	11.30	0.40	-0.29	7.75	6.06	<b>38.99</b>	0.21	-2.04
	DRY	3.06	1.56	0.66	0.49	-1.12	2.66	1.74	0.92	<b>7.07</b>	1.68
	GLS	3.41	2.05	5.87	0.05	0.18	0.80	-1.04	-0.49	2.26	<b>6.03</b>
Indirect	Modified Base Index	4.87	9.67	21.61	0.85	0.29	11.40	3.97	23.31	1.89	2.94

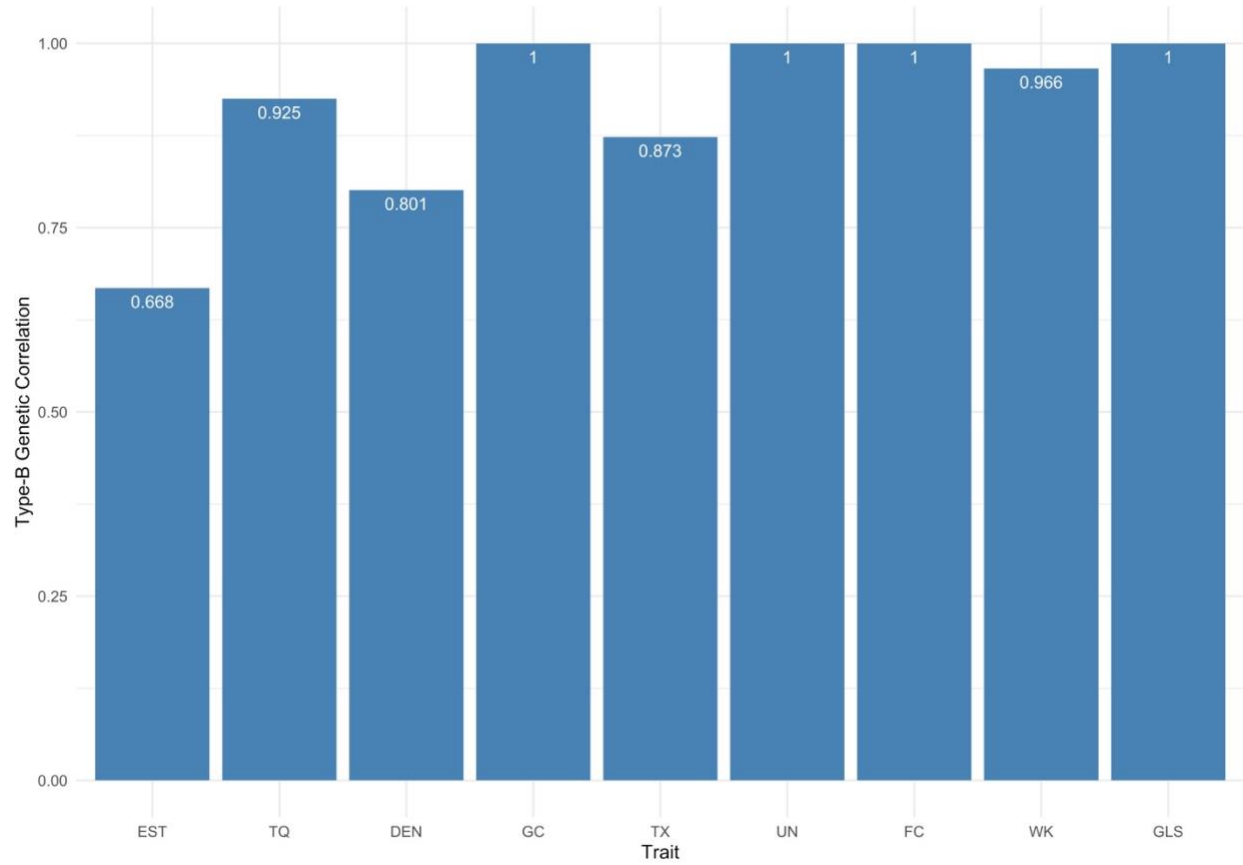
<sup>a</sup> Indicates the type of selection as either direct or indirect as well as the selection criteria. For each selection criteria the top 10 genotypes were selected.

<sup>b</sup> Estimated genetic gain (%) for each trait under the selection scheme indicated in <sup>a</sup>.

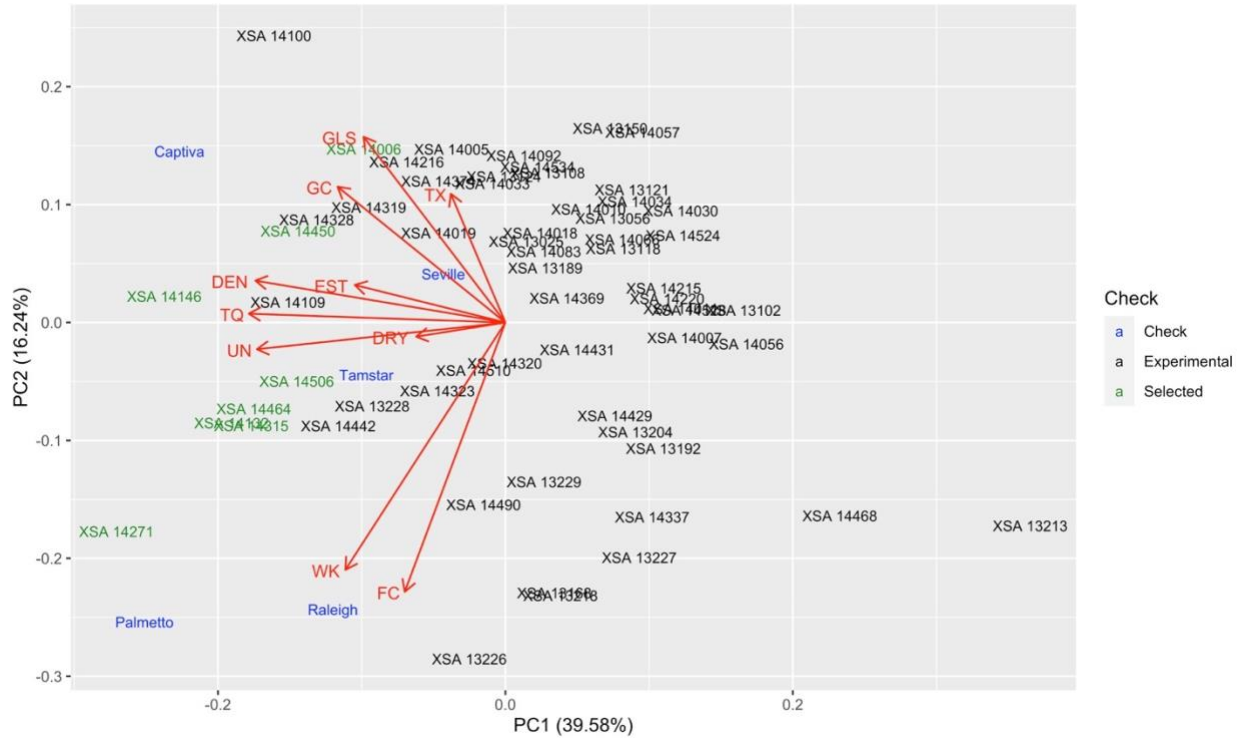
EST = establishment, TQ = turf quality, DEN = density, GC = genetic color, TX = texture, UN = uniformity, FC = fall color, WK = winterkill, DRY = drought, GLS = gray leaf spot.



**Figure 2.1:** Pairwise Spearman rank correlation between traits. The lower triangle displays the correlation coefficients. In the upper triangle, the size of the circle corresponds to the absolute value of the correlation coefficient, and the intensity of color indicates a positive or negative correlation. Dark blue indicates a correlation coefficient, of 1 and dark red of -1. Black stars indicate significance as ‘\*\*\*’ < 0.001 ‘\*\*’ < 0.01 ‘\*’ < 0.05 correlations. EST = establishment, TQ = turf quality, DEN = density, GC = genetic color, TX = texture, UN = uniformity, WK = winterkill, FC = fall color, DRY = drought.



**Figure 2.2:** Type-B genetic correlations for St. Augustinegrass breeding lines evaluated in multi-environment trials in North Carolina. EST = establishment, TQ = turf quality, DEN = density, GC = genetic color, TX = texture, UN = uniformity, WK = winterkill, FC = fall color.



**Figure 2.3:** Genotype by Trait Biplot. Principal components analysis generated using the normalized predicted values for each trait. Traits are listed in green and genotypes are listed in blue. Principal component 1 is on the x-axis and represents 39.58% of the overall variation, and principal component 2 is on the y-axis and represents 16.24% of total variation. EST = establishment, TQ = turf quality, DEN = density, GC = genetic color, TX = texture, UN = uniformity, WK = winterkill, FC = fall color, DRY = drought, GLS = gray leaf spot.



**Figure 2.4:** Studentized-Blups of selected St. Augustinegrass breeding lines and commercial checks evaluated in multi-environment trials in North Carolina 2017-2020 for EST = establishment, TQ = turf quality, FC = fall color, WK = winterkill, DRY = drought, 1, GLS = Gray leaf spot. \* denotes significance at 5% probability by t-Student test.

### **Chapter 3**

#### **Validation of QTL Associated with Freezing Tolerance across Two St. Augustinegrass [*Stenotaphrum secundatum* (Walt.) Kuntze] Mapping Populations**

**Validation of QTL Associated with Freezing Tolerance across Two St. Augustinegrass  
[*Stenotaphrum secundatum* (Walt.) Kuntze] Mapping Populations**

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## ABSTRACT

St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntz) is a warm-season turfgrass commonly used in home lawns in the southern United States. Despite the identification of freeze-tolerant germplasm, limited progress has been made in increasing the available pool of St. Augustinegrass freeze-tolerant cultivars. In order to elucidate the genetic control of this trait, an SSR-based linkage map was previously developed for a freeze-tolerant ‘Raleigh’ by freeze-susceptible ‘Seville’ mapping population (biparental population). The map was used in conjunction with field- and lab-based freezing data to identify QTL on multiple linkage groups. The objectives of the present study were to 1) improve the resolution of previously identified freeze tolerance QTL using a high-density SNP-based linkage map and 2) validate these QTL in a ‘Raleigh’ selfing population. Utilization of the high-density biparental linkage map resulted in 39 QTL being identified, including 17 not previously detected. Additionally, a linkage map containing 119 SSR markers with a total genetic distance of 1641 cM was developed for the selfing population, and 16 putative QTL were identified using lab-based freezing data. The linkage maps from both populations were aligned with a draft reference genome of parent cultivar ‘Raleigh’ as means to compare freeze tolerance QTL identified in the different populations. Five genomic regions with significant overlap were found on chromosomes 1, 3, 4, 6, and 7. Gene annotation in these regions identified auxin response factors, transcription factors, and enzymes that affect freeze tolerance, including diacylglycerol kinases and cycloartenol synthase. These QTL and their associated markers may be used in future breeding efforts to develop a broader pool of freeze-tolerant St. Augustinegrass cultivars.

**Keywords:** QTL Mapping, St. Augustinegrass, Linkage Mapping, Freeze Tolerance

**Abbreviations:** ANOVA, analysis of variance; BLAST, basic local alignment search tool; BLUEs, best linear unbiased estimates; CA, cold acclimation treatment; CIM, composite interval mapping; cM, centimorgan; CP, cross-pollinator population type; GC5, percent green tissue at day 5 post-freeze relative to pre-freeze green cover; GC26, percent green cover at day 26 post-freeze relative to pre-freeze green cover; DS, number of days survival post-freeze; F2, freezer #2; F3, freezer #3; GBS, genotype-by-sequencing; LG, linkage group; LOD, logarithm of odds; LSMean, least-squares mean; MQM, multiple QTL model; NA, no cold acclimation treatment; NCBI, National Center for Biotechnology Information; PAGE, polyacrylamide gel electrophoresis; QTL, quantitative trait locus; RG, regrowth; SGT, surviving green tissue; SGU, spring green-up; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; VWC, volumetric water content; WK, winterkill.

## INTRODUCTION

St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntz) is a popular warm-season turfgrass commonly used in home lawns in the southern United States. It is characterized by its dense canopy, aggressive growth via stolons, and good shade tolerance. However, a lack of freeze tolerance had limited its use into the transition zone of the United States, including widespread use in North Carolina.

Freeze tolerance, a plant's ability to withstand subzero temperatures, is a complex, quantitative trait. While many plants have a constitutive but often low level of freeze tolerance, it can be significantly increased in response to the environment via cold acclimation (Arora, 2010). This change is triggered by periods of low temperatures or short photoperiods and can be controlled by genetic factors (Kalberer et al., 2006; Thomashow, 1999). Winter survival, measured by the amount of winterkill, is the loss of turfgrass stand over winter and may be due to direct cold temperature injury, desiccation, anoxia, winter diseases, or any combination of these (Frank, 2015). Elucidating the extent of freeze tolerance – and therefore the source – is a challenge confounded by the complexity of the trait and evaluation methods.

Variation among genotypes of St. Augustinegrass for winter survival and freeze tolerance is well reported. A large-scale trial in Arkansas reported 0-10.8% winter survival for 30 genotypes, with cultivar 'Raleigh' being the most winter hardy and 'Seville' among the least (Moseley et al., 2012). Milla-Lewis et al. (2013) reported that 'Raleigh' is significantly more freeze tolerant than 'Seville', but that the latter benefited more from cold acclimation. A field trial in northern Mississippi showed cultivars varied significantly in winter survival and that diploid genotypes had greater winter survival than polyploidy ones (Phillely et al., 1996). Field evaluations in North Carolina identified several genotypes with comparable winter survival to 'Raleigh', but found survival varied by location (Reynolds et al., 2009). In addition to field evaluations, whole container

freeze tests showed significant differences between genotypes across four freezing temperatures and identified ‘Raleigh’ among the most freeze tolerant (Kimball et al., 2017a). Despite the identification of germplasm with improved winter survival and freeze tolerance, ‘Raleigh,’ released in the early 1980s (Bateman, 1980), remains the industry standard to date. An improved understanding of the genetic and physiological control of freeze tolerance will help breeders develop a broader pool of St. Augustinegrass cultivars with improved winter survival.

Many factors influence freeze tolerance, including acclimation, de-acclimation, and freeze injury. Cold acclimation is the increase in freezing tolerance as plants adapt to low, non-freezing temperatures and is characterized by upregulation and downregulation of cellular components in response to cold stress (Fowler and Thomashow, 2002). Cool-season turfgrasses are well known for their ability to cold acclimate, while warm-season turfgrasses vary in that ability. In St. Augustinegrass, the response to cold acclimation varies by genotype (Kimball et al., 2017b; Li et al., 2010; Maier et al., 1994; Milla-Lewis et al., 2013). Cultivar ‘Floritam’ shows little to no response to cold acclimation (Busey, 2003; Fry et al., 1991), while ‘Raleigh’ shows a significant acclimation effect (Kimball et al., 2017b; Li et al., 2010; Maier et al., 1994). In controlled lab-based freezing tests, cultivar ‘Seville,’ known for its lack of freeze tolerance, significantly improved performance when acclimated (Kimball et al., 2017b). Genotypes with the best winter survival often have high acclimation ability indicating cold acclimation may play a role in freeze tolerance (Kimball et al., 2017a).

Most important traits such as yield, disease resistance, and stress response are controlled by multiple genomic regions and have a range of phenotypes as a result. Quantitative trait locus (QTL) mapping aims to identify regions of a genome that are correlated with a trait of interest. When QTL associated with a phenotype are identified, the objective is ultimately for them to be used in marker-assisted selection (MAS). Markers can be used to help pyramid together several

QTL to improve the trait of interest more rapidly than with traditional breeding (Collard et al., 2005). Other advantages of MAS over conventional selection include: there are no confounding effects from the environment, it can be carried out on young plants, and the undesirable plants can be discarded early on, saving resources. In addition, single plants can be selected, which may be unreliable with traditional evaluations (Collard and Mackill, 2008). MAS can be a cost-effective tool, particularly in turfgrass breeding, where selection cycles tend to be longer because it can reduce the need for multi-year field evaluations, thus speeding up the breeding pipeline (McClure et al., 2014). MAS for improved freeze tolerance has been successfully implemented in sorghum and bread wheat (Knoll et al., 2008; Michel et al., 2019) and, although MAS is more complex in outcrossing populations, it has the potential to increase genetic gain in turf and forage grasses (Brummer and Casler, 2007).

Previously, the first complete linkage map for St. Augustinegrass was developed by Kimball et al. and used to identify QTL associated with freezing tolerance and turf quality traits (2018). Overlapping QTL were identified for winterkill and spring green-up; turf quality, turf density, and leaf texture; and surviving green tissue. Furthermore, QTL for both field and lab freeze traits that co-localized were identified on linkage group 3 (Kimball et al., 2018). More recently, a linkage map for the same population was developed with higher marker density using SNPs (Yu et al., 2018). High-density linkage maps allow for fine mapping of QTL and improved implementation of MAS. High-density linkage maps can also be utilized in reference genome assembly to indicate errors in the assembly (Hedgecock et al., 2015). On the other hand, reference genomes are a helpful tool in QTL mapping and marker-assisted selection because they offer a standard for linkage map comparison, and once the reference genome is developed, marker development and identification are more streamlined (He et al., 2014).

While freeze tolerance QTL have been identified, they may be population dependent and thus must be validated prior to utilization in MAS. Additionally, with recent advances in technology, it is possible to identify narrower QTL regions and markers more tightly linked to these QTL. Thus, this project will focus on validating the freeze tolerance QTL previously identified by Kimball et al. (2018). The objectives of this study include i) utilize the phenotypic data from Kimball et al. (2018) in conjunction with the high-density SNP map developed by Yu et al. (2018) to fine map freeze tolerance QTL in the ‘Raleigh’ x ‘Seville’ population, ii) validate the identified QTL using a ‘Raleigh’ x ‘Raleigh’ population.

## MATERIALS AND METHODS

### Plant Material

Two mapping populations were developed to elucidate the genetic control of freezing tolerance in St. Augustinegrass. The first is a population of 120 F<sub>1</sub> lines from a cross between ‘Raleigh’ and ‘Seville’ as described and evaluated in Kimball et al. (2018). This pseudo-F<sub>2</sub> population will be known as the biparental population for the remainder of this chapter.

Kimball et al. (2018) evaluated 117 lines from the biparental population in two field locations in North Carolina; the Lake Wheeler Turfgrass Field Laboratory in Raleigh (LW, Lat. 35.72816° Long. 78.67981°, 116.434m elevation, 11.34°C to 22.58°C average temperature range, average rainfall = 4.06 in monthly), and the Upper Mountain Research Station in Laurel Springs (LS, Lat. 36.40232°, Long. 81.29711°, 917m elevation, 6.36°C to 16.82°C average, average rainfall = 5.77 in monthly). A randomized complete block design with three replications of the population was planted at each location. Plots were 0.91x 0.91 m with 0.46 m alleys between plots. Plots were mowed weekly to a height of 6.35 cm, fertilized monthly at a rate of 0.23 kg ha<sup>-1</sup> from May to October, and irrigated as needed to avoid drought stress.

