ABSTRACT

HOLLOWAY, HELEN MCCAMY PRUITT. Genomic Approaches for Improving Freeze Tolerance in Zoysiagrass. (Under the direction of Dr. Susana Milla-Lewis and Dr. David Livingston)

Zoysiagrasses (*Zoysia* spp. Willd.) are warm season perennial turfgrasses originating in the Pacific Rim and well-adapted for use in the warm-humid and transitional climatic zones of the United States as both ornamental and recreational turfgrasses. Their low growth habit and general tolerance of many abiotic stresses such as drought, shade, and salinity make them desirable for home and commercial use. However, the use of zoysiagrasses is limited to warm-humid climates because of their relative lack of freezing tolerance compared to cool-season grasses. Limited progress has been made in the development of new winter hardy cultivars which is due in part to the complexity of the trait and a lack of efficient selection criteria. Further investigation into physiological processes that influence freeze tolerance, such as cold acclimation, is needed to improve understanding of this complex trait. In addition, the identification of molecular markers linked to genomic regions controlling freeze tolerance would allow the application of marker assisted selection strategies for the development of zoysiagrass cultivars with improved winter survival.

To investigate the relationship between cold acclimation and freeze tolerance in zoysiagrasses, selected cultivars with a reported range of freeze susceptibility were exposed to two cold acclimation treatments and evaluated in four controlled freezing chambers at -8, -9, -10, and -11ºC. Results indicated that cold acclimation has a significant influence on the freeze response of these cultivars. The interaction between cultivar and acclimation treatment was highly significant ($p=0.0037$). While significant differences in cultivar response were recorded for acclimated plants ($p=0.0300$), there were no significant differences among cultivars for

non-acclimated plants ($p>0.05$). Lethal temperatures were calculated for all cultivar by acclimation treatment combinations using logistical regression modeling. Overall, these results support previous reports that cold acclimated plants are able to tolerate lower temperatures than non-acclimated plants.

To identify genomic regions that influence freeze tolerance in zoysiagrass, a pseudo-F_2 mapping population and a high-density genetic map were utilized for quantitative trait loci (QTL) analysis and mapping. A population of 175 progenies obtained from crossing freeze-tolerant 'Meyer' by freeze-susceptible 'Victoria' was evaluated in field trials at Laurel Springs, NC, and West Lafayette, IN in 2014-2016. Winter survival data was collected through digital imaging analysis and significant variation in winter injury was observed within the population, including eight lines that performed as well as or better than Meyer on average across all environments. Additionally, 112 simple sequence repeat (SSR) markers and 2,306 sequencing-derived single nucleotide polymorphism (SNP) markers were used to construct the first SNP-based high density map of *Zoysia japonica*. The map covers 323 mega basepairs (Mbp) and 1973.1 centimorgans (cM) as well as all 20 chromosomes of the zoysiagrass allotetraploid genome. This map was used in conjunction with winter injury data to identify putative quantitative trait loci (QTL) associated with freeze tolerance in zoysiagrass. One hundred seven QTL were identified in this manner across six environments. Nine QTL observed in two or more environments had major effects (R^2 values $\geq 10\%$). These QTL and associated markers could be valuable for implementing marker assisted selection for winter hardiness in a zoysiagrass breeding program.

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Genomic Approaches for Improving Freezing Tolerance in Zoysiagrass

by
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DEDICATION

To my husband, Rick, for all your love, support, patience and partnership.

Thank you for always bringing out the best in me.

BIOGRAPHY

McCamy Pruitt Holloway was born in 1992 and grew up in Augusta, Georgia with her parents, Dr. Jerry Ned Pruitt, II and Ellen Neal Pruitt, and her two younger siblings, Ned and Julia Pruitt. McCamy graduated as the valedictorian from Westminster Schools of Augusta in 2010.

McCamy attended Auburn University for her undergraduate education where she majored in Agronomy and Soil Sciences. While at Auburn, she was involved in undergraduate recruiting at the university and college levels and grew to love the relationship between society and agriculture. While at Auburn, McCamy had the opportunity to spend time in Gulu, Uganda and Yangling, China, doing community outreach and studying international agricultural practices. During her sophomore year at Auburn, McCamy began dating her husband Rick, an electrical engineer, who is a constant example of God's provision and faithfulness.

McCamy began her Masters work under Dr. Susana Milla-Lewis at NCSU in 2014. When she's not in the lab, McCamy volunteers as the youth ministry director at Christ the King Presbyterian Church in downtown Raleigh and loves to hike, cook, and spend time with friends and family.

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And lastly, to the Lord, my Creator and Sustainer, who imagined the zoysiagrass genome with all its intricacies and yet cares about the struggles of my heart. I am humbled to be your daughter.

"Though the fig tree should not blossom,
nor fruit be on the vines,
the produce of the olive fail
and the fields yield no food,
the flock be cut off from the fold
and there be no herd in the stalls,
yet I will rejoice in the Lord;
I will take joy in the God of my salvation.
God, the Lord, is my strength;
he makes my feet like the deer's;
he makes me tread on my high places."
Habakkuk 3:17-18

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CHAPTER I.

LITERATURE REVIEW

ZOYSIAGRASS

Zoysiagrasses (*Zoysia* spp. Willd.) are warm season turfgrasses grown in the warm-humid and transitional climatic zones of the United States as both ornamental and recreational turfgrasses. Zoysiagrass's low growth habit and general tolerance of many abiotic stresses such as drought, shade, and salinity make it desirable for home and commercial use (Li et al., 2009). These attributes also make zoysiagrass a generally low input turfgrass requiring less maintenance and inputs than many other turfgrasses.

Zoysiagrasses are native to the Pacific Rim and have been used as turfgrasses in that region since the 1700s (Yaneshita et al., 1999). Speciation of the genus into today's eleven recognized species is attributed to geographic distribution throughout this region (Guo et al., 2014). Species of zoysiagrasses are differentiated based on color, leaf width, texture, and florescence characteristics (Ma et al., 2007). Zoysiagrass species are all allotetraploids with 40 chromosomes (2n=4x=40) and a relatively small genome size (334 Mb for *Z. japonica* Steud.) (Forbes 1952; Arumuganathan et al., 1999; Tanaka et al., 2016). Zoysiagrass was first introduced in the United States in the early 1900s and has been widely grown as turfgrass since the 1930s (MacDonald and Copeland, 1997; Engelke and Anderson, 2003). Today, *Z. japonica* Steud., *Z. matrella* (L.) Merr. and *Z. pacifica* (Goudswaard) M. Hotta & Kuroki are the three most popular zoysiagrass species in the United States.

The 1999 North Carolina Turfgrass Survey (NCTS, 1999) reported 19,220 acres of zoysiagrass grown for home, recreational, and commercial use in North Carolina. Although a more recent report of zoysiagrass use in the state is not available, it is safe to assume that zoysiagrass has grown in popularity with the new cultivar releases of the last 15 years. Within the North Carolina Sod Producers Association (2016), there are 21 sod producers growing 13

different varieties of zoysiagrass. Zoysiagrass's marketability lies in its low maintenance requirements, its shade and drought tolerance, and its weed resistance. Zoysiagrass is also well adapted for use on golf courses, and with less input and water requirements, it is an environmentally sustainable option for sports turf (Patton and Reicher, 2007a). However, zoysiagrass is generally lacking in winter hardiness in relation to cool-season grasses which limits its reliability and use in the transition zone and farther north. Therefore, there is a need for zoysiagrass varieties with improved freeze tolerance (Fry et al., 2008).

BREEDING FOR WINTER HARDINESS IN ZOYSIAGRASS

The turfgrass transitional climatic zone is the geographic area of the United States that is suitable for both warm and cool season grasses. This zone falls roughly between Interstate 70 as it runs through Maryland to eastern Kansas and the southern borders of North Carolina, Kentucky, and Tennessee (Fry et al., 2008). Of the warm season grasses, zoysiagrass is second in winter hardiness, with a reported LT_{50} (temperature resulting in 50% death of tillers) range of -8.4º to -11.5ºC (Patton and Reicher, 2007) which is behind only buffalograss (*Bouteloua dactyloides*) with a reported LT_{50} range -14.0º to -21.7ºC (Qian et al., 2001). Of the three zoysiagrass species commonly used in the United States, *Z. japonica* is the most winter hardy but has course texture and poor establishment rates compared to *Z. matrella* and *Z. pacifica*. *Zoysia pacifica*'s use is restricted to Florida and southern California because of poor freeze tolerance, and *Z. matrella* typically does not survive north of the southern border of the transition zone.