For the lab-based freeze tests conducted by Kimball et al. (2018), twelve additional sets of the biparental population were propagated into 7.6 x 7.6 cm plastic pots filled with Fafard 4P potting mix (Conrad Fafard Inc., Agawam, MA) using nine single node stolons per pot. These pots were fertilized at a rate of 0.45 kg of nitrogen per 1000 square feet every month using Scotts® Starter® Fertilizer (The Scotts Company LLC, Marysville, OH) and maintained in a greenhouse until the pots were completely filled (Kimball et al., 2018).

The second population contains 115 individuals derived from self-pollination of freeze-tolerant parent 'Raleigh' for one generation to develop a pseudo-F<sub>2</sub> mapping population. This population will be herein referred to as the selfing population. A subset of 78 genotypes from the selfing population were propagated into 5.4 x 4.8 x 6 cm containers filled with Fafard 2 potting mix (Conrad Fafard Inc., Agawam, MA). In total, twenty containers of each genotype were propagated with one single node cutting per pot in batches between April - August 2020. The plants were maintained in the greenhouse and fertilized at a rate of 0.45 kg of nitrogen per 1000 square feet every month using Scotts® Starter® Fertilizer (The Scotts Company LLC, Marysville, OH) for an average of 90 days to fully establish.

## **Freezing Evaluations**

### *Biparental Population*

The biparental population was evaluated at LW and LS from 2012-2015 for spring green-up (SGU) and winterkill (WK). Spring green-up was scored from 1–9 where 1 = no green-up to 9 = complete green-up and winterkill was scored on a 1–9 scale where 1 = complete winterkill to 9 = complete survival (Kimball et al., 2018). Herein this will be referred to as the field study.

Kimball et al. (2018) performed freeze tests for the biparental population according to Kimball et al. (2017b) with two acclimation treatments: cold acclimation (CA) and no acclimation

(NA), and two target freezing temperatures:  $-3^{\circ}\text{C}$  and  $-4^{\circ}\text{C}$ . Post-freeze visual ratings were conducted at 3 and 6 weeks after freezing for surviving green tissue (SGT) and regrowth (RG) (Kimball *et al.*, 2018).

For both the field study and freeze tests, least squares means (LSmeans) were calculated for all traits using the MIXED procedure in SAS 9.4 (SAS Institute, Cary NC). For the field study, each year and location combination was considered as a separate environment (i.e., Laurel Springs in 2014). The mixed model for type 3 analysis of variance (ANOVA) and best linear unbiased estimates (BLUEs) considered entry, environment, and entry\*environment as fixed effects and replication within environments as a random effect. BLUEs (in the form of LSmeans) were generated for entry and for entry\*environment. For the lab-based freeze tests, the mixed model included entry, acclimation, temperature, and two- and three-way interactions as fixed effects. Replication, rep\*temperature, rep\*acclimation, and rep\*temperature\*acclimation were random effects; LSmeans were calculated by entry.

### *Selfing population*

Freezing tests for 78 genotypes of the selfing population were conducted using two vertical freezers (Kenmore 20.3 cu. ft. upright freezers model 253.9260410 with cooling fans and FE Micro-controllers PXR4 (FujiElectronic Systems Co., Ltd., Tokyo, Japan)), which were considered two separate environments. Due to the size of the population and space limitations in the freezers, each replication of the freeze test was split into two runs over the course of a single week. Individual pots were randomly assigned to their run ahead of acclimation treatment. In total, five replications – each consisting of two runs – were conducted for each genotype by treatment by freezer combination (Figure 3.1).

Pots were randomly assigned to cold acclimation treatment (CA) or no acclimation treatment (NA). For CA, established pots were placed in a growth chamber at 13°C with 600  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light and a 12-hour photoperiod for one week. After one week, the pots were moved to a 3°C growth chamber with the same light conditions for an additional week. Pots assigned to NA were placed in a growth chamber at 24°C (NA) with 600  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light and a 12-hour photoperiod for 2 weeks. Pots were watered as needed until saturated with water seven and five days prior to freezing for CA and NA treatments, respectively.

After the acclimation treatment and prior to freezing, pots were randomized in a completely randomized design and placed into custom-sized trays (each containing 45 pots) for freezing. In total, each replication contained 8 trays with 4 trays per run and 2 trays per freezer. Six pots (3 CA and 3 NA) of cultivar ‘Raleigh’ were included in each tray. Volumetric water content (VWC) of each pot was measured using a Turf-Tec Field Scout Handheld Digital Moisture Sensor TDR 100 (Turf-Tech International, Tallahassee, FL). Additionally, a visual establishment rating was taken where 0 = dead plant, 1=poorly established, and 5 = completely filled pot surface. A pre-freeze photo of each pot was taken using a Samsung Tab 3 on a PVC frame -to ensure constant distance from pots- fit with a black background and with curtains to prevent shadows. Prior to freezing, each tray was placed inside a trash bag to prevent desiccation. In order to detect malfunctions and measure realized freezer temperatures, a SensorPush HT1 Temperature and Humidity Smart Sensor (SensorPush) that recorded data every fifteen minutes was placed inside each bag.

Trays were then placed in freezers set with the following temperature profile: 3°C for two hours, decrease 1°C/hr to 0°C, hold at 0°C for one hour, and then decrease 1°C/hr until reaching the target temperature of -11°C, which showed good separation of genotypes in preliminary tests. The temperature was held at the target temperature for 3 hours and then increased 2°C/hr until

reaching 10°C. At that point, plants were removed and placed on the lab bench overnight before being moved to the greenhouse.

Evaluations of freeze response were conducted using both visual and image-based approaches. Each pot was scored daily for 26 days post-freeze on a binary scale where 0 = no green tissue/dead and 1 = any amount of green tissue. Images of individual pots were also taken on days 1, 5, 12, 19, and 26 post-freeze with a Samsung Tab 3 as described above. All images (including pre-freeze images) were batch analyzed using ImageJ-Fiji to segment the images into green tissue and background (including brown tissue) and record pixel counts for each category (Schindelin et al., 2012). Segmentation was done using the HSB (hue-saturation-brightness) color threshold selecting for hue values from 35-194, saturation values of 70-255, and brightness values of 60-255 for pre-freeze and day 1 images, which were taken indoors (Holloway et al., 2018). Hue, saturation, and brightness values were set to 40-194, 68-255, and 126-255, respectively, for day 5, 12, 19, and 26 images, which were taken in the greenhouse. Post-freeze green cover values for each day were calculated as the number of green pixels in the post-freeze image over the number of green pixels in the pre-freeze image.

Visual ratings were used to calculate the number of days of survival (DS) -- the total number of days for which green tissue was recorded. Relative green cover on day 5 (GC5) and day 26 (GC26) were used in QTL analysis because they correspond with the least and most amount of green tissue, respectively. Furthermore, these traits were expected to approximate the SGT and RG ratings taken in the biparental population study by Kimball et al. (2018). Data were analyzed for all traits using the GLM procedure in SAS 9.4 (SAS Institute, Cary, NC). The check cultivar 'Raleigh' was analyzed independently prior to the full analysis to determine if run affected freeze response. The 'Raleigh' model for each trait included replication, treatment, freezer, and run nested within the interaction of replication\*freezer as random effects and VWC and establishment as

covariates (simplified code available in Appendix 5). For the full analysis, all factors (replication, genotype, treatment, freezer) and their interactions were considered random effects, and VWC and establishment were included in the model as covariates. LSmeans were calculated for genotype, genotype\*freezer, and genotype\*treatment (code available in Appendix 5).

## **Genotyping and Linkage Mapping**

### *Biparental Population*

A high-density linkage map for the ‘Raleigh’ x ‘Seville’ population developed by Yu et al. (2018) was used in this study. The map was developed using a genotype-by-sequencing (GBS) strategy and sequenced on Illumina HiSeq 2500. Using JoinMap 4.0, separate linkage maps were first created for each parent, and then a consensus map was developed. Linkage groups were numbered in accordance with the foxtail millet genome since no *St. Augustinegrass* reference was available at the time. Yu et al. (2018) were also able to integrate previously identified simple sequence repeat (SSR) markers, and the consensus linkage map contained 2871 single nucleotide polymorphism (SNP) and 81 SSR markers on nine linkage groups. The total genetic distance was 1241.7 cM, with an average distance of 0.4 cM between markers.

### *Selfing population*

Genomic DNA was extracted according to Kimball et al. (2012) using a modified CTAB method. Progeny lines were genotyped with 142 SSR primer pairs (Supplementary Table 4.4) using the PCR conditions described in Mulkey et al. (2014). Amplified bands were separated by polyacrylamide gel electrophoresis (PAGE) on a LI-COR 4300 DNA Analyzer Sequencer (LI-COR Biosciences, Lincoln, NE) on 25 cm 8% v/v denaturing polyacrylamide gels.

A total of 142 SSR markers were used to generate the linkage map using *Onemap*, a package designed to accommodate linkage mapping of outcrossing populations in R studio (Margarido et al., 2007; RStudio Team, 2020). Redundant markers and those that showed segregation distortion were dropped, and then the remaining markers were assigned to linkage groups based on the population specific suggested logarithm of odds (LOD) calculated within *Onemap*. The order of markers within each linkage group was determined using the `order_seq` function, and the `ripple` function was used to check for alternate orders. Genetic distances were estimated with the Kosambi mapping function, and the linkage groups were numbered according to the linkage map of Yu et al. (2018).

### **QTL Analysis**

For the biparental population, QTL mapping was performed using the phenotypic data generated by Kimball et al. (2018) and the consensus high-density map generated by Yu et al. (2018). QTL analysis was done in R Studio using the *r/qrtl* package (Broman et al., 2003; RStudio Team, 2020). Data were imported as cross-pollinator (CP) type and a stepwise-QTL model-fitting method using a normal model, 2cM step, and Hayley-Knott regression was used to identify QTL (Poudel et al., 2019). The logarithm of odds (LOD) threshold and penalties were calculated using a 1000 permutation test for each trait. The width of the genomic region for each QTL was determined with a 1.5 LOD confidence interval. A drop one at a time ANOVA test was conducted, and only QTL with significance values of 0.05 or higher were reported. QTL analysis for the selfing population was conducted using *R/qrtl* in the same manner. The linkage map and genotype information were manually converted into MapQTL format and imported into *r/qrtl* as a CP-type population, and then the analysis followed the same procedure as above.

## **QTL Validation & Sequence Annotation**

Comparing QTL across linkage maps poses a unique challenge since linkage map units are not necessarily indicative of physical position. Furthermore, discrepancies in marker order are more likely to occur with a limited number of markers. However, some of the SSR markers used were conserved across both populations, and a draft reference genome for cultivar ‘Raleigh’ was recently developed using both long and short reads technology (Schoonmaker, unpublished).

In order to identify genomic regions that overlap across the two populations, Bowtie 2 was used to align the SNP and SSR primer sequences for both populations with the reference genome resulting in the physical location of these markers (Langmead and Salzberg, 2012). The flanking markers for each QTL were used to identify the physical QTL position, but when the flanking SNP or SSR location could not be determined, the region was expanded to the next marker.

For regions with several overlapping QTL, the sequence information was then entered into the National Center for Biotechnology Information’s (NCBI) basic local alignment search tool (BLAST). The markers which flanked the QTL as well as the peak marker, if applicable, were the basis of a BLASTx search. Each query sequence was 2000bp, 1000bp in both the forward and backward direction from each marker. Aligned sequences with an E value < .0001 were used to determine potential gene function for regions of interest. Predicted, hypothetical, and unknown protein sequences were not reported.

## **RESULTS**

### **Freezing Evaluations**

#### *Biparental population*

In the field study, significant effects were found for entry for both SGU and WK. Environment was only a significant factor for SGU, but the entry-by-environment interaction was

significant for both traits (Supplementary Table 4.5). For the lab-based freezing tests, entry was significant for both SGT and RG, while temperature was significant for RG only (Supplementary Table 4.6). No other factors were determined to have significant effects on either SGT or RG. Despite slightly different analyses of the same phenotypic data for the biparental population, both analyses found entry as well as entry-by-environment interaction to be significant. However, the analysis in Kimball et al. (2018) considered replication as a fixed effect and found environment to be a significant factor.

### *Selfing population*

While the freezer settings were constant, the outside air temperature and other factors influenced the temperature within the freezers during the individual runs. The minimum temperature within a bag in a single freezer during a run ranged from  $-8.7^{\circ}\text{C}$  to  $-11.4^{\circ}\text{C}$ , but only in replication 2 did freezers fail to get below  $-10^{\circ}\text{C}$ . Freezer #2 had an average minimum temperature of  $-10.6^{\circ}\text{C}$  while freezer #3 had an average minimum temperature of  $-11.0^{\circ}\text{C}$ .

Preliminary analysis of plants of check cultivar ‘Raleigh’ only indicated run was not a significant factor for any of the traits (Supplementary Table 4.7). Therefore, run was not included in the model for the whole population. While treatment was significant for DS and GC26, it was not a significant factor for GC5. The adjusted DS average for NA and CA ‘Raleigh’ was 18.1 and 17.8, respectively. On day 26, NA ‘Raleigh’ had an adjusted mean of 27% of the pre-freeze green coverage, while CA ‘Raleigh’ had 50% of the pre-freeze green cover.

For the full selfing population freeze analysis, genotype was only a significant factor for GC5 ( $p= 0.05$ ). Meanwhile DS and GC26 were not significant ( $p= 0.0561$  and  $0.6498$ , respectively) (Table 3.1). Replication was a significant ( $p= <.0001$ ) source of variation for all traits. Treatment was a significant factor for DS ( $p= 0.0451$ ) and GC26 ( $p= <.0001$ ), but not

significant for GC5 ( $p= 0.9958$ ). Freezer and replication(freezer) each had significant effects on DS ( $p= <.0001$  and  $p= 0.0001$ ) and GC5 ( $p= <.0001$  and  $p= <.0001$ ) but not on GC26 ( $p= 0.0567$  and  $p= 0.1649$ ). While ‘Raleigh’ performed well across traits, there were progeny genotypes which outperformed ‘Raleigh’ for all traits, trait by treatment, and trait by freezer subsets (Table 3.2).

## **Genotyping and Linkage Mapping**

For the biparental population, the previously constructed consensus linkage map by Yu et al (2018) was used for analysis (Yu et al., 2018). For construction of the selfing population map, of the 140 genotyped SSR markers, eleven were found to be redundant and thus removed. An additional three markers were removed due to segregation distortion. Therefore, 126 markers were initially included in the linkage analysis. The grouping function resulted in 10 total linkage groups and four unlinked markers. One linkage group had only two markers and was not included in further analysis. The final linkage map contained 119 SSR markers, spanned nine linkage groups, and had a total genetic distance of 1641cM with an average distance of 13.8cM between markers (Table 3.3). LG4 was both the longest and the least dense, spanning 247.8cM with an average distance between markers of 20.7cM. Conversely, LG6 was the shortest and the densest, totaling 123.9cM, and had an average marker distance of 9.5cM.

## **QTL Analysis**

### *Biparental Population*

A total of 39 putative QTL with 1.5 LOD confidence intervals were identified across all nine linkage groups for the biparental population (Table 3.4). The QTL explained between 5.2 and 30.0 percent of the phenotypic variance ( $R^2$ ), and the average size of confidence intervals was 2.1cM. No QTL were identified for SGU across all years or SGU2014, but all other traits had

associated QTL, including SGU2013 and SGU2015. Linkage groups 1 and 3 each housed seven putative QTL more than any other LG. Overlapping QTL were identified on LG 3: for RG and WK2015 at position 0.0-8.0 cM, and for WK2013, WK2014, SGU2013, and WK at position 132.0-140.0 cM. Additionally, WK and SGU2013 peaks were co-localized on LG4 at position 31.3cM. Genomic regions associated with SGU2015 and WK2015 overlapped on LG 5 and LG 9. Because several overlapping QTL were identified on LG 3, the markers associated with these QTL were used for sequence annotation.

### *Selfing population*

Few QTL were initially identified for the selfing population. Therefore, BLUEs were generated for genotype-by-freezer and genotype-by-treatment to improve the resolution of QTL detection. In total, 16 putative QTL were identified for the selfing population, but no QTL were identified for GC26, across or within freezers (Table 3.5). The putative QTL were identified on linkage groups 2-9 and explained between 6.8 and 24.5 percent of the variance with an average confidence interval length of 8.8cM. Overlapping QTL were identified on LG5 for GC5, GC5-freezer2, GC5-NCA, and GC5-freezer3. However, the confidence interval for GC5-freezer3 did not contain the peak position for the QTL detected in GC5 and GC5-freezer2. Additionally, genomic regions associated with DS, GC5-CA, and GC5-freezer2 overlapped on LG7.

### **QTL Validation & Sequence Annotation**

The sequences of flanking SNPs and SSRs were used to identify the physical position of many of the QTL on parent 'Raleigh' (Tables 3.4 - 3.6). In some cases, the location of the sequence could not be identified because it 1) aligned several times to the reference genome, 2) did not have any high-quality alignments to the reference genome, or 3) was aligned to a scaffold sequence that

was not associated with a chromosome. In any of these cases, the flanking marker region was expanded to the next marker according to the linkage map if possible or if not, the QTL was dropped from further analysis. Physical regions for some of the QTL spanned much of the chromosome due to imperfect collinearity of the linkage maps and the reference genome, or to the necessary expansion to neighboring markers with physical location information.

#### *Biparental Population QTL in low-density vs. high-density maps*

The SNP-based map was able to identify more QTL associated with freezing tolerance than the initial SSR map. Additionally, analysis in the high-density map found QTL which explained a larger percentage of the variance and had higher LOD scores. Furthermore, the QTL were associated with much smaller genomic regions when the higher-density map was utilized. There is strong overlap among the QTL identified between the two biparental maps on chromosomes 1, 4, and 6, but the high-density linkage map was able to detect additional QTL on chromosomes 5 and 7 (Figure 3.2). Due to the overlap between the QTL identified by the high- and low- density maps, regions on chromosomes 1, 4, and 6 were identified as candidate gene regions and used for sequence annotation.

#### *Biparental Population vs. Selfing population QTL*

The biparental and selfing populations had strong overlapping regions on chromosomes 5 and 7 (Figure 3.2). On chromosome 5, several QTL associated with GC5 co-localized with one another and with SGU2015 from the biparental population. On chromosome 7, many GC5 QTL overlapped with both SGT and RG QTL identified from the biparental population. Because of the high level of colocalization between the two mapping populations on chromosome 7, this region was included for gene annotation.

### *Gene Annotation*

Five genomic regions on chromosomes 1, 3, 4, 6, and 7 were used for gene annotation, and ten homologous proteins were identified through the BLAST database (Table 3.7). Three proteins were associated with chromosome 1, only one protein each with chromosomes 3 and 4, two with chromosome 6, and finally three with chromosome 7. These matching protein sequences were found in other grasses: *Oryza sativa* (rice), *Setaria italica* (foxtail millet), *Setaria viridis*, *Panicum miliaceum* (proso millet), and *Brachypodium distachyon*. From these, *Setaria italica* has been regarded as the closest relative to St. Augustinegrass, but genomic comparisons have also been conducted with *Oryza sativa* (Yu et al., 2018).