Zoysiagrass's limited winter hardiness was first reported in 1947 (Forbes and Ferguson, 1947). In this study, both *Z. japonica* and *Z. matrella* genotypes survived winter low

temperatures in a Maryland field trial, but *Z. japonica* genotypes had significantly less winter injury and faster spring green-up. Zoysiagrass's usefulness and popularity increased in the 1950s with the introduction of the remarkably winter hardy cultivar, 'Meyer' (*Z. japonica*) released jointly by the United States Department of Agriculture and the United States Golf Association (Grau and Radko, 1951). Meyer's abiotic stress tolerance is exceptional, exhibiting heat, freeze, and drought tolerance that was lacking from other turfgrasses in the transition zone at the time of its release (Grau, 1952). This variety, which is well adapted to the transition zone, made zoysiagrass a predominant golf course turfgrass. However, Meyer is slow to establish and is lacking the fine texture that is available in many other zoysiagrass cultivars. Although many *Z. matrella* cultivars including 'Cavalier' (Engelke et al., 2002a), 'Diamond' (Engelke et al., 2002b) and 'Zorro' (Engelke and Reinert, 2003) have since been released that are superior to Meyer in color, texture, and overall turf quality, Meyer remains the most consistently winter hardy cultivar available (Okeyo et al., 2011).

Evaluation of winter hardiness of available zoysiagrass cultivars may help show the range of responses for this trait present in zoysiagrass species and varieties. Although field evaluation of winter hardiness provides the most reliable indication of how a turfgrass variety will perform through a winter season, variable environmental conditions can make consistent and reproducible winter stress very difficult to attain (Anderson and Taliaferro, 2002). In order to avoid problems associated with the unpredictability of winter conditions, freeze tests in temperature controlled chambers have proved useful to test freeze tolerance and provide more efficient selection methods. Controlled environment freeze tests can be a more cost-effective and efficient way to quickly assess cold acclimation and freeze tolerance of turfgrass species

and has generally corresponded well with field screenings (Anderson and Taliaferro, 2002; Qian et al., 2001).

Early evaluation of zoysiagrass winter hardiness began in a field test of Meyer in Missouri where Rogers et al. (1975) found an LT_{50} between -11.1ºC and -12.8ºC. Rogers et al. (1977) also evaluated several zoysiagrass genotypes using freezing tests of stolons and rhizomes, determining that Meyer had better freeze tolerance than *Z. matrella* cultivars. In zoysiagrass, similar freezing chamber studies have been successfully used to evaluate the low temperature tolerance and estimate LT_{50}s for a variety of genotypes. Dunn et al. (1999) used controlled freeze testing of rhizomes to measure freezing tolerance of a selection of zoysiagrasses and found that 'Belair', 'Korean Common', and Meyer rhizomes survived temperatures as low as -18ºC. In this study, 'Sunburst' rhizomes survived at -14ºC while 'Cavalier', 'Crowne', 'Palisades', 'Emerald', and 'El Toro' did not survive when exposed to -10ºC temperatures. Patton and Reicher (2007) evaluated whole plants in conetainers in controlled freeze chambers to determine LT_{50}s of thirteen different *Z. japonica* and *Z. matrella* genotypes. In this study Meyer had an LT_{50} of -11.5±0.8ºC while 'Zenith' (*Z. japonica*) was similar at -11.5±0.5ºC. *Zoysia japonica* varieties had less winter injury and better freeze tolerance than *Z. matrella* varieties. Additionally, seeded varieties of *Z. japonica* were generally more freeze tolerant. Most recently, Hinton et al. (2012) evaluated four *Z. japonica* and five *Z. matrella* cultivars in freeze chamber tests. Reported *Z. japonica* LT_{50}s ranged from -9.6ºC for Palisades to -10.5ºC for Empire and *Z. matrella*'s ranged from -5.6ºC for Pristine to -9.8ºC for Cavalier. Furthermore, Dunn et al. (1999), Patton and Reicher (2007a), and Hinton et al. (2012) found significant correlations between plant survival in controlled freeze testing and survival in simultaneously conducted field trials. This consistency demonstrates that

plants most likely undergo similar physiological changes during cold acclimation, freezing, and deacclimation in temperature controlled chambers and in the field. These extensive evaluations of current zoysiagrass cultivars have helped to separate useful breeding material and more closely examine factors influencing zoysiagrass winter hardiness.