On chromosome 1, a cycloartenol synthase was detected in association with both QTL SGT4CA-2 and RG4CA-2 reported by Kimball et al. (2018) (Table 3.7). Both an auxin response factor 6 isoform and an F-box protein were identified for QTL 1@37.4 for both WK2013 and WK2014 using the high-density map. On chromosome 3, a translation initiation factor was identified for QTL 3@135.1 and 3@136.0 for SGU2013 and WK. A splicing factor subunit was identified on chromosome 4 for QTL SGU-2 from LW2013. Transcription factor NIGTH1 and diacylglycerol kinase 5-like were detected on chromosome 6, for QTL 6@64.0 for GC5-freezer3 from the selfing population and QTL SGT3NCA-1, SGT3-1, SGTNCA-2 from the previous study, respectively. On chromosome 7, a cyclic pyranopterin monophosphate synthase mitochondrial isoform was identified at QTL 7@52.8, and an amino acid transporter was identified at QTL 7@54.0 both for SGT using the high-density map. Finally, a very-long-chain aldehyde decarbonylase GL1-7-like was identified at 7@75.7 for DS in the selfing population.

## DISCUSSION

While genomic tools for St. Augustinegrass have been rapidly advancing, few QTL studies have been conducted. Furthermore, the absence of a reference genome has made the comparison of linkage maps and identified QTL across mapping populations challenging. The availability of a reference genome opens the door to further advancements allowing quick development of additional genetic markers and providing a standard for comparison (Dossa, 2016; Kim et al., 2020). This could allow improved detection of QTL, better implementation of marker-assisted selection including gene pyramiding, and an increased understanding of the molecular functions which control traits. In turn, the linkage maps can be used to improve reference genome scaffolding (Perez-de-Castro et al., 2012). This study represents the first comparison of QTL based on a reference genome for St. Augustinegrass, which enabled the identification of overlapping QTL across populations and linkage maps.

The multiple QTL model utilized for the biparental population identified 39 total QTL with an average 1.5 LOD confidence interval length of 2.2cM. Of the 39 QTL, 17 were identified as large effect QTL, which explained greater than 10% of the variance for the trait. While only 10 QTL were associated with the lab-based phenotypic data, two of those associated with SGT explained 33 and 26.6% of the phenotypic variance, respectively, the largest values for any trait in the study. This suggests fewer large effect QTL may be associated with such controlled freeze studies since the conditions are limited relative to field studies. Furthermore, there are only two genomic regions where QTL from the field study and freeze study of the biparental population overlap, further suggesting that these two analyses are detecting different aspects of freeze response. This has been observed in winter wheat and underscores the quantitative nature of freezing tolerance (Kruse et al., 2017). Within the biparental population, field traits for individual

years often co-localized with one another, including on linkage group 3, indicating this method is viable to detect QTL across environments and supporting gene annotation for this region.

Consistent with genomic analysis in other species (Robledo et al., 2018; Zhang, S. et al., 2019), the high-density linkage map utilized in this study was able to detect QTL that were missed with the low-density linkage map of Kimball et al. (2018). In total, 39 putative QTL associated with freezing tolerance were identified as compared to the 27 identified previously in the SSR map. High-density linkage maps have the power to resolve tightly linked QTL and provide more precise QTL localization and effect estimates according to simulated data. Thus, the increase in the number of QTL could be due to resolving closely linked QTL (Stange et al., 2013). Additionally, the high-density map was able to identify more narrow genetic regions associated with them compared to the SSR-based map, a phenomenon also detected in alfalfa (Zhang, F. et al., 2019). Overlapping QTL were identified on both the high- and low- density maps on chromosomes 1, 4, and 6, which indicates strong support that these are true QTL, making them good candidates for gene annotation.

The linkage map developed for the selfing population had the longest genetic distance (1641cM) of the three linkage maps that were considered. This map was developed with the multipoint likelihood mapping algorithm available in *Onemap*, which may have increased the overall length relative to the regression algorithm used by Yu et al. (2018) for the biparental population. Similar patterns have been found in other species (De Keyser et al., 2010), and the dramatic expansion in length is due to genotyping errors, even when the error level is low (Lincoln and Lander, 1992). Despite the slightly increased genetic distance, QTL analysis for the selfing population identified 16 total QTL, primarily for GC5, which was the only trait for which genotype was a significant factor. Additionally, it has been well reported that both marker number and population size impact QTL detection (Darvasi et al., 1993; Li et al., 2006). Thus, the limited

number of QTL detected is a consequence of both the selfing population being small, and the low marker density of the linkage map. Darvasi et al. (1993) used simulated backcross data to show that increasing marker density can improve localization of QTL up to a certain point at which the population size needs to be increased to see further improvement. Furthermore, only lab-based freeze test data was available for the selfing population, so fewer traits were evaluated. Despite this, overlapping QTL for GC5 and DS were detected on linkage group 7, and multiple QTL for GC5 under different treatments and environments were detected on linkage group 5. Furthermore, alignment of this linkage map with that of ‘Raleigh’ x ‘Seville’ allowed for the identification of overlapping QTL across populations. Unfortunately, not all markers were aligned with the reference genome, resulting in an artificial expansion of the physical region, and imperfect collinearity between the physical and genetic maps also resulted in some warping of the physical locations of QTL. However, overlapping QTL across the two populations were identified on chromosomes 6, and 7 and these regions were used for gene annotation. A challenge of QTL identification in plant breeding is that QTL are often only detectable in the population and environment in which they are mapped, but identification of QTL in two populations – even when they share one parent – provides support for the broader application of the trait as a molecular tool across populations (Feltus et al., 2006).

Freeze tolerance QTL have been identified in many turfgrass species, including zoysiagrasses (*Zoysia japonica*) (Brown et al., 2020; Holloway et al., 2018), bermudagrass (*Cynodon dactylon*) (Fan et al., 2019), and ryegrass (*Lolium perenne*) (Paina et al., 2016). While the comparison of QTL across studies is not straightforward, there is a strong indication that markers associated with genetic control of freeze tolerance can be identified and potentially utilized in a breeding program. Furthermore, QTL associated with acclimation treatment were

detected on LG 2 in the biparental population, consistent with other studies which have been able to detect treatment-specific QTL from freeze-tests (Kimball et al., 2018; Brown et al., 2020).

Gene annotation identified several proteins which have documented roles in freeze tolerance (Table 3.7). Cycloartenol synthase is the enzyme that catalyzes sterol biosynthesis beginning with the conversion of 2,3-oxidosqualene to cycloartenol and is a requirement for plant viability (Babiychuk et al., 2008). While sterols are essential to many plant functions, they also have been implicated in extreme temperature tolerance in the fungus *Pythium* when added to the growth medium (Haskins, 1965). Additionally, sterols play an essential role in the membrane lipid bilayer. A study in two *Solanum* species found that a decrease in sterol to phospholipid ratio resulted in an increase in freezing tolerance following cold acclimation (Rogowska and Szakiel, 2020). Mitochondrial initiation factor IF3 is a subunit that is required for translation in prokaryotic systems and controls the green light pathway in cyanobacteria (Andrian Gutu et al., 2013). Furthermore, in *E. coli*, cold shock resulted in an increase in the IF3 to ribosome ratio, implicating IF3 in cold tolerance response (Giuliodori et al., 2007). Diacylglycerol kinases are signaling enzymes that phosphorylate diacylglycerol (DAG) to produce phosphatidic acid. In turn, phosphatidic acid is associated with overcoming plant stress events with cell responses (Escobar-Sepúlveda et al., 2017). In *Arabidopsis thaliana*, diacylglycerol kinases, including DGK5, are associated with cold tolerance, and *dgk5* knockouts had improved cold tolerance (Tan et al., 2018). Aldehyde decarbonylases catalyze the conversion of fatty aldehydes to alkanes. In plants, these can produce very-long-chains that prevent desiccation of the plant, thus improving drought resistance and perhaps playing a role in freeze tolerance as well (Marsh and Waugh, 2013).

Other proteins identified via QTL analysis have roles in plant response which can include cold response, including auxin response factors (ARFs), which are regulators of plant aspects of plant growth and development through transcription. In particular, ARF6, among other ARFs, are

thought to be transcriptional activators (Tabata et al., 2010). In *Arabidopsis thaliana*, ARF6 mutants were associated with decreased jasmonic acid and a delay in flowering (Tabata et al., 2010). While ARF6 targets many genes, it also interacts with PIF4, which has been implicated in coordinating light, cold, and phytohormone signaling (Shi et al., 2015). F-box proteins are the site of protein-protein interactions as part of SCF (Skp I, Cullin, and an F-box protein) ubiquitin-ligase complexes (Kipreos and Pagano, 2000). While the extent of f-box protein functions is not known, they have been associated with both low-temperature and drought signaling (Bu et al., 2014; Venkatesh et al., 2020). Transcription factor NIGTH1 is a transcription factor that likely has a role in plant development and metabolism. While a thorough characterization of this transcription factor has not been performed, in general, it has been implicated in cold tolerance as well as other stress-induced plant responses (Sharma et al., 2020). Amino acid transporters (AATs) are membrane-bound proteins that facilitate the transfer of amino acids. These AATs play many cellular roles, including development regulation, abiotic stress response, and defense response (Yang et al., 2020).

Additionally, two of the proteins that were implicated via QTL analysis have little documentation and no known association with temperature response. In humans, splicing factor 3B subunit 2 is a subunit of the splicing factor 3B complex, which, along with splicing factor 3a and a 12S RNA, bind pre-mRNA in a sequence-independent manner (Prados-Carvajal et al., 2018). Its role in plants and its relationship to temperature stress is not documented. Cyclic pyranopterin monophosphate synthase catalyzes the biosynthesis of molybdopterin, a cofactor with a role in several pathways but no documented association with freeze tolerance (Kruse et al., 2018).

One caveat of this gene annotation is the assumption that the QTL is tightly linked with the marker since only 2000bp regions were annotated. Additionally, further validation is needed as 'Raleigh' is a parent and likely the source of freezing tolerance in both populations. Further studies

are needed to confirm the identified genomic regions and proteins impact freeze tolerance in St. Augustinegrass. However, results of this study lay the groundwork for future marker-assisted selection breeding and improved understanding of freezing tolerance in this economically important warm-season turfgrass species.

### **Acknowledgments**

This research was supported by funding provided by the North Carolina Crop Improvement Association, the North Carolina State University Center for Turfgrass Environmental Research and Education, and Specialty Crop Research Initiative grant [2015-51181-24291] from the USDA National Institute for Food and Agriculture.

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**Table 3.1:** Type 3 Analysis of Variance for freeze tolerance traits for the selfing population. DF = degrees of freedom, Green Cover Day 5 = green cover at day 5 post-freeze relative to the pre-freeze green cover from digital imaging, Green Cover Day 26 = green cover at day 26 post-freeze relative to the pre-freeze green cover from digital imaging, Days Survival = sum of the days where green tissue was present post-freeze, SS = sum of squares, VWC = volumetric water content.

Source	Days Survival						Green Cover Day 5				
	DF	Type III SS	Mean Square	F Value	Pr > F		DF	Type III SS	Mean Square	F Value	Pr > F
Establishment	1	184.5741	184.5741	1.7700	0.1842		1	0.0419	0.0419	1.3100	0.2518
VWC	1	4276.5918	4276.5918	40.9100	<.0001		1	0.5152	0.5152	16.1600	<.0001
Reps	3	5585.7104	1861.9035	17.8100	<.0001		3	3.2975	1.0992	34.4700	<.0001
Geno	78	7583.9378	97.2300	0.9300	0.6498		78	4.3223	0.0554	1.7400	0.0001
Treatment	1	420.6170	420.6170	4.0200	0.0451		1	0.0000	0.0000	0.0000	0.9958
Freezer	1	9558.0538	9558.0538	91.4300	<.0001		1	0.8876	0.8876	27.8400	<.0001
Geno*Freezer	78	8363.6074	107.2257	1.0300	0.4202		78	2.2336	0.0286	0.9000	0.7225
Geno*Treatment	78	6243.2729	80.0420	0.7700	0.9335		78	3.0326	0.0389	1.2200	0.1004
Geno*Treatme*Freezer	79	6507.3245	82.3712	0.7900	0.9115		79	1.9589	0.0248	0.7800	0.9228
Reps(Freezer)	3	2200.5135	733.5045	7.0200	0.0001		3	1.4498	0.4833	15.1600	<.0001
Green Cover Day 26											
Source	DF	Type III SS	Mean Square	F Value	Pr > F						
Establishment	1	3.8599	3.8599	11.9100	0.0006						
VWC	1	13.1254	13.1254	40.5000	<.0001						
Reps	3	37.7633	12.5878	38.8400	<.0001						
Geno	78	32.3406	0.4146	1.2800	0.0561						
Treatment	1	12.4924	12.4924	38.5500	<.0001						
Freezer	1	1.1793	1.1793	3.6400	0.0567						
Geno*Freezer	78	25.5006	0.3269	1.0100	0.4597						
Geno*Treatment	78	25.9829	0.3331	1.0300	0.4150						
Geno*Treatme*Freezer	79	27.2041	0.3444	1.0600	0.3375						
Reps(Freezer)	3	1.6545	0.5515	1.7000	0.1649						

**Table 3.2:** Adjusted means for number of days of survival, post-freeze green cover on day 5 and post-freeze green cover on day 26 measured in lab based freeze tests of a cultivar ‘Raleigh’ selfing population. Number of days survival can range from 0 to 26 where 26 indicates the plant had green tissue every day after freezing. Post-freeze green cover values are a proportion of pre-freeze values where 0 indicates no change in the amount of green tissue, 1 indicates the same amount of green tissue, and negative values indicate loss of green tissue after freezing. F2 = freezer 2, F3 = freezer 3, CA = cold acclimated, NA = non-cold acclimated.

	Number Days Survival					Green Cover Day 5 (GC5)					Green Cover Day 26 (GC26)				
	Across	F2	F3 <sup>a</sup>	CA	NA <sup>b</sup>	Across	F2	F3	CA	NA	Across	F2	F3	CA	NA
Progeny Min	9.709	10.862	4.763	9.650	5.083	0.018	0.020	0.009	0.013	0.012	0.012	0.003	-0.034	0.040	-0.047
Progeny Max	20.910	24.766	20.192	23.361	20.474	0.291	0.420	0.288	0.392	0.364	1.280	0.574	2.257	2.374	0.848
Progeny Avg	14.799	17.533	12.065	15.390	14.209	0.128	0.155	0.102	0.129	0.128	0.258	0.287	0.228	0.357	0.158
Raleigh	17.983	21.822	14.145	17.694	18.272	0.117	0.145	0.089	0.109	0.125	0.41	0.509	0.312	0.542	0.278

<sup>a</sup> F2 and F3 indicate progeny and parent values for freeze tolerance traits for each freezer but across acclimation treatment.

<sup>b</sup> CA and NA indicate progeny and parent values for freeze tolerance traits for each acclimation treatment but across freezers.

**Table 3.3:** Summary of the distribution of markers on the Raleigh selfing population linkage map. The map was developed using the package r/Onemap and the genetic distances estimated using the Kosambi function. cM = centimorgan, LG = linkage group.

Linkage Group	Number of Markers	Genetic Distance (cM)	Average Distance between markers (cM)
LG1	10	156.26	15.63
LG2	17	195.92	11.52
LG3	16	213.59	13.35
LG4	12	247.84	20.65
LG5	11	144.98	13.18
LG6	13	123.87	9.53
LG7	12	147.42	12.29
LG8	14	206.82	14.77
LG9	14	204.27	14.59
Total	119	1640.96	13.79

**Table 3.4:** Quantitative Trait Loci associated with Freeze tolerance traits for the Raleigh x Seville biparental population based on a high-density SNP linkage map and field and lab-based freeze test data. QTL mapping was conducted using *r/QTL* using the stepwise model function and QTL identified as significant ( $p > 0.05$ ) are presented here. Physical positions were identified using the sequences of the flanking markers mapped to the ‘Raleigh’ reference genome. When these sequences could not be aligned to the reference the region was expanded to the next marker to identify a physical position, these are denoted with \*. When the flanking markers could not be used for alignment the markers in the expand to next marker were used. CHR = chromosome, LOD = logarithm of odds, RG = regrowth, SGT = surviving green tissue, SGU = spring green-up, WK = winterkill.

QTL	Trait	CHR	Peak Position	LOD	% var	Flanking Markers	1.5 LOD Confidence Interval	Peak Physical Position (bp)	Physical Position Range	Expand to next marker
1@37.4	WK2013	1	37.41	8.54	17.55	SNP6815, SNP21579	36.70 - 38.00	1964279	4024870 - 7951778*	SNP6815, SNP22998
1@37.4	WK2014	1	37.41	8.54	17.55	SNP6815, SNP21579	36.70 - 38.00	1964279	4024870 - 7951778*	SNP6815, SNP22998
1@100.0	SGU2013	1	99.97	5.60	8.90	SNP58875, SNP56842	99.33 - 100.07	38344058	7005395 - 38404344	
1@102.6	WK	1	102.58	4.09	7.39	SNP60477, SNP16765	102.08 - 103.05	-	39005540 - 39101553	
1@104.9	WK	1	104.93	3.94	7.10	SNP45737, SNP53464	104.82 - 105.12	33871657	35187089 - 38833657	
1@122.5	WK2013	1	122.53	4.94	9.30	SNP28177, SSR10345	120.00 - 126.00	38800115	33690062 - 40355223	
1@122.5	WK2014	1	122.53	4.94	9.30	SNP28177, SSR10345	120.00 - 126.00	38800115	33690062 - 40355223	
2@72.0	WK2013	2	72.00	4.96	9.34	SNP5948, SSR11067	70.17 - 72.61	-	1724504 - 15733910	
2@72.0	WK2014	2	72.00	4.96	9.34	SNP5948, SSR11067	70.17 - 72.61	-	1724504 - 15733910	
2@142.2	RG	2	142.25	6.64	8.55	SNP54898, SSR12233	141.20 - 143.75	44018096	43864849 - 44088499	
2@187.1	SGU2013	2	187.06	3.77	5.74	SSR16939, SSR21056	184.00 - 187.06	-	36293153 -	
3@2.0	RG	3	2.00	4.29	5.24	SNP36429, SNP35780	0.00 - 6.00	-	0 - 20914*	SNP29714

**Table 3.4** (continued).

3@4.0	WK2015	3	4.00	5.63	7.91	SNP36429, SNP35780	2.00 - 8.00	-	0 - 20914*	SNP29714
3@61.2	RG	3	61.19	5.63	7.09	SNP44932, SNP12463	60.80 - 61.78	14453165	12749256 - 15186823*	SNP3224, SNP12463
3@134.0	WK2013	3	134.00	3.66	6.68	SNP16045, SNP22983	132.00 - 135.06	-	43672689 - 44496196	
3@134.0	WK2014	3	134.00	3.66	6.68	SNP16045, SNP22983	132.00 - 135.06	-	43672689 - 44496196	
3@135.1	SGU2013	3	135.06	6.64	10.80	SNP16045, SNP11728	134.00 - 138.00	44496196	43672689 - 47116538	
3@136.0	WK	3	136.00	6.16	11.61	SNP22983, SNP11728	135.06 - 140.00	-	44496196 - 47116538	
4@12.0	RG	4	11.99	8.53	11.44	SSR2037, SNP59548	10.00 - 13.90	354101	1359635 - 26691493	
4@31.1	SGU2013	4	31.06	8.44	14.32	SNP61522, SNP24764	30.39 - 31.07	-	235904 - 16074411	
4@31.1	WK	4	31.06	4.49	8.17	SNP61522, SNP24764	30.39 - 31.07	-	235904 - 16074411	
4@60.3	WK2015	4	60.28	5.61	7.87	SNP33270, SNP60527	60.00 - 60.51	10171254	9456037- 14019325*	SNP3700, SNP60527
5@57.6	RG	5	57.63	5.17	6.45	SNP11143, SNP22332	56.82 - 58.00	-	5851305 - 6949737*	SNP27507, SNP15585
5@74.2	SGU2015	5	74.24	5.63	10.93	SNP12837, SNP54683	74.00 - 74.82		12558399 - 37498202*	SNP37865, SNP54683
5@74.8	WK2015	5	74.82	8.04	11.87	SNP13029, SNP36171	74.24 - 75.30	37498202	12558399 - 14946448*	SNP37865, SNP61427
6@66.0	WK2015	6	66.00	6.18	8.77	SNP21645, SNP49288	65.74 - 66.62	-	27859141 - 31922236*	SNP21645, SNP8728
6@72.0	WK2015	6	71.98	7.36	10.72	SNP53137, SNP56467	71.74 - 72.25	4856730	989545 - 28815495	
7@37.9	RG	7	37.91	6.14	7.82	SNP5415, SNP45406	37.38 - 37.94	15544599	15743416 - 30811132	
7@44.7	RG	7	44.71	8.25	10.99	SNP5444, SNP42947	44.47 - 45.01	-	27845856 - 35099182	

**Table 3.4** (continued).

7@52.8	SGT	7	52.78	8.17	26.63	SNP57283, SNP2748	52.62 - 53.33	22252247	21835559 - 22437529	
7@54.0	SGT	7	54.00	9.78	33.03	SNP39730, SNP32672	53.69 - 54.59	-	22418038 - 31801574*	SNP39730, SNP46771
8@8.0	WK	8	8.00	5.66	10.55	SNP11680, SNP10828	7.47 - 10.00	-	0 - 1834339*	
8@28.0	SGU2013	8	28.00	8.33	14.09	SSR9943, SNP2733	27.10 - 29.66	-	5257621 - 49794429	
8@38.1	WK	8	38.08	3.99	7.20	SNP29656, SNP7787	37.26 - 38.31	-	5840582 - 6061123	
9@0.0	WK2015	9	0.00	15.31	26.49	SNP10984, SNP53760	0.00 - 2.00	-	0 - 655150*	
9@0.0	SGU2015	9	0.00	7.37	14.85	SNP10984, SNP53760	0.00 - 2.00	-	0 - 655150*	
9@105.2	SGU2015	9	105.16	4.40	8.33	SNP30612, SNP25080	104.65 - 105.95	16800905	17260942 - 39902733*	SNP57529, SNP25080
9@154.6	RG	9	154.61	9.29	12.66	SNP22178, SNP57836	153.68 - 156.00	52775419	50957361 - 53214998*	SNP53664, SNP57836
9@157.0	SGU2013	9	156.97	5.68	9.04	SNP15515, SNP15190	156.00 - 158.00	53214998	52775419 - 53267212	

**Table 3.5:** Significant Quantitative Trait Loci associated with Freeze Tolerance for the Raleigh selfing population. QTL mapping was conducted using *r/QTL* using the stepwise model function and QTL identified as significant ( $p > 0.05$ ) are presented here. Physical positions were identified using the sequences of the flanking markers mapped to the ‘Raleigh’ reference genome. When these sequences could not be aligned to the reference the region was expanded to the next marker to identify a physical position. CHR = chromosome, DS = sum of the number of days post freeze where green tissue was observed, GC5 = green tissue at day 5 post-freeze, F2 = freezer #2, F3 = freezer #3, LOD = logarithm of odds. \*indicates region was expanded to next markers to identify QTL physical position.

QTL	Trait	CHR	Peak Position	LOD	% var	Flanking Markers	1.5 LOD Confidence Interval	Peak Physical Position (bp)	Physical Position Range	Expand to next marker
2@96.0	GC5-CA	2	96.00	4.68	13.37	SSR17189, SSR28514	88.37 - 96.00	-	22096982 - 23961738*	SSR01288 - SSR28514
3@9.8	GC5-F3	3	9.77	4.13	9.40	SSR11218, SSR27743	6.00 - 14.00	2360446	1935337 - 4039040	
3@34.0	GC5-F3	3	34.00	4.39	10.06	SSR27743, SSR03677	32.00 - 38.00	-	3547484 - 4039040	
3@98.0	GC5-CA	3	98.00	7.77	24.49	SSR28896, SSR03460	92.00 - 98.00	-	18997359 - 23483619	
4@156.0	GC5-F2	4	156.00	4.31	10.97	SSR10023, SSR03614	152.00 - 158.00	-	29308803 - 32361887	
5@88.0	GC5-F2	5	88.00	4.57	11.72	SSR04450, SSR00276	86.00 - 92.00	-	23779075 - 30466589	
5@90.0	GC5	5	90.00	3.72	19.74	SSR02330, SSR01952	64.00 - 126.00	-	17223613 - 36602828	
5@96.0	GC5-NCA	5	96.00	3.82	20.17	SSR11492, SSR01952	72.26 - 136.00	-	15635215 - 36602828	
6@64.0	GC5-F3	6	64.00	5.52	13.11	SSR29525, SSR06099	60.00 - 66.00	-	2694070 - 4464443	
7@75.7	DS	7	75.74	2.91	15.81	SSR27447, SSR03592	66.19 - 94.00	18449991	2694070 - 25638621	
7@92.0	GC5-CA	7	92.00	4.25	12.01	SSR29093, SSR21192	82.00 - 92.00	-	18449991 - 30991443	
7@95.7	GC5-F2	7	95.68	4.36	11.12	SSR29093, SSR06868	90.00 - 98.00	25638621	18449991 - 29507194*	SSR29093 - SSR01073
8@52.0	GC5-F2	8	52.00	2.81	6.82	SSR01466, SSR21225	46.46 - 58.00	-	15451919 - 29938686	

**Table 3.5** (continued).

9@94.0	GC5-CA	9	94.00	4.48	12.74	SSR01356, SSR17841	90.00 - 102.00	-	8050821 - 9294532	
9@162.0	GC5-F2	9	162.00	4.77	12.32	SSR10863, SSR15541	157.25 - 166.00	-	27304686 - 34336136	
9@168.1	GC5-F2	9	168.12	3.89	9.76	SSR10863, SSR28745	162.00 - 170.00	34336136	27304686 - 35633989	

**Table 3.6:** Physical location of previously reported freeze tolerance QTL. Freeze tolerance related QTL identified by Kimball et al. (2018) assigned to physical positions on the reference genome according to the flanking markers. CA = cold acclimation, CHR = chromosome, Env = environment, LG = linkage group, NCA = no cold acclimation, RG = regrowth, SGT = surviving green tissue, SGU = spring green-up, tmt = treatment, WK = winterkill. \* indicates the flanking markers used to identify the physical region were not the flanking markers originally reported, regions were expanded if the original markers could not be aligned with the reference genome.

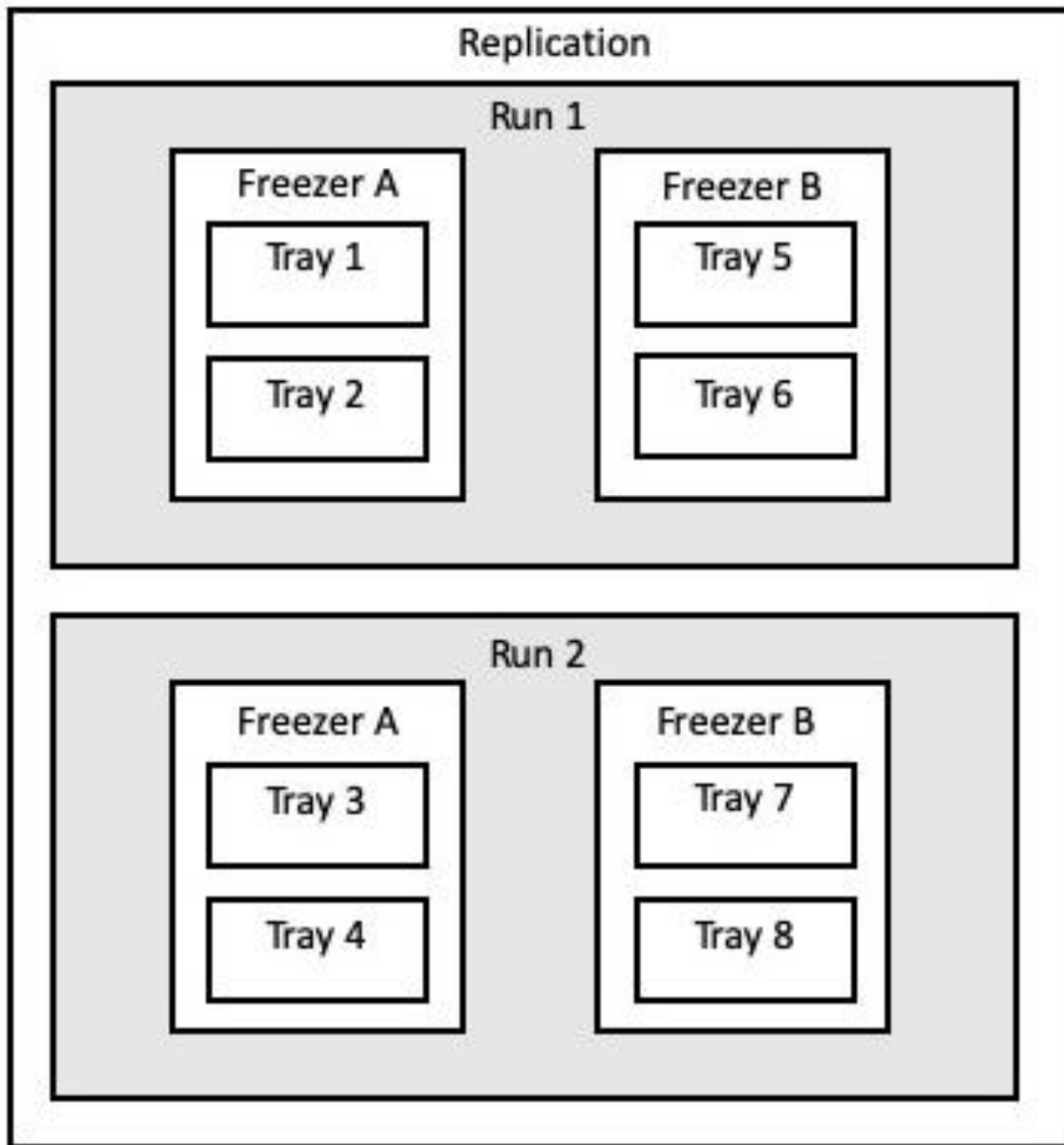
QTL Name	Trait	Env or Tmt	Genetic LG	Genetic Position (cM)	Flanking Markers	Physical CHR	Physical Region
RG3CA-3	RG	CA at -3°C	6	114.8	SSR16877, SSR20919	1	22213154 - 35678765
SGT4CA-2	SGT	CA at -4°C	6	49.51	SSR16446, SSR21772	1	2790204 - 7348550
RG4CA-2	RG	CA at -4°C	6	49.51	SSR16446, SSR21772	1	2790204 - 7348550
RG4NCA-1	RG	NCA at -4°C	6	68.41	SSR21772, SSR22738*	1	7348550 - 11566467
SGT4CA-1	SGT	CA at -4°C	1	38.31	SSR23374, SSR17849	2	33443012 - 33973299
RG3CA-1	RG	CA at -3°C	1	38.31	SSR23374, SSR17849	2	33443012 - 33973299
SGTCA-1	SGT	CA	3	77.7	SSR03460, SSR21486*	3	23483680 - 34427426
SGT-1	SGT	Across	3	77.7	SSR03460, SSR21486*	3	23483680 - 34427426
SGTNCA-1	SGT	NCA	3	86.32	SSR21486, SSR03677	3	3547484 - 34427426
WK-2	WK	All Environments	3	99.21	SSR03677, SSR17039*	3	3547484 - 44234510
SGU-1	SGU	All Environments	3	99.21	SSR03677, SSR17039*	3	3547484 - 44234510
SGU-1	SGU	LW2013	3	99.21	SSR03677, SSR17039*	3	3547484 - 44234510
SGT4-1	SGT	-4°C	3	68.47	SSR03460, SSR27205	3	5385792 - 23483680

**Table 3.6** (continued).

RG4CA-1	RG	CA at -4°C	3	96.6	SSR27205, SSR17039	3	5385792 - 44234510
SGT3CA-1	SGT	CA at -3°C	9	34.2	SSR30340, SSR22229	4	13607834 - 36261395
RG3CA-4	RG	CA at -3°C	9	34.2	SSR30340, SSR22229	4	13607834 - 36261395
SGU-2	SGU	LW2015	9	57.9	SSR25018, SSR04046	4	27736461 - 36261395
RG3CA-2	RG	CA at -3°C	4	45.81	SSR04527, SSR23320	6	23354478 - 25104204
SGT3NCA-1	SGT	NCA at -3°C	4	64.81	SSR02442, SSR03140	6	4876694 - 5708895
SGT3-1	SGT	-3°C	4	64.81	SSR02442, SSR03140	6	4876694 - 5708895
SGTNCA-2	SGT	NCA	4	64.81	SSR02442, SSR03140	6	4876694 - 5708895
RG3NCA-2	RG	NCA at -3°C	5	105	SSR02460, SSR04381	8	28755716 - 47121754
WK-1	WK	All Environments	2	12.33	SSR20662, SSR02877*	9	1059591 - 4343455
WK-1	WK	LW2015	2	12.33	SSR20662, SSR02877*	9	1059591 - 4343455
SGT3-2	SGT	-3°C	6	34.5	SSR00770, SSR16446	Not Aligned	-
RG3NCA-1	RG	NCA at -3°C	1	71.31	SSR24167, SSR02502	Not Aligned	-

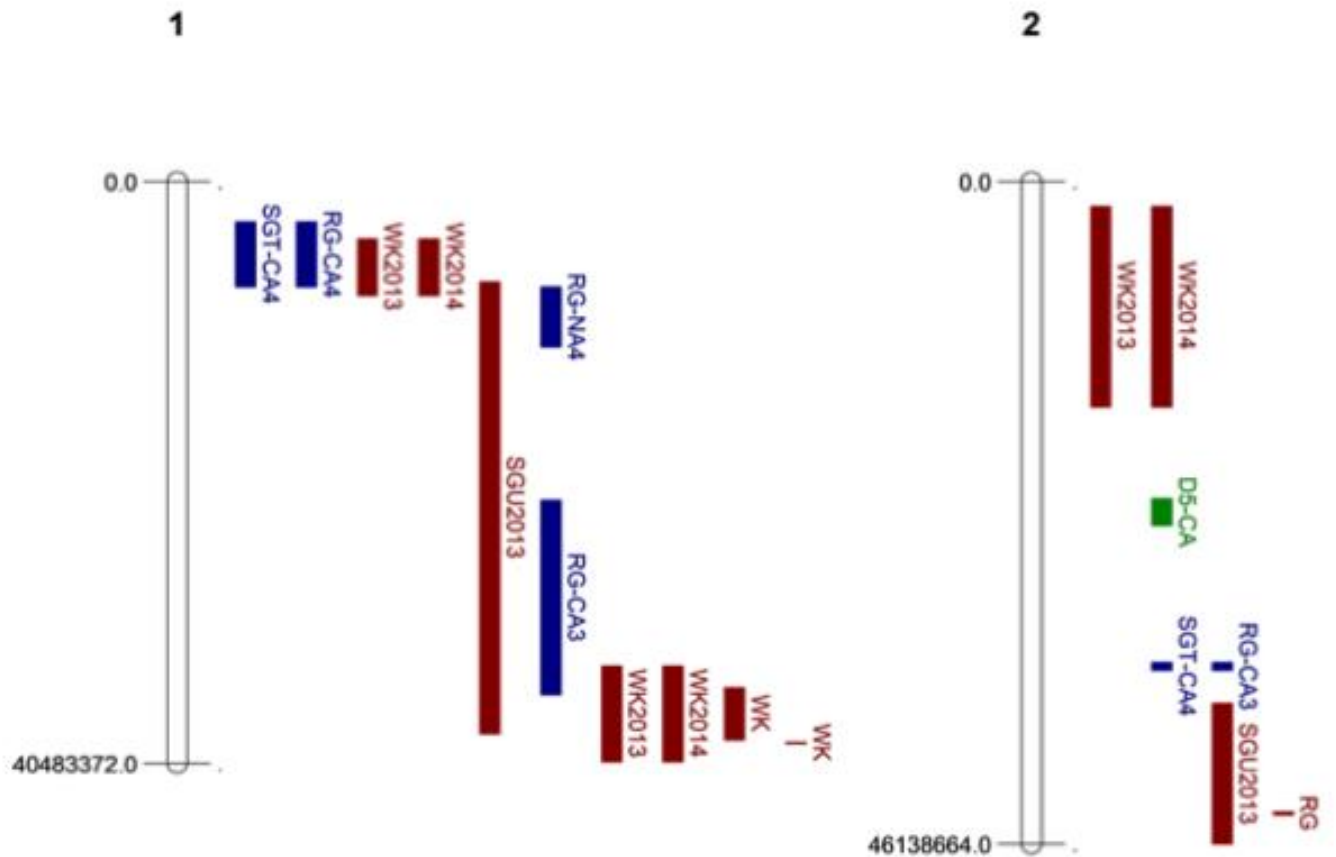
**Table 3.7:** Annotated *St. Augustinegrass* sequences associated with overlapping freeze tolerance QTL. The top result from BLASTx is presented for the 2000bp region surrounding genetic markers identified through QTL analysis across populations. Marker position and query sequence region indicate the number of base pairs from the beginning of the respective chromosome. Markers with no significant results or no known protein coding sequence alignments are not presented.

Chr	Associated Marker	Marker Position (bp)	Query Sequence Region (bp)	Aligned Sequence	Species	E-value	Accession #
1	SSR16466	2790204	2789204-2791204	cycloartenol synthase	<i>Setaria viridis</i>	2.0E-12	XP_034588580.1
1	SNP6815	4024870	4023870-4025870	auxin response factor 6 isoform X1	<i>Setaria italica</i>	2.0E-73	XP_004951871.1
1	SNP22998	7951778	7950778-7952778	F-box protein At5g10340	<i>Oryza sativa Japonica Group</i>	5.0E-20	XP_015611253.1
3	SNP11728	47116538	47115538-47117538	translation initiation factor IF3-1, mitochondrial	<i>Setaria italica</i>	7.0E-13	XP_004963283.1
4	SSR04046	36261395	36260395-36262395	splicing factor 3B subunit 2	<i>Setaria italica</i>	7.0E-41	XP_004966563.2
6	SSR29525	2694005	2693005-2695005	transcription factor NIGTH1	<i>Brachypodium distachyon</i>	7.0E-13	XP_003573358.1
6	SSR02442	5708895	5707895-5709895	diacylglycerol kinase 5-like	<i>Panicum miliaceum</i>	8.0E-13	RLN03606.1
7	SNP39730	22418038	22417038-22419038	amino acid transporter AVT11-like	<i>Setaria viridis</i>	3.0E-102	XP_034605567.1
7	SNP2748	22437529	22436529-22438529	cyclic pyranopterin monophosphate synthase, mitochondrial isoform X1	<i>Setaria italica</i>	7.0E-30	XP_012702633.1
7	SSR03592	25638621	25637621-25639621	very-long-chain aldehyde decarboxylase GL1-7-like	<i>Setaria viridis</i>	8.0E-49	XP_034602793.1



**Figure 3.1:** Schematic of the experimental design for the 'Raleigh' selfing population controlled freezing tests. The experimental design featured a completely nested design with trays within freezers within runs within replications. Each tray contained 45 pots including 3 acclimated 'Raleigh' and 3 not-acclimated 'Raleigh.Raleigh' as controls. Freezers can be considered analogous to the environment. Five replications were conducted in total. Trays 1-4 contain all 78 genotypes by treatment combinations as do trays 5-8.

**Figure 3.2:** Identified freeze tolerance QTL aligned to the physical map of 'Raleigh'. The flanking markers of each QTL were used to identify the physical position of each. If a flanking marker did not align, the region was expanded based on the next marker on the respective genetic map (refer to tables 3-6). QTL presented for all St. Augustinegrass chromosomes (1-9). CA = cold acclimated; GC5 = green cover at day five post-freeze; DS = number of days of survival; F2 = freezer 2; F3 = freezer 3; NA= Not-acclimated; RG= regrowth; SGT = surviving green tissue; SGU = spring green-up; WK = winterkill.

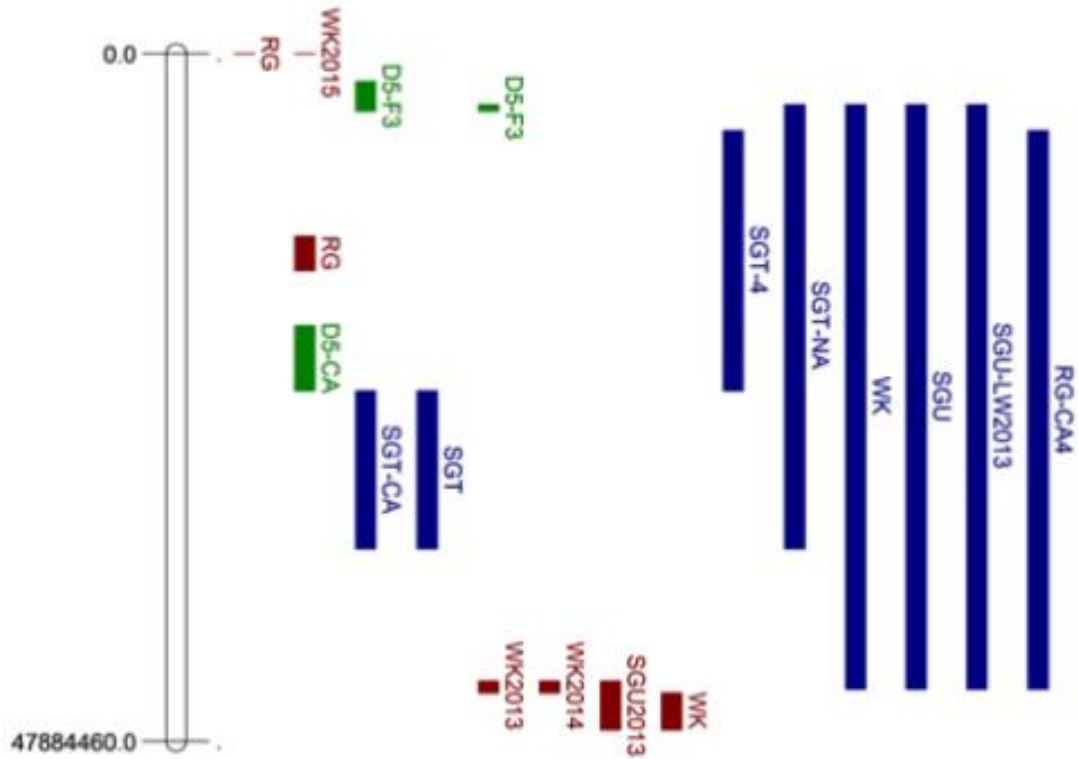


**Legend:** ■ QTL identified using a SSR map for the biparental population (Kimball et al. 2018)

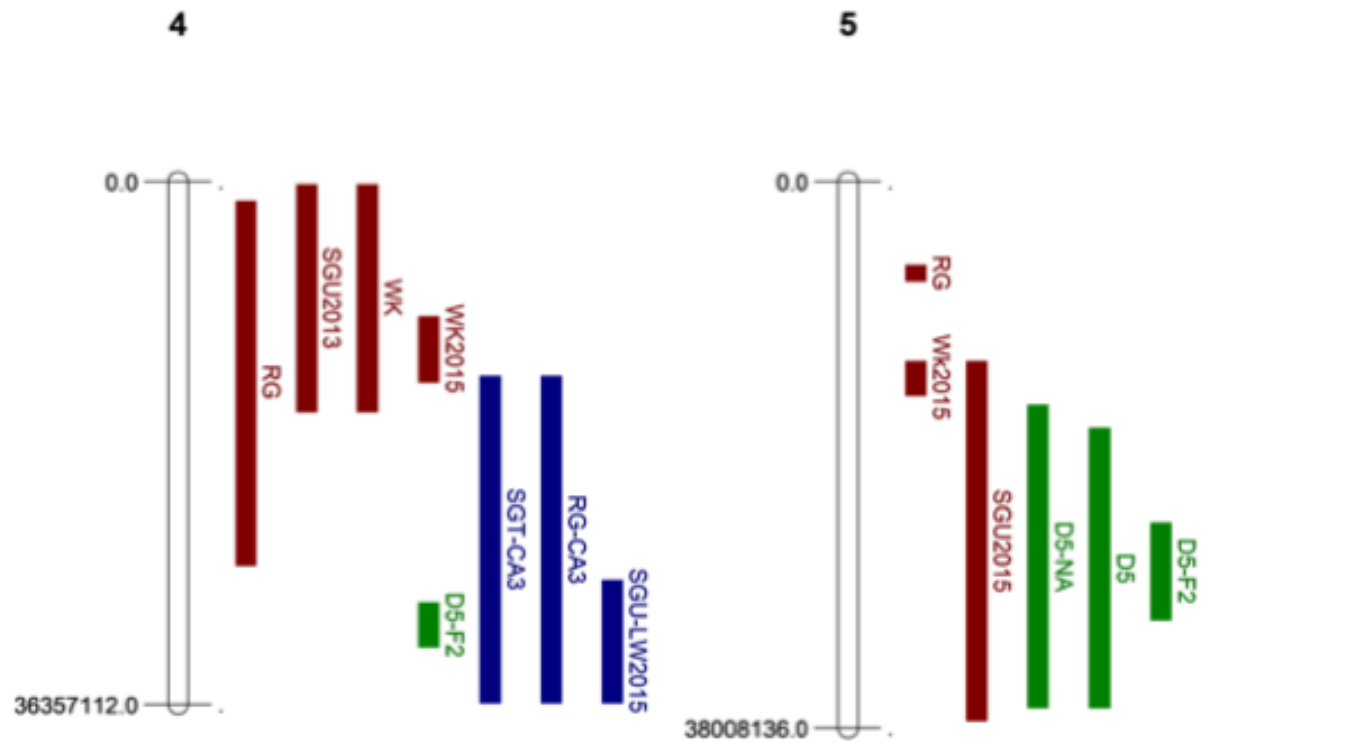
■ QTL identified using a SNP map for the biparental population

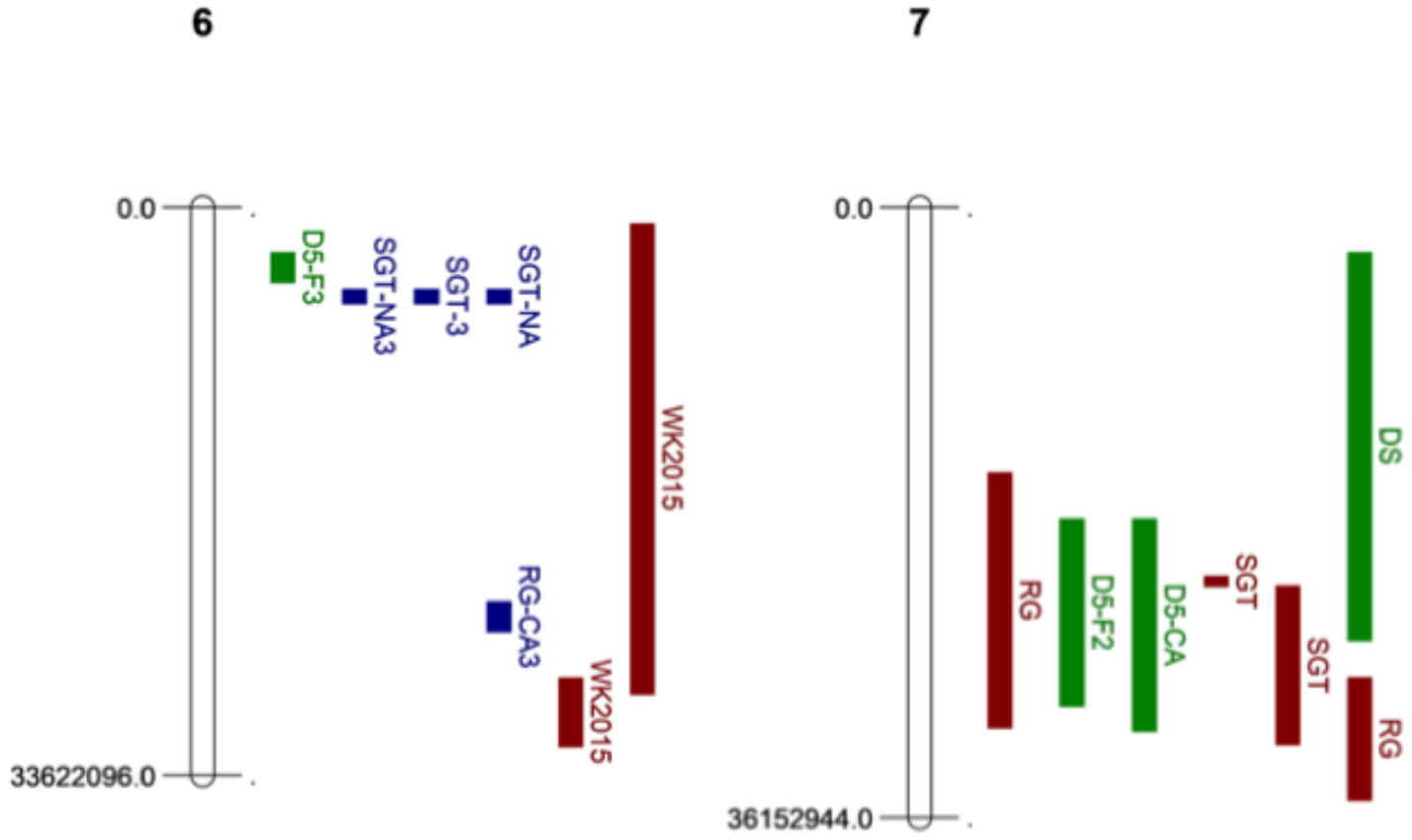
■ QTL identified using a SSR map for the self population

3

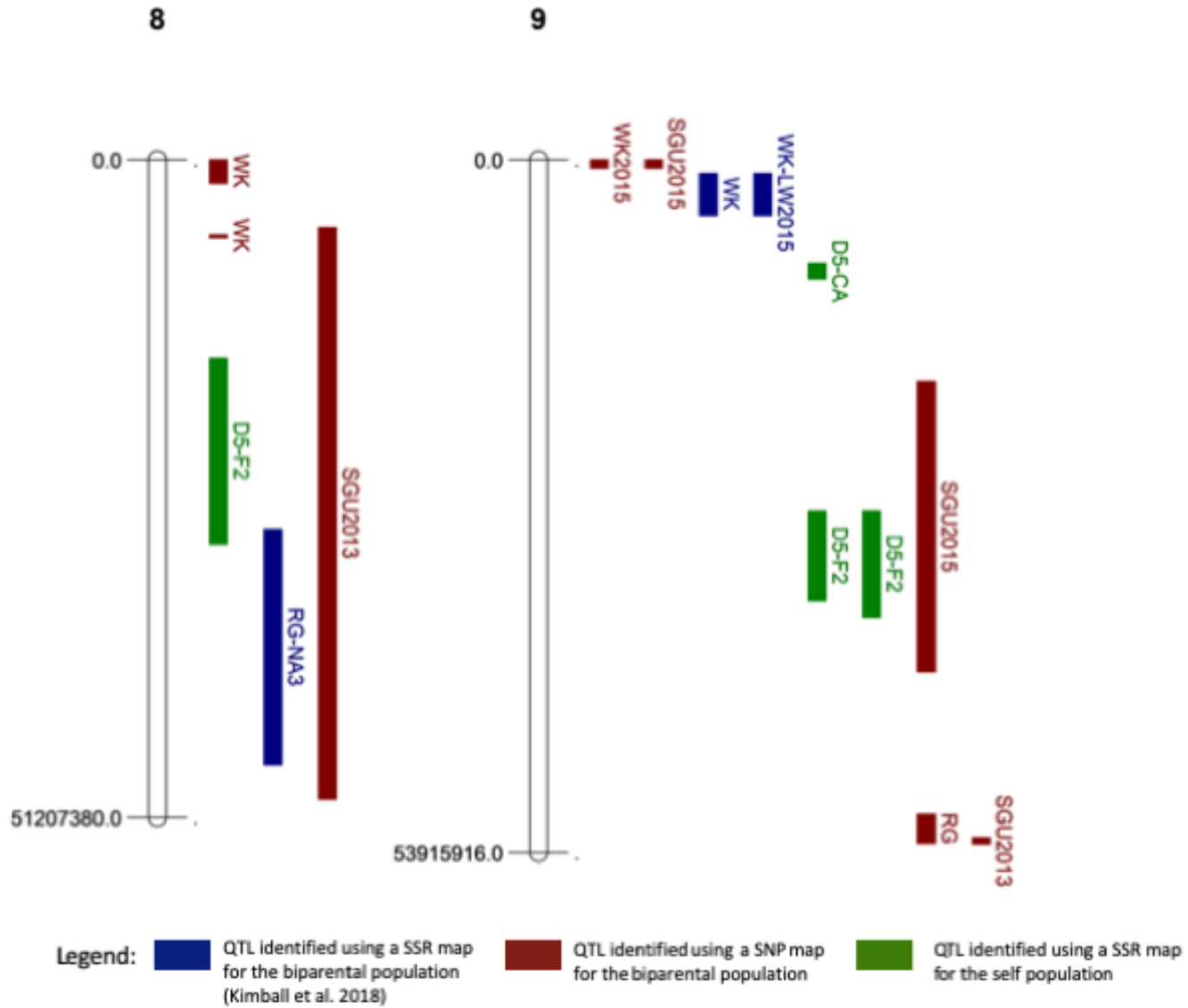


Legend: ■ QTL identified using a SSR map for the biparental population (Kimball et al. 2018) ■ QTL identified using a SNP map for the biparental population ■ QTL identified using a SSR map for the self population





Legend: ■ QTL identified using a SSR map for the biparental population (Kimball et al. 2018) ■ QTL identified using a SNP map for the biparental population ■ QTL identified using a SSR map for the self population



## APPENDICES

## APPENDIX 1: Supplementary Tables for Chapter II

**Table 4.1:** Bayesian information criterion (BIC) for multi-environment analysis models of eight St. Augustinegrass traits using compound symmetry (CS) for the genetic (G) variance-covariance matrix and different structures in the residual (R) variance-covariance matrix. The lowest value for each trait is indicated in bold.

Matrix <sup>a</sup>		Traits <sup>b</sup>							
R <sup>c</sup>	G	TQ	DEN	GC	TX	UN	WK	FC	GLS
DIAG $R_l \otimes$ CS $R_t$	CS	1515.16	1163.7	870.2	521.64	1026.9	2127.4	874.39	4131.7
DIAG $R_l \otimes$ CSH $R_t$	CS	DNC	DNC	DNC	DNC	DNC	DNC	DNC	DNC
DIAG $R_l \otimes$ AR1 $R_t$	CS	1516.44	1163.7	870.2	521.64	1026.9	2122.8	874.39	4131.7
DIAG $R_l \otimes$ AR1H $R_t$	CS	DNC	DNC	DNC	DNC	DNC	DNC	DNC	DNC
DIAG $R_l \otimes$ US $R_t$	CS	DNC	DNC	DNC	DNC	DNC	DNC	DNC	DNC
ID $R_l \otimes$ CS $R_t$	CS	1521.47	1137.7	881.0	579.56	1021.4	2121.6	857.32	4271.1
ID $R_l \otimes$ CSH $R_t$	CS	DNC	1133.0	893.3	DNC	1041.4	2066.0	860.85	DNC
ID $R_l \otimes$ AR1 $R_t$	CS	1522.66	1137.7	881.0	579.56	1021.4	2120.3	857.32	4271.1
ID $R_l \otimes$ AR1H $R_t$	CS	1464.94	DNC	893.3	DNC	1004.9	2063.8	871.52	DNC
ID $R_l \otimes$ US $R_t$	CS	DNC	DNC	DNC	DNC	DNC	DNC	DNC	DNC

<sup>a</sup> AR1, first-order autoregressive matrix; AR1H, first-order autoregressive heterogeneous matrix; CS, compound symmetry matrix; CSH, compound symmetry heterogeneous matrix; DIAG, diagonal matrix; US, unstructured.

<sup>b</sup>TQ = turf quality, DEN = density, GC = genetic color, TX = texture, UN = uniformity, WK = winterkill, FC = fall color, GLS = Gray leaf spot, DNC = did not converge.

<sup>c</sup> $R_l$  is the covariance matrix for locations and  $R_t$  is the covariance matrix for years. The Kronecker product is denoted by  $\otimes$ .

**Table 4.2:** Significance tests and variance component estimates for each trait. The significance of each factor was evaluated by likelihood ratio test (LRT) for random effects (entry, entry\*location, entry\*year, entry\*year\*location, rep(location)) and by Wald tests for fixed effects (location, year, location\*year). SS = sum of squares, LR = likelihood ratio, EST = establishment, TQ = turf quality, DEN = density, GC = genetic color, TX = texture, UN = uniformity, FC = fall color, WK = winterkill, DRY = drought, GLS = gray leaf spot.

	EST				TQ			
	SS	Wald/LR Statistic	P-Value	Var. Comp	SS	Wald/LR Statistic	P-Value	Var. Comp
Intercept	244842.0000	3305.7000	0.0000	-	3132.9800	3132.9800	0.0000	-
Entry	-	21.3750	0.0000	63.0541	-	30.5630	0.0000	0.2488
Location	4666.0000	63.0000	0.0000	-	36.5700	18.2900	0.0001	-
Year	-	-	-	-	11.4700	5.7300	0.0569	-
Location*Year	-	-	-	-	64.1400	21.3800	0.0001	-
Entry*Location	-	20.2600	0.0000	31.3636	-	-12.0070	0.5000	0.0202
Entry*Year	-	-	-	-	-	0.8259	0.1817	0.0360
Entry*Location*Year	-	-	-	-	-	23.0970	0.0000	0.2273
Rep(Location)	-	6.0229	0.0071	3.3136	-	18.6460	0.0000	0.0332
	DEN				GC			
	SS	Wald/LR Statistic	P-Value	Var. Comp	SS	Wald/LR Statistic	P-Value	Var. Comp
Intercept	2479.2900	2479.2900	0.0000	-	9312.9000	9312.9000	0.0000	-
Entry	-	25.6710	0.0000	0.3974	-	10.8500	0.0005	0.1135
Location	56.9800	28.4900	0.0000	-	9.5000	4.7000	0.0938	-
Year	4.6200	4.6200	0.0316	-	19.4000	19.4000	0.0000	-
Location*Year	43.5900	21.8000	0.0000	-	40.1000	20.1000	0.0000	-
Entry*Location	-	2.1505	0.0713	0.0991	-	0.0000	0.5000	0.0000
Entry*Year	-	0.0023	0.4809	0.0025	-	0.0000	0.5000	0.0000
Entry*Location*Year	-	8.1967	0.0021	0.2189	-	5.7375	0.0083	0.1145
Rep(Location)	-	0.0000	0.5000	0.0000	-	3.8652	0.0247	0.0203

**Table 4.2** (continued).

	<b>TX</b>				<b>UN</b>			
	SS	Wald/LR Statistic	P-Value	Var. Comp	SS	Wald/LR Statistic	P-Value	Var. Comp
Intercept	11032.8000	11032.8000	0.0000	-	4663.9000	4663.9000	0.0000	-
Entry	-	19.7110	0.0000	0.1493	-	23.9390	0.0000	0.2080
Location	115.4000	57.7000	0.0000	-	144.3000	72.1000	0.0000	-
Year	224.8000	224.8000	0.0000	-	71.8000	71.8000	0.0000	-
Location*Year	119.0000	59.5000	0.0000	-	108.9000	54.4000	0.0000	-
Entry*Location	-	0.4335	0.2551	0.0218	-	0.0000	0.4988	0.0000
Entry*Year	-	0.2698	0.3017	0.0115	-	0.0000	0.5000	0.0000
Entry*Location*Year	-	3.4267	0.0321	0.0676	-	8.0347	0.0023	0.1732
Rep(Location)	-	4.4451	0.0175	0.0116	-	0.1056	0.3726	0.0027
	<b>WK</b>				<b>FC</b>			
	SS	Wald/LR Statistic	P-Value	Var. Comp	SS	Wald/LR Statistic	P-Value	Var. Comp
Intercept	655.3500	655.3500	0.0000	-	949.2900	949.2900	0.0000	-
Entry	-	79.0510	0.0000	0.8726	-	6.8639	0.0044	0.1921
Location	16.1600	8.0800	0.0176	-	1.4800	0.7400	0.6908	-
Year	113.0200	56.5100	0.0000	-	0.0500	0.0500	0.8214	-
Location*Year	8.0700	2.6900	0.4421	-	16.5000	16.5000	0.0000	-
Entry*Location	-	0.3251	0.2843	0.0309	-	0.0000	0.5000	0.0000
Entry*Year	-	0.0000	0.5000	0.0000	-	0.0000	0.5000	0.0000
Entry*Location*Year	-	35.9290	0.0000	0.3571	-	22.0550	0.0000	0.4368
Rep(Location)	-	30.2660	0.0000	0.0606	-	54.2870	0.0000	0.1655

**Table 4.2** (continued).

	<b>DRY</b>				<b>GLS</b>			
	SS	Wald/LR Statistic	P-Value	Var. Comp	SS	Wald/LR Statistic	P-Value	Var. Comp
Intercept	1034.4300	598.1	0.0000	-	2555.5900	2555.5900	0.0000	-
Entry	-	5.438	0.0099	0.5062	-	21.9920	0.0000	25.1274
Location	-	-	-	-	48.9900	24.4900	0.0000	-
Year	-	-	-	-	53.2700	53.2700	0.0000	-
Location*Year	-	-	-	-	-	-	-	-
Entry*Location	-	-	-	-	-	0.0000	0.5000	0.0000
Entry*Year	-	-	-	-	-	0.0000	0.5000	0.0000
Entry*Location*Year	-	-	-	-	-	7.2935	0.0035	19.2106
Rep(Location)	-	0.20771	0.3243	0.0201	-	22.2640	0.0000	12.0063

**Table 4.3:** Normalized BLUPs and Modified Base Index Values. Normalized best linear unbiased prediction values for each trait used in the modified base index calculation (Eq. 7). All genotypes in the study listed in order of rank using the selection index.

<b>Entry</b>	<b>EST</b>	<b>TQ</b>	<b>WK</b>	<b>FC</b>	<b>DRY</b>	<b>GLS</b>	<b>Index</b>	<b>Rank</b>
XSA 14271	0.893	2.897	1.357	0.823	-0.142	-0.232	5.597	1
Palmetto*	0.254	1.681	2.075	0.855	0.262	-0.009	5.117	2
XSA 14506	1.326	0.933	0.807	0.138	0.396	0.362	3.962	3
XSA 14450	0.988	1.174	-0.083	0.263	0.458	1.162	3.961	4
XSA 14464	0.478	1.492	0.921	0.330	0.531	0.148	3.899	5
XSA 14146	-0.024	1.592	1.065	0.272	0.396	0.245	3.546	6
XSA 14132	0.545	1.292	1.759	0.167	-0.411	0.059	3.411	7
XSA 14315	0.230	1.242	0.894	0.450	-0.007	0.334	3.143	8
Tamstar*	1.175	0.851	0.442	-0.044	-0.142	0.659	2.941	9
XSA 14006	1.214	1.509	-0.518	-0.180	0.396	0.490	2.912	10
Raleigh*	-0.523	0.356	1.894	0.581	0.666	-0.103	2.871	11
XSA 14100	0.929	1.304	0.281	-0.591	0.127	0.700	2.750	12
XSA 14328	0.525	0.717	-0.478	0.243	1.069	0.537	2.613	13
Captiva*	-0.305	3.128	-0.248	0.090	-0.398	0.011	2.278	14
XSA 14323	0.517	-0.098	0.558	0.095	0.935	0.044	2.051	15
XSA 14109	0.452	0.971	0.808	-0.155	-0.007	-0.140	1.929	16
XSA 14442	-0.409	1.055	1.569	-0.090	-0.007	-0.208	1.909	17
XSA 13226	-0.026	-0.170	1.565	0.916	-0.276	-0.124	1.884	18
XSA 14510	0.594	-0.022	0.315	0.068	0.666	0.198	1.818	19
XSA 13228	-0.360	1.141	0.415	0.558	-0.020	-0.003	1.731	20
XSA 13168	0.590	0.111	1.206	0.388	-0.007	-0.683	1.605	21
XSA 13229	1.011	-0.466	0.635	0.323	0.127	-0.148	1.483	22
XSA 14019	0.717	0.470	-0.023	-0.316	0.262	0.203	1.313	23
XSA 13218	0.775	-0.159	0.960	0.700	-0.546	-0.436	1.295	24
XSA 14431	0.436	-0.521	0.624	-0.172	-0.007	0.792	1.152	25
XSA 14216	0.134	0.632	0.181	-0.336	0.127	0.378	1.117	26
XSA 14490	0.226	0.147	1.129	0.082	0.262	-0.739	1.107	27
XSA 14320	0.621	-0.379	-0.007	0.141	0.127	0.576	1.078	28
XSA 14319	0.163	0.368	0.267	-0.268	-0.007	0.521	1.044	29

**Table 4.3** (continued).

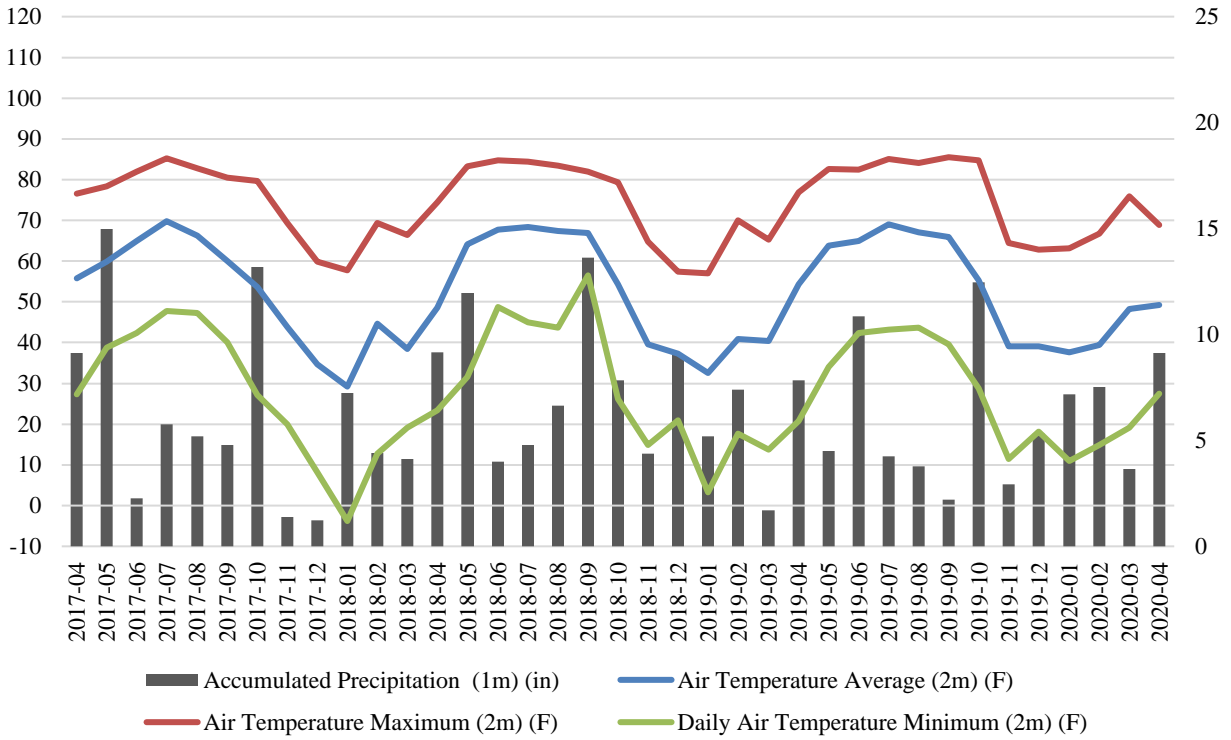
XSA 13189	0.343	0.348	0.297	-0.402	0.262	-0.158	0.690	30
XSA 14369	0.390	-0.979	0.098	0.047	0.262	0.594	0.412	31
XSA 14005	-0.289	0.847	-0.717	-0.157	0.139	0.470	0.293	32
XSA 13025	0.690	-0.188	-0.899	0.049	0.458	0.052	0.161	33
XSA 13227	0.245	-0.619	0.616	0.513	-0.180	-0.482	0.094	34
XSA 14429	0.918	-0.636	0.339	-0.126	-0.142	-0.266	0.087	35
XSA 14010	1.145	-0.232	-0.519	-0.297	-0.276	0.163	-0.018	36
XSA 14018	0.694	-0.009	0.071	-0.419	-0.546	0.178	-0.031	37
XSA 14534	0.777	-0.010	-0.591	-0.792	0.262	0.202	-0.151	38
XSA 14092	0.701	-0.176	-0.907	-0.287	0.006	0.483	-0.180	39
Seville*	-0.251	0.673	-0.419	0.132	-0.481	-0.210	-0.555	40
XSA 14374	-0.099	-0.271	-0.053	-0.132	-0.339	0.298	-0.596	41
XSA 13204	-0.282	-0.195	0.024	0.445	-0.546	-0.383	-0.936	42
XSA 13124	0.867	-0.785	-0.934	-0.154	-0.276	0.297	-0.985	43
XSA 13108	0.649	-0.851	-0.736	-0.365	-0.202	0.332	-1.173	44
XSA 14337	0.019	-1.449	-0.263	0.320	0.531	-0.618	-1.461	45
XSA 14083	-0.465	1.130	-1.164	0.064	-0.398	-0.636	-1.469	46
XSA 13192	-0.467	-0.354	0.087	0.055	-0.007	-0.785	-1.471	47
XSA 14066	0.004	-0.645	-0.717	-0.119	-0.411	0.105	-1.782	48
XSA 13150	0.130	-0.720	-0.961	-0.406	-0.142	0.233	-1.866	49
XSA 13056	-0.094	-0.588	-1.248	0.142	-0.409	0.232	-1.965	50
XSA 13118	-0.054	-1.121	-1.102	-0.418	0.794	-0.073	-1.974	51
XSA 14011	-0.091	-0.980	-0.520	-0.162	-0.322	0.004	-2.070	52
XSA 14030	0.663	-1.151	-1.005	-0.404	-0.202	-0.338	-2.438	53
XSA 14215	-0.941	-0.964	-0.102	-0.233	-0.276	-0.103	-2.619	54
XSA 14528	-0.339	-0.708	-0.551	-0.434	-0.153	-0.477	-2.663	55
XSA 14007	-0.619	-1.019	-0.226	-0.070	-0.339	-0.409	-2.682	56
XSA 13121	-0.339	-0.326	-0.884	-0.517	-0.181	-0.516	-2.763	57
XSA 14524	0.113	-1.366	-1.349	-0.151	0.000	-0.254	-3.006	58
XSA 14033	-2.189	0.170	-1.284	-0.128	0.000	0.370	-3.062	59
XSA 14057	-1.488	-0.934	-0.808	-0.246	-0.202	0.561	-3.118	60
XSA 14220	-0.837	-0.816	0.101	-0.426	-0.800	-0.438	-3.216	61

**Table 4.3** (continued).

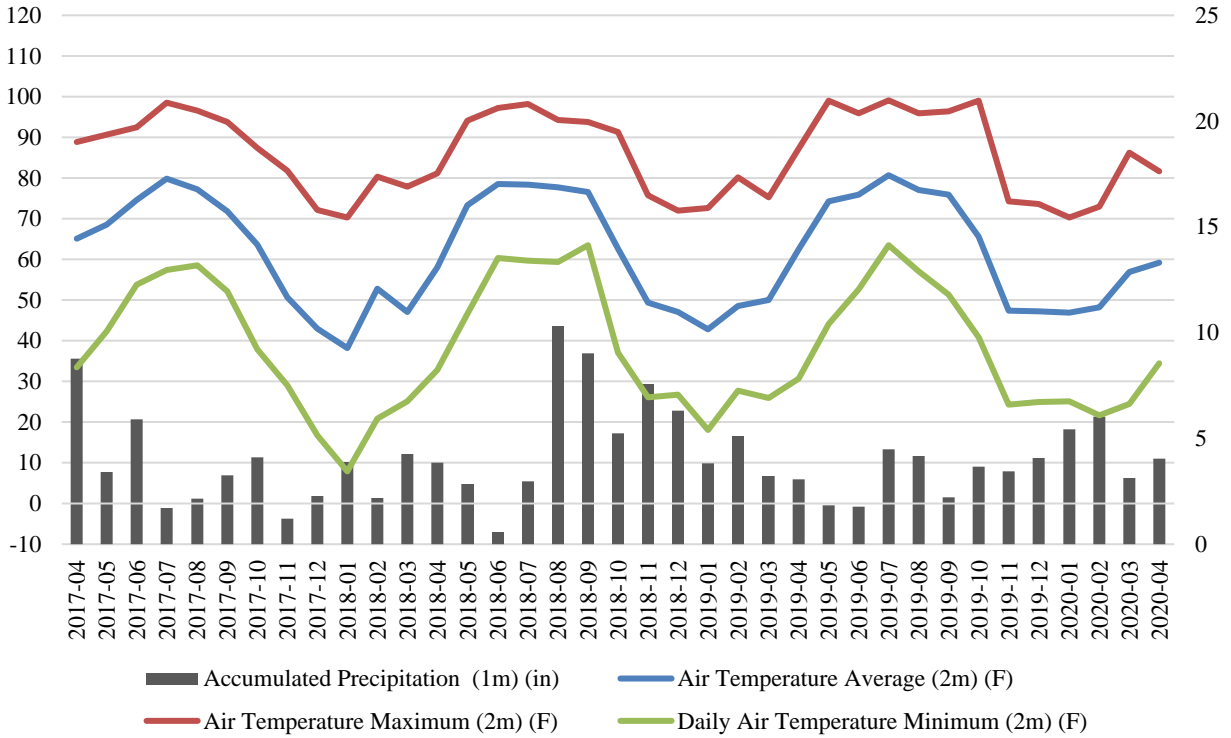
XSA 13102	-0.870	-1.048	-0.918	-0.212	-0.313	-0.293	-3.654	62
XSA 14034	-1.715	-1.025	-1.339	-0.117	0.000	-0.029	-4.225	63
XSA 14056	-2.620	-1.793	-1.104	0.259	0.000	0.458	-4.800	64
XSA 14468	-2.526	-2.019	0.080	-0.053	-0.398	-1.223	-6.138	65
XSA 13213	-4.919	-2.440	-0.757	-0.133	0.000	-1.937	-10.187	66

\* = check cultivar; all other lines are experimental entries. EST = establishment, TQ = turf quality, WK = winterkill, FC = fall color, DRY = drought, GLS = gray leaf spot.

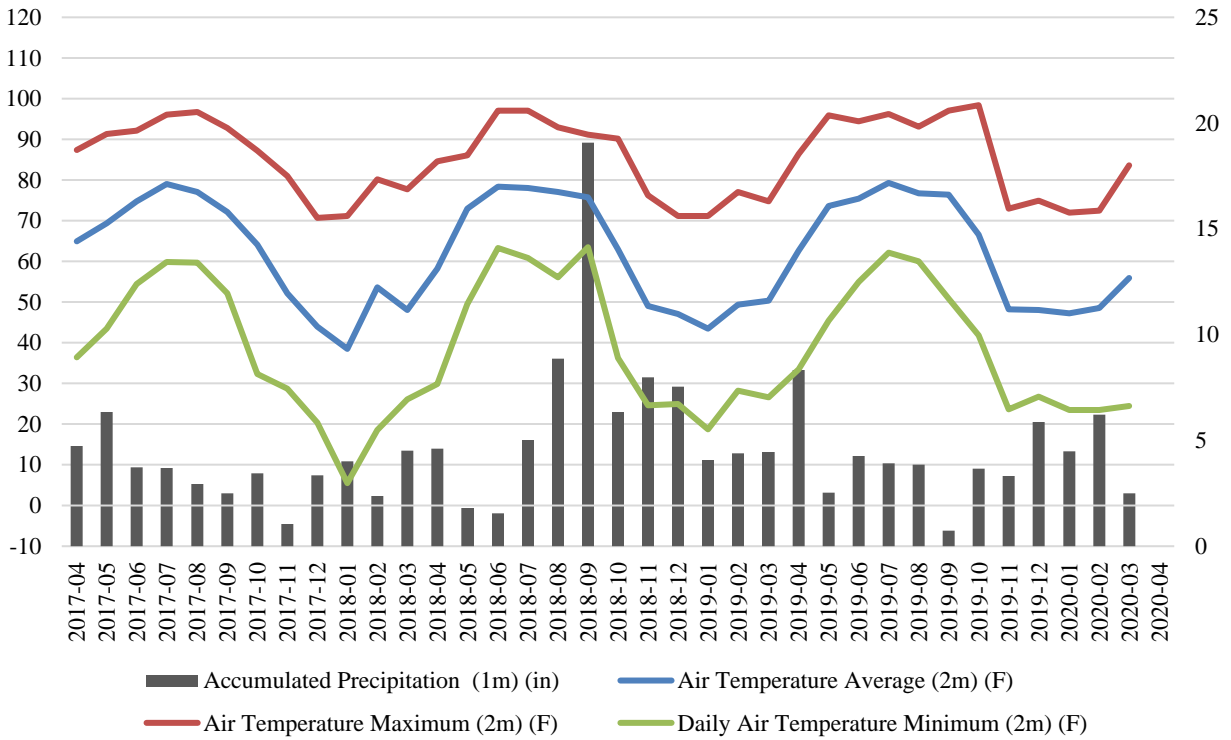
## APPENDIX 2: Supplementary Figures for Chapter II



**Figure 4.1:** Monthly Temperature and Rainfall at the Upper Mountain Research Station in Laurel Springs, NC. Temperature and precipitation data from the North Carolina State Climate Office from April 2017 to April 2020. Minimum, average, and maximum temperature are presented in °F along the primary axis (left), and accumulated precipitation is measured in inches and follows the secondary axis (right).



**Figure 4.2:** Monthly Temperature and Rainfall at the Turfgrass Field Laboratory in Raleigh, NC. Temperature and precipitation data from the North Carolina State Climate Office from April 2017 to April 2020. Minimum, average, and maximum temperature are presented in °F along the primary axis (left), and accumulated precipitation is measured in inches and follows the secondary axis (right).



**Figure 4.3:** Monthly Temperature and Rainfall at the Sandhills Research Station in Jackson Springs, NC. Temperature and precipitation data from the North Carolina State Climate Office from April 2017 to April 2020. Minimum, average, and maximum temperature are presented in °F along the primary axis (left), and accumulated precipitation is measured in inches and follows the secondary axis (right). No data was collected in April 2020.

### APPENDIX 3: Abbreviated ASReml-R Code for Chapter II

EST:

```
Model1 <- asreml(fixed= Value ~ Location,  
  random= ~ Location:Rep + Entry + Location:Entry,  
  maxiter=100, workspace=64e6,data=dataST)
```

TQ, UN, WK:

```
Model2 <- asreml(fixed= Value ~ Location + Year + Year:Location,  
  random= ~ Year:Location:Rep + Entry + Location:Entry + Year:Entry +  
  Location:Year:Entry,  
  residual = ~id(Location):ar1h(Year):Rep:Entry,  
  maxiter=100, workspace=64e6,data=dataST)
```

GC, TX, GLS:

```
Model3 <- asreml(fixed= Value ~ Location + Year + Year:Location,  
  random= ~ Year:Location:Rep + Entry + Location:Entry + Year:Entry +  
  Location:Year:Entry,  
  residual = ~dsum(~corv(Year):id(Rep):id(Entry) | Location),  
  maxiter=100, workspace=64e6,data=dataST)
```

DN:

```
Model4 <- asreml(fixed= Value ~ Location + Year + Year:Location,  
  random= ~ Year:Location:Rep + Entry + Location:Entry + Year:Entry +  
  Location:Year:Entry,  
  residual = ~id(Location):corh(Year):Rep:Entry,  
  maxiter=100, workspace=64e6,data=dataST)
```

FC:

```
Model5 <- asreml(fixed= Value ~ Location + Year + Year:Location,  
  random= ~ Year:Location:Rep + Entry + Location:Entry + Year:Entry +  
  Location:Year:Entry,  
  residual = ~id(Location):corv(Year):Rep:Entry,  
  maxiter=100, workspace=64e6,data=dataST)
```

DRY:

```
Model6 <- asreml(fixed= Value ~ Location,  
  random= ~ Rep + Entry,  
  maxiter=100, workspace=64e6,data=dataST)
```

#### APPENDIX 4: Supplementary Tables for Chapter III

**Table 4.4:** SSR primer sequences for 'Raleigh' x 'Raleigh' population. T<sub>m</sub> = melting temperature.

Pair No.	Primer Name	Sequence	Length (bp)	T <sub>m</sub> (°C)	Expected product size (bp)
1	SSR00102_F	CACGACGTTGTAAAACGACCACCATTTTAAAGCATGTCCAA	41	59.9	101
	SSR00102_R	GTGAGAAGCGACATCCTCTTG	21	60	
2	SSR00276_F	CACGACGTTGTAAAACGACTGTTTCTATGTTTCACGCACA	40	57.8	119
	SSR00276_R	TTGATCTATTATTTGTCAACTTTAACC	27	56.4	
3	SSR00332_F	CACGACGTTGTAAAACGACAACATGGTGCTTGGTGCATA	39	60	77
	SSR00332_R	GTTCAATTGCTGTCAAGTGATTTTC	23	58.8	
4	SSR00491_F	CACGACGTTGTAAAACGACCCTGCCACTCGCAAACAC	37	61.5	100
	SSR00491_R	CAATAATTCGGGGCCAGTT	19	59.8	
5	SSR00557_F	CACGACGTTGTAAAACGACTGCTTGCTTGTCGCAGAG	37	60	122
	SSR00557_R	GTACACGCTGCCCCCTCTC	18	59.4	
6	SSR00933_F	CACGACGTTGTAAAACGACACAAAAATCAGAGCCGCAAT	39	59.7	111
	SSR00933_R	CTCGCTCCCATGAAATAAAAA	21	57.2	
7	SSR01073_F	AGGCTGCTGCATGATCAAA	19	60	115
	SSR01073_R	CACGACGTTGTAAAACGACCCGCTGATTTCAAGATGGAT	39	60.5	
8	SSR01288_F	TGTGTCGTAGTACAGAATACAGATTACC	28	60.3	90
	SSR01288_R	CACGACGTTGTAAAACGACACGACATCTTTCGCAACCA	38	59.1	
9	SSR01349_F	GCGAGAGGAGTTGAGAGCAG	20	60.3	116
	SSR01349_R	CACGACGTTGTAAAACGACTGAGACCACATCATCTTCTTGC	41	60.4	
10	SSR01356_F	CAGCAAAGAAGCTAGCAGCA	20	59.6	108
	SSR01356_R	CACGACGTTGTAAAACGACCGGATTGGTGAGAGGATCA	38	59.7	
11	SSR01445_F	CACGGAATGGTTTGAATGG	19	59.9	121
	SSR01445_R	CACGACGTTGTAAAACGACCGAATGTAGCTGATATGTGAAACC	43	59.8	

**Table 4.4** (continued).

12	SSR01466_F	CGAATTCTTGGAGGCGAAAT	20	61.7	102
	SSR01466_R	CACGACGTTGTAAAACGACATCGAGGTCGTGTCCGACGTA	39	61.4	
13	SSR01512_F	GTACCCACTTGCATCCCTTG	20	60.7	110
	SSR01512_R	CACGACGTTGTAAAACGACGAACTAAAAAGGAGGCCTCAGC	41	60.4	
14	SSR01842_F	TTCATGTAGACAGCCTTTTCTCA	23	58.5	91
	SSR01842_R	CACGACGTTGTAAAACGACTTCGCTTAGCACACAGGATT	39	59	
15	SSR01952_F	CACGACGTTGTAAAACGACCATGCATCCGTGTTACATGC	39	61	90
	SSR01952_R	GCAGGAGGAGGAGGAGAGG	19	61.4	
16	SSR02037_F	CACGACGTTGTAAAACGACACACCACCAGACCCTACCAA	39	60.3	101
	SSR02037_R	GGGTTCAATTCGGTGAGAGAA	20	60	
17	SSR02279_F	CACGACGTTGTAAAACGACGGTGTGTTGAAACTGCCGAC	39	60	107
	SSR02279_R	ATCCGGTTGATTGCCCTACT	20	58.8	
18	SSR02330_F	CACGACGTTGTAAAACGACGTGGCGCGTCTGTTCACT	37	61.1	110
	SSR02330_R	AAATACAACAAGCTAGGCTTAACAGC	26	60.5	
19	SSR02339_F	CACGACGTTGTAAAACGACAGAATTTGATGTGCATGTTTGG	41	59.9	110
	SSR02339_R	CTCAAAATCCCATAAGTTGAAGTTG	25	60.3	
20	SSR02389_F	CACGACGTTGTAAAACGACTGTGCACTTAGGATGACCGA	39	58.7	95
	SSR02389_R	GTAGGGCTGAATATGCTACGA	21	57.1	
21	SSR02442_F	CACGACGTTGTAAAACGACCTCGCCTCACCTCCATTC	37	59.3	103
	SSR02442_R	CCATTCGGCTCATCAAGTG	19	60.2	
22	SSR02460_F	CACGACGTTGTAAAACGACGCTTTAGTGCGTGCTTTTT	39	59.5	112
	SSR02460_R	GACTGGGTGCAACCGATAG	19	59.1	
23	SSR02502_F	CACGACGTTGTAAAACGACAAGAACAAGGGTTTCAAAGTCAA	42	59.2	110
	SSR02502_R	TTCCATTCCAAGTGAATGTCC	21	59.8	
24	SSR02503_F	CACGACGTTGTAAAACGACACCTGGTACTGCAACCGTCT	39	59.6	116
	SSR02503_R	TCTCTCTAGTCTCTCTGAACTGAGC	25	57.9	
25	SSR02522_F	CACGACGTTGTAAAACGACCCACCACACCATGACGACA	38	59.9	70
	SSR02522_R	CACCCAAAAGACGCGTGC	18	60.1	

**Table 4.4** (continued).

26	SSR02573_F	CACGACGTTGTAAAACGACTCTACGGTAACGTCCGGTTT	39	59.5	112
	SSR02573_R	GGTAGCATAATTGTTCTAGATGTTGG	26	59.4	
27	SSR02759_F	TTCCTATGCATTTGCTTGGTT	21	59.7	110
	SSR02759_R	CACGACGTTGTAAAACGACCAACATTGCCGGTGATATTTT	40	59.6	
28	SSR02819_F	TCCACGACCTGCCTATCTAAC	21	57.2	114
	SSR02819_R	CACGACGTTGTAAAACGACTCACAGATACAAATGACACAAAATTTT	46	59	
29	SSR02902_F	GGCTTCTGCCTCTCTCCTCT	20	59.1	100
	SSR02902_R	CACGACGTTGTAAAACGACCGTTGCTGGCTGACTACG	37	60.2	
30	SSR03084_F	TTGTTTGTTTCATTGCTATCCATT	23	59.3	81
	SSR03084_R	CACGACGTTGTAAAACGACTCGATGCATCAAACCAAATAAT	41	58.6	
31	SSR03133_F	TGAACACAGCATACCTCGG	19	56	113
	SSR03133_R	CACGACGTTGTAAAACGACACCTGGCAGTCTGATTTAATTT	41	57.2	
32	SSR03140_F	GAGAAAACCGCGCCAAC	17	60.1	81
	SSR03140_R	CACGACGTTGTAAAACGACTGTTGTGATGGAGAGGGACA	39	60.3	
33	SSR03460_F	CACGACGTTGTAAAACGACTTTCTTGTAGAGAAGTGCTATGTTACA	46	58.6	90
	SSR03460_R	TGCAACTCTTATGGTAGATTTCTGC	25	59.4	
34	SSR03592_F	CACGACGTTGTAAAACGACGTTTGGCTAGTAAAGTATCAACCATGC	45	59.6	99
	SSR03592_R	GGCCTTGGCAGACACTACAT	20	60.1	
35	SSR03614_F	CACGACGTTGTAAAACGACCGCCGTGGCCATCCATC	36	61	100
	SSR03614_R	GAGGGAAGAAGGAGCTCCGG	20	61.7	
36	SSR03677_F	CACGACGTTGTAAAACGACCTTCCCAAATCCCTCGCCG	38	60.8	117
	SSR03677_R	GAGACGGAGGCGGGAGG	17	61.2	
37	SSR03956_F	CACGACGTTGTAAAACGACATCCTCCCTCAAGCCTTCC	38	60.5	102
	SSR03956_R	AGGCCAACCCAGCATAAC	18	60.9	
38	SSR03957_F	CACGACGTTGTAAAACGACATTTGCCAACTACTACCTATTTTGG	44	59	90
	SSR03957_R	AAAATTAGCAATAAAGCAATGGAA	24	58.4	
39	SSR04170_F	CCAGCATGCTCCTCTTCATC	20	60.1	113
	SSR04170_R	CACGACGTTGTAAAACGACTTCCTCGAAAAACAAAGGAAAA	41	60.9	

**Table 4.4** (continued).

40	SSR04205_F	TGTGGTGTTTTTGCCTCTAATG	22	59.8	112
	SSR04205_R	CACGACGTTGTAAAACGACAAAATTATACTGGAAGGAGTGGAAGA	45	60	
41	SSR04343_F	CTTCTTCGGAGTTCCCACTG	20	59.2	120
	SSR04343_R	CACGACGTTGTAAAACGACCTGAGACGCTGAGAGAGCTG	39	59.8	
42	SSR04381_F	GGGTAATTTAGTTGGCTTACACACA	25	59.7	111
	SSR04381_R	CACGACGTTGTAAAACGACAAGAACGACTGAAGTAACACAACCT	44	60.5	
43	SSR04408_F	GGCTGCATTTGCCCTTT	17	59.3	112
	SSR04408_R	CACGACGTTGTAAAACGACGGATGGCACCGGTTTTT	36	59.7	
44	SSR04432_F	ACACACAGCTCGGTCTTCTAT	21	58.7	110
	SSR04432_R	CACGACGTTGTAAAACGACACCCTCTAATGGAGGAAGAGG	40	56.5	
45	SSR04450_F	GGGTTTGGATCGGAGGGTTC	20	59.8	104
	SSR04450_R	CACGACGTTGTAAAACGACGATCTCGCAGGCAGAAGTGA	39	60.4	
46	SSR04527_F	CCATCCATCTTTCCATCCAT	20	59.8	94
	SSR04527_R	CACGACGTTGTAAAACGACTCCGGGATAAGTTTTAAAAAGAAGT	44	59.6	
47	SSR04549_F	CTCTCCATCTTTGCCCATTG	20	60.2	95
	SSR04549_R	CACGACGTTGTAAAACGACCATGCCCCCACTTTTATCAC	39	60.6	
48	SSR04584_F	CAAAGATTGAGCCTTCAAATCA	23	59.7	111
	SSR04584_R	CACGACGTTGTAAAACGACCATGTTAATCTTTATAAAAAGCGCTAGA	47	60.6	
49	SSR05455_F	GCACAGGAGAGAGATGGTGTG	21	61.1	-
	SSR05455_R	CACGACGTTGTAAAACGACGTGGGCGTAGGTCCGTCT	37	60.9	
50	SSR06099_F	TCGAAGACGACGCACTTG	18	61	86
	SSR06099_R	CACGACGTTGTAAAACGACAACGGGACATCCGGAGAG	37	59.7	
51	SSR06868_F	AAACCAAAGCCAGGTAACACC	21	60.4	87
	SSR06868_R	CACGACGTTGTAAAACGACCGATACGATGGTTTTGTTTCG	40	60.3	
52	SSR07483_F	CACGACGTTGTAAAACGACGACGTGGCAGCCTGTACTCT	39	60.5	86
	SSR07483_R	ATTCCGGACGAGCACATCT	19	60.6	
53	SSR08101_F	CACGACGTTGTAAAACGACGCCCGCTAACGCACTCT	36	60.1	82
	SSR08101_R	CCACCATTCTCAACCGTAT	20	59.7	

**Table 4.4** (continued).

54	SSR08347_F	CACGACGTTGTAAAACGACCTCCGCCTCCTCTGCTTC	37	61.2	115
	SSR08347_R	CTTCCTCTCTCCTTCGCTGTC	21	60.7	
55	SSR08674_F	CACGACGTTGTAAAACGACCCTCTTGACAAGTAAACAATCCA	42	58.3	91
	SSR08674_R	ACTACTAGTCCTTTGCCTTATTTGA	25	57	
56	SSR09002_F	CACGACGTTGTAAAACGACACCACCAGGTCGGGCTA	36	60.1	110
	SSR09002_R	CGAGGACCGGGTAGGG	16	60	
57	SSR09747_F	CACGACGTTGTAAAACGACGGACACGCATCGTTCAGAT	38	59.7	116
	SSR09747_R	AACAAGACAATTTGAGATGTGCTC	24	59.7	
58	SSR09951_F	CACGACGTTGTAAAACGACCGTCTCTGCATCGCCTATC	38	59.5	110
	SSR09951_R	CATCACCTCACCGAGCAAG	19	60.4	
59	SSR10023_F	CACGACGTTGTAAAACGACTGTAAGCTTGGGTGCAAGG	38	59.8	96
	SSR10023_R	GAACCAGGGCCTCACTCTTA	20	59.3	
60	SSR10212_F	CACGACGTTGTAAAACGACGTGAAACACGACACTCCTTCC	40	59.6	110
	SSR10212_R	TCTTTCTGAACCATTGTTATCTGAG	25	58.9	
61	SSR10259_F	CACGACGTTGTAAAACGACCCACATACCTCAATATACAAACGTC	44	58.8	113
	SSR10259_R	TGTTAACTGCAGCTTATTAGGTGAT	25	58.5	
62	SSR10345_F	CACGACGTTGTAAAACGACGTGGGTGCTGTGCTTGTG	37	59.9	90
	SSR10345_R	TAAACAAACACATTTGAGCCGTA	23	59.6	
63	SSR10668_F	CACGACGTTGTAAAACGACACCAAAAAGCCATCACAACC	39	59.8	102
	SSR10668_R	ACGGACGTACATGCTCTGG	19	59.7	
64	SSR10863_F	CACGACGTTGTAAAACGACCGCTCCTCCCGTCACTC	36	60.5	115
	SSR10863_R	CTTGTTCCCTGAGCTCTCTCTCC	22	59.8	
65	SSR11067_F	AACCGAGCTCTGCCACCT	18	60.5	90
	SSR11067_R	CACGACGTTGTAAAACGACCCCTGATCCATTCTCTCTACCA	41	61	
66	SSR11218_F	TGGACTAATGGTTGTAGTAAGCAAC	25	59.6	120
	SSR11218_R	CACGACGTTGTAAAACGACGCCGTCAACGAAAATAATTG	40	58.8	
67	SSR11328_F	GACAACAGTGATGGCTCCATTT	22	60.8	110
	SSR11328_R	CACGACGTTGTAAAACGACCAAACATGCGTGCGTTCTAA	39	61.3	

**Table 4.4** (continued).

68	SSR11381_F	TATTTGGGCGACGCTGTTGA	20	60.2	91
	SSR11381_R	CACGACGTTGTAAAACGACACGCAAGCACACAACGTTG	38	60.3	
69	SSR11402_F	TCTCTATTCGTTAGATTCCCAAAT	24	57	93
	SSR11402_R	CACGACGTTGTAAAACGACAATTTGTTGTGTTTGTATGTAATCTC	46	57.5	
70	SSR11424_F	CGCATGGGAGCTCTTTACC	19	59.5	114
	SSR11424_R	CACGACGTTGTAAAACGACTTAATTGCTCAGCTTCCAGATG	41	60.8	
71	SSR11435_F	GGCCGTGTCATGGCTAGTAA	20	61.4	89
	SSR11435_R	CACGACGTTGTAAAACGACGGGACCCGCCTAAGCTAAA	38	61.1	
72	SSR11492_F	CGAAAATGAAGGGAGAGAATG	21	58.7	94
	SSR11492_R	CACGACGTTGTAAAACGACATGTGAAGATCGCCGTTTC	38	58.8	
73	SSR11704_F	CACGACGTTGTAAAACGACTTCAGAATCATTTCGTAAATGC	40	55.5	117
	SSR11704_R	GAGTGACTGAGCCGAAATTTTA	22	58.5	
74	SSR12104_F	CACGACGTTGTAAAACGACCGTGACAGTAATCTGCAAGAGAG	42	59.2	116
	SSR12104_R	CTGCTCCTTCCCCATGC	17	60.3	
75	SSR12198_F	CACGACGTTGTAAAACGACGGACCCAGAGGATGACAG	38	60.5	104
	SSR12198_R	CGACACCCCTTCCCTTTTAT	20	60.2	
76	SSR12750_F	CACGACGTTGTAAAACGACGAACCGACCCTTGAGCCG	37	60.4	92
	SSR12750_R	GTGATTGGGAAGGGATGCTGA	21	60.1	
77	SSR13043_F	TTTGAACCGACTGCTACTGT	20	56.6	110
	SSR13043_R	CACGACGTTGTAAAACGACAAAGTTAAGTATATATGCATCCGAAG	45	56	
78	SSR14922_F	TCGTAATGTTTGCTAGTTCTTCTGT	25	58.8	112
	SSR14922_R	CACGACGTTGTAAAACGACCCAGCCAAACTGTTCTGTGT	39	58.6	
79	SSR15446_F	GGGGTTCGCAATTTCTATGAT	21	60.6	71
	SSR15446_R	CACGACGTTGTAAAACGACCAAGCTCGCCACAAGGTC	37	60.2	
80	SSR15541_F	CAAAGGCACCAAGAAACGCA	20	61.1	100
	SSR15541_R	CACGACGTTGTAAAACGACCCGACGACAACGGTGAGC	37	59.9	
81	SSR15639_F	CGATCCTCATCTAGCTTATTTTGC	24	60.2	116
	SSR15639_R	CACGACGTTGTAAAACGACCAGCCCAGGATCGACATC	37	60.6	

**Table 4.4** (continued).

82	SSR16446_F	CACGACGTTGTAAAACGACCGAGGGGGAGAGGAAGG	36	60.7	95
	SSR16446_R	ACCCACCACATTCCAAATCA	20	61	
83	SSR16728_F	CACGACGTTGTAAAACGACTCCAGAAGAGAGAAGATGAAATGA	43	59.5	114
	SSR16728_R	GCCAGTCCGAGATGGTTAAG	20	59.7	
84	SSR16866_F	CACGACGTTGTAAAACGACGGATGGATCGATCGGAAT	37	58.2	112
	SSR16866_R	AAGGGCTCACTGACCTCTCA	20	60	
85	SSR16939_F	CACGACGTTGTAAAACGACCAGTAGCGTTGCGTTGCC	37	59.8	100
	SSR16939_R	TTGCATGTGCCCGTGTTTTT	20	59.8	
86	SSR17039_F	TCCTTTGTTTCATTCTCCATGT	22	59.1	105
	SSR17039_R	CACGACGTTGTAAAACGACATCCGACGGCCACTTG	35	58.6	
87	SSR17049_F	ACAGAGTCGTGGTGGAGATG	20	56.6	115
	SSR17049_R	CACGACGTTGTAAAACGACGAGATTTATTTTCAGTGCAATCAT	43	58.7	
88	SSR17189_F	GGCCCGCTCTCCATT	16	60	100
	SSR17189_R	CACGACGTTGTAAAACGACAAATATTAATGGCCGGAGGTG	40	59.7	
89	SSR17353_F	GTACTCCCCTGGACTGCTGA	20	60.4	93
	SSR17353_R	CACGACGTTGTAAAACGACGCTGAGCAATTTTTCGTTTT	39	60.3	
90	SSR17356_F	AGACTCGAGCGATGGTGATT	20	60.1	113
	SSR17356_R	CACGACGTTGTAAAACGACATTGTTGAGCGGCATACACA	39	59.8	
91	SSR17684_F	CACGACGTTGTAAAACGACCTGCGCGAGCTTCCTCT	36	60.5	110
	SSR17684_R	GGCTGGATCCGGTTACTTTT	20	60.3	
92	SSR17841_F	CACGACGTTGTAAAACGACCGCCTTGGATAGCTTGCTT	38	60.5	121
	SSR17841_R	GGACAAATCATAACATACCTTTTCCA	25	60.3	
93	SSR17849_F	CACGACGTTGTAAAACGACCTTGATGCCGGGAGAAATAA	39	60	106
	SSR17849_R	AGGGAATTTGCTCTCGATTCT	21	59.3	
94	SSR17952_F	CACGACGTTGTAAAACGACAGAAGTCTTTGCTGCCTCGG	39	60.3	100
	SSR17952_R	GAGGAGGGGAGCTGGGGAT	19	62.4	
95	SSR18316_F	CACGACGTTGTAAAACGACCTCGGATCGCCTTCCTC	36	59.4	91
	SSR18316_R	ATTTAGGGCCCGAGAACTTG	20	60.4	

**Table 4.4** (continued).

96	SSR18593_F	CATATGCATTACGATCGACTATGA	24	58.6	106
	SSR18593_R	CACGACGTTGTAAAACGACGCATGATGAGTATTTTCCCTTG	41	59	
97	SSR19047_F	TTTCTTGAACACGTAGGAGAACTG	24	59.9	116
	SSR19047_R	CACGACGTTGTAAAACGACACCCTACCACAATCCAGCAC	39	59.9	
98	SSR19552_F	CAGGGTGGGGAGATGGTTA	19	60.2	95
	SSR19552_R	CACGACGTTGTAAAACGACTGTCAGTGGCAAGAAATGGA	39	60.7	
99	SSR20662_F	CTCACGCTGCCACCACAT	18	61.2	91
	SSR20662_R	CACGACGTTGTAAAACGACCCACCATCTTTGTCTTGAGCA	40	62	
100	SSR21056_F	TTGTGCAACTTTGGACCTTG	20	60.7	82
	SSR21056_R	CACGACGTTGTAAAACGACTCATTACGGGCCCAAATTC	38	59.7	
101	SSR21192_F	TCAGGTTATCAGGGTAACTGGGTA	24	60.7	115
	SSR21192_R	CACGACGTTGTAAAACGACTAGACCCACTGATGGGGATG	39	61	
102	SSR21225_F	AATTGTGTATTTTCATGCCAGCTC	23	60.9	90
	SSR21225_R	CACGACGTTGTAAAACGACGGATGCCATGCACGAGTAAT	39	60.4	
103	SSR21266_F	CACGACGTTGTAAAACGACATTCCAGCGGGCAGCATA	37	62.7	83
	SSR21266_R	CGCCGTCGTCTTTACATGAG	20	61.8	
104	SSR21486_F	CACGACGTTGTAAAACGACTCACCACCACAACCCTATTA	39	56.3	110
	SSR21486_R	TGCGCGCGTAAAAGATT	17	59.1	
105	SSR21978_F	CACGACGTTGTAAAACGACGCGAGTTACCACCCCTTCG	38	60.4	90
	SSR21978_R	CCACCTCAAACAAGAAGATAAGCA	24	59.2	
106	SSR22229_F	ATTATTCCAAATCAGACTCGAGCC	24	59.9	100
	SSR22229_R	CACGACGTTGTAAAACGACCACGGCGAGAGATTCATCAGA	40	58.9	
107	SSR23208_F	CACGACGTTGTAAAACGACCAAGGTCTTCCTTGGTGCTT	39	59.3	99
	SSR23208_R	GATGTCTGCCAGGCCATT	18	59.6	
108	SSR23320_F	CACGACGTTGTAAAACGACGCGATGGTGGATCAAGTTTT	39	59.9	111
	SSR23320_R	CGCCACCTATTGGATGCTA	19	59.7	
109	SSR23374_F	CACGACGTTGTAAAACGACGATCGTTCGCATGAGGTGAT	39	61	93
	SSR23374_R	AGTGGTAAACAGCCATAAATGAA	23	57.8	

**Table 4.4** (continued).

110	SSR23382_F	CACGACGTTGTAAAACGACTCCTTCCACTCCTCTCCTCTC	40	59.9	111
	SSR23382_R	TGTGAGCATCTATGTGTTGTACTATTT	27	58.4	
111	SSR24167_F	CACGACGTTGTAAAACGACTTGGTACGACGCACAATAGAA	40	59.2	112
	SSR24167_R	TTATGTTGGGATTGGGGAAC	20	59.5	
112	SSR25120_F	CACGACGTTGTAAAACGACTTACGACAAACATTTTCATGAGG	41	57.7	86
	SSR25120_R	GAGCCAAGCATGACCACA	18	59.3	
113	SSR25882_F	CACGACGTTGTAAAACGACCATTTAGGTACTGGGAAAACACG	42	59.8	92
	SSR25882_R	TGTATCGCTATCCAAGAATATCACA	25	59.9	
114	SSR27269_F	CACGACGTTGTAAAACGACCCCTGCCATGCTTGTTT	37	62	90
	SSR27269_R	ATCAGCATGTGCCCAACT	19	61.5	
115	SSR27447_F	CACGACGTTGTAAAACGACTGCTTTTAAGGTTAGATCCATGC	42	59.7	71
	SSR27447_R	TCTACGCAAATCCACCCAAT	20	60.3	
116	SSR27743_F	CACGACGTTGTAAAACGACATGTTAAATTTTCTATGTGGCAACT	44	57.5	90
	SSR27743_R	GCATAAGAAAGGATGGGCATA	21	59	
117	SSR28482_F	CACGACGTTGTAAAACGACTCATTGTTGTACCTTTTTAGTCC	43	56.6	86
	SSR28482_R	TCTGAAAGAAAGGTAACAAGAGC	23	57	
118	SSR28514_F	CACGACGTTGTAAAACGACCACGGAAATGTGGTTCATGTTT	41	61.9	80
	SSR28514_R	CTTGAATCGCACGGTCCATA	20	62	
119	SSR28745_F	CACGACGTTGTAAAACGACTGGCAGAAATTACTTCATGTGG	41	60	100
	SSR28745_R	GGAAAATGAGCCTTAGTCATGG	22	60	
120	SSR28896_F	CACTGCTCAAGACTGAAGAGC	21	59	93
	SSR28896_R	CACGACGTTGTAAAACGACACCGACCTCATTTTCCTGTC	39	58	
121	SSR29093_F	CCACTTTACATGTTGTTGTTACCTG	25	59.7	96
	SSR29093_R	CACGACGTTGTAAAACGACCCTAGCATCTCCAGGTTTGG	39	59.8	
122	SSR29118_F	CATAAGCGCAAACCCTAGCC	20	62.2	118
	SSR29118_R	CACGACGTTGTAAAACGACTTACGGATCGCCGGTCA	36	62	
123	SSR29233_F	CCAAAAACCTTTGTTTGTCCA	21	60	116
	SSR29233_R	CACGACGTTGTAAAACGACAGAAACAGCGAGGGAGTTCA	39	59.9	

**Table 4.4** (continued).

124	SSR29525_F	GGTATGGCTGCTCGTGTCA	19	60.3	84
	SSR29525_R	CACGACGTTGTAAAACGACAGCACGTTAGGCTTCCACAC	39	60.9	
125	SSR29567_F	AGTCCGAGCTCCACATCATC	20	59.6	103
	SSR29567_R	CACGACGTTGTAAAACGACCCACGAAGCTACCACTACC	39	60.2	
126	SSR30108_F	CTCGATGACGGCAGGAGC	18	59.7	90
	SSR30108_R	CACGACGTTGTAAAACGACTAAGAGCAAGTACAACAATTGGCAA	44	60.3	
127	SSR30340_F	GAGGGTGCTGCCGATG	16	59.8	105
	SSR30340_R	CACGACGTTGTAAAACGACGGACAAGCCTCCTTCCTTCT	39	59.9	

**Table 4.5:** Type 3 Tests of Fixed Effects for the Biparental Field Study. Significance at 0.05 is indicated by \*. DF = degrees of freedom, Env = environment.

Effect	Spring Green-up (SGU)				Winterkill (WK)			
	Numerator DF	Denominator DF	F Value	Pr > F	Numerator DF	Denominator DF	F	Pr > F
Env	2	6.04	9.94	0.0123*	2	6.1	1.90	0.2287
Entry	120	704	14.39	<.0001*	120	698	6.10	<.0001*
Env*Entry	237	704	2.90	<.0001*	237	698	1.29	0.0069*

**Table 4.6:** Type 3 Tests of Fixed Effects for SGT for the Biparental lab-based freeze study. Significance at 0.05 is indicated by \*. DF = degrees of freedom, Accl = acclimation treatment (either cold acclimated or not-acclimated), RG = regrowth, SGT = surviving green tissue, Temp = freezing temperature.

Effect	SGT				RG			
	Numerator DF	Denominator	F Value	Pr > F	Numerator	Denominator	F Value	Pr > F
Temp	1	1.93	16.85	0.0581	1	6.09	7.95	0.0299*
Accl	1	2.04	1.19	0.3867	1	6.15	3.87	0.0955
Temp*Accl	1	2.02	0.15	0.7365	1	6.09	1.01	0.3528
Entry	119	974	1.38	0.0062*	119	976	1.56	0.0003*
Temp*Entry	116	975	1.08	0.2670	116	976	0.75	0.9752
Accl*Entry	118	975	1.05	0.3563	118	976	0.94	0.6477
Temp*Accl*Entry	116	975	1.09	0.2638	116	976	0.70	0.9921

**Table 4.7:** Type 3 Analysis of Variance for ‘Raleigh’ checks within the selfing population lab-based freeze study. DF = degrees of freedom, Green Cover Day 5 = green cover at day 5 post-freeze relative to the pre-freeze green cover from digital imaging, Green Cover Day 26 = green cover at day 26 post-freeze relative to the pre-freeze green cover from digital imaging, Days Survival = sum of the days where green tissue was present post-freeze, SS = sum of squares, VWC = volumetric water content. Significance at 0.05 indicated by \*.

Source	Days Survival					Green Cover Day 5				
	DF	Type III SS	Mean Square	F Value	Pr > F	DF	Type III SS	Mean Square	F Value	Pr > F
Establishment	1	5.7789	5.7789	0.0700	0.7965	1	0.0049	0.0049	0.2000	0.6542
VWC	1	355.9063	355.9063	4.1100	0.0442*	1	0.0959	0.0959	3.9000	0.0498*
Reps	3	538.8639	179.6213	2.0700	0.1055	3	0.2297	0.0766	3.1200	0.0276*
Treatment	1	3.8400	3.8400	0.0400	0.8335	1	0.0149	0.0149	0.6000	0.4377
Freezer	1	2885.2394	2885.2394	33.3100	<.0001*	1	0.1395	0.1395	5.6800	0.0183*
Run(Reps*Freezer)	11	914.8376	83.1671	0.9600	0.4847	11	0.2421	0.0220	0.9000	0.5454
Green Cover Day 26										
Source	DF	Type III SS	Mean Square	F Value	Pr > F					
Establishment	1	0.0265	0.0265	0.2200	0.6387					
VWC	1	1.3534	1.3534	11.3100	0.0009*					
Reps	3	2.8104	0.9368	7.8300	<.0001*					
Treatment	1	3.1591	3.1591	26.4000	<.0001*					
Freezer	1	1.6462	1.6462	13.7500	0.0003*					
Run(Reps*Freezer)	11	2.1221	0.1929	1.6100	0.0989					

## APPENDIX 5: Abbreviated SAS Code for Chapter III

*SAS code used to generate LSmeans for the biparental population field study*

```
proc mixed data = dataset ; *method = type3;
class Env entry Rep Year;
Model Trait = env Entry env*Entry /ddfm =kr alpha = 0.05 outpm=outmnx2; * cl ;
Random Rep(env);
Lsmeans Entry;
Lsmeans env*Entry
run;
```

*SAS code used to generate LSmeans for the biparental population controlled freeze test study*

```
proc mixed data=data method=Type3;
class Temp Accl Entry Rep;
model response = Temp Accl Temp*Accl Entry Temp*Entry Accl*Entry Temp*Accl*Entry /
ddfm=kr;
random Rep Rep*Temp Rep*Accl Rep*Temp*Accl;
lsmeans entry;
run;
```

*SAS code used to generate LSmeans for the selfing population controlled freeze study*

```
Title "GLM for Raleigh only";
proc glm data=Raleigh_glm;
where Reps ne '2';
class Reps Run Treatment Freezer;
model Trait = Establishment VWC Reps Treatment Freezer Run(Reps*Freezer);
random Reps Treatment Freezer Run(Reps*Freezer);
run;
```

```
Title "GLM on all entries";
proc glm data=glm;
where Reps ne '2';
class Reps Geno Treatment Freezer;
model Trait = Establishment VWC Reps Geno Treatment Freezer Geno*Freezer
Geno*Treatment Geno*Treatment*Freezer Reps(Freezer);
random Reps Geno Treatment Freezer Geno*Freezer Geno*Treatment Geno*Treatment*Freezer
Reps(Freezer);
lsmeans Geno;
lsmeans Geno*Freezer;
lsmeans Geno*Treatment;
run;
```