Physiological factors such as cold acclimation have an effect on the winter hardiness of turfgrass species. Cold acclimation refers to the natural physiological processes that take place in a plant when it is exposed to low but not freezing temperatures. Studies in zoysiagrass (Patton and Reicher, 2007a; Hinton et al., 2012), as well as saltgrass (*Distichlis spicata* (L.) Greene) (Shahba et al., 2003), buffalograss (*Bouteloua dactyloides* (Nutt.) Engelm.) (Qian et al., 2001), bermudagrass (*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* Burt-Davy) (Anderson et al., 1993; Anderson et al., 2003) and St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntze) (Milla-Lewis et al., 2013; Kimball et al. 2016) have indicated that warm-season grasses respond well to cold acclimation resulting in better freeze tolerance. Hinton et al., (2012) found that across nine different zoysiagrass cultivars with varying LT_{50}s, samples collected in the winter after cold acclimation were consistently more freeze tolerant in freezing tests than non-acclimated samples collected in the spring. In addition, Patton et al. (2007a; 2007b), used cold chambers to acclimate thirteen different zoysiagrass genotypes in order to evaluate the physiological changes in carbohydrates, proline, and proteins occurring in zoysiagrasses during cold acclimation. Concentrations of soluble sugars and proline increased during acclimation while starch concentrations decreased. Additionally, in cold acclimated plants, there was a positive correlation between starch levels and LT_{50}s as well as negative correlations between starch/sugar ratios, glucose, total reducing sugars, and proline and LT_{50}s (Patton et al., 2007b). In the evaluation of protein concentration

in cold acclimated plants, Patton et al., (2007a) found that dehydrin polypeptides increased in concentration during cold acclimation. The concentration of a particular 23kDa dehydrin was found to be significantly correlated with freezing tolerance. Zhang et al. (2009) examined the influence of cold acclimation on membrane polar lipid levels in rhizomes and their association with freeze tolerance in two cultivars, Meyer and 'Cavalier' (*Z. matrella*). Eleven major polar lipid groups were identified in both cultivars. Phosolipids were consistently found at a higher concentration than galactolipids in both cultivars, suggesting that phosolipids play a more active role in cold acclimation. However, the concentrations of lipids between the two cultivars were variable and there were no consistent associations between polar lipid levels and freeze tolerance. More information on the response of polar lipids to freeze stress is needed to determine their role in cold acclimation and freeze tolerance in zoysiagrass. Further evaluation of proteomic and other metabolic changes that occur in zoysiagrass during cold acclimation may shed light on phenotypic traits useful in the breeding of more winter hardy varieties.

LINKAGE AND QTL MAPPING IN ZOYSIAGRASS

Molecular markers are powerful tools for a variety of genetic applications including the examination of genotypic variation between individuals in a population, the construction of linkage maps, and the identification and mapping of quantitative trait loci (QTL). DNA-based molecular markers including restriction length polymorphisms (RFLP) (Yaneshita et al., 1999), amplified length polymorphisms (AFLP) (Cai et al., 2004), sequence related amplified polymorphisms (SRAP) (Guo et al. 2012), microsatellites or simple sequence repeats (SSR) (Tsuruta et al., 2005; Cai et al., 2005; Ma et al., 2007; Li et al., 2009), and single nucleotide polymorphisms (SNP) (Huang et al., 2016) have been used to evaluate genetic diversity

(Kimball et al., 2012), construct linkage maps (Yaneshita et al., 1999; Cai et al., 2004, 2005; Li et al., 2009) and identify QTL associated with traits of interest (Guo et al., 2014; Huang et al., 2016) in zoysiagrass. Microsatellites are generally easier to use than RFLPs or AFLPs and are highly variable, highly polymorphic, codominant, and abundant making them desirable and significant as genetic markers. In addition, SSRs are PCR-based and therefore easily detected and reproduced, as well as conducive for high-throughput sampling (Kalia et al., 2011).

The first linkage map of zoysiagrass was developed by Yaneshita et al. (1999) and included 115 RFLP loci covering 1506cM in 22 linkage groups. Cai et al. (2004) used 471 AFLP markers to construct a high-density linkage map of zoysiagrass, and Cai et al. (2005) continued to analyze the genetic structure of zoysiagrass by adding 161 SSR markers to the existing linkage map. Li et al., (2009) reported 447 SSR markers in 22 linkage groups for *Zoysia japonica* Steud. Additional SSR markers have been developed for zoysiagrass and characterized by Tsuruta et al. (2005) and Ma et al. (2007). The characterization of additional molecular markers to identify and tag genes of interest in zoysiagrass will assist in selecting superior plant material for developing improved zoysiagrass cultivars.

In order for molecular markers to be useful for marker-assisted selection (MAS) they must be located in close proximity to a gene of interest. Markers that are linked in this way to specific traits can be used to identify plant material with useful alleles and desirable traits without the use of phenotypic information. Linkage information can be especially useful in traits that show continuous variation (i.e. plant height, yield, winter hardiness etc.) due to influences from multiple genes (QTL) (McCouch et al., 1995; Paterson,1996). To identify markers linked to QTL, a linkage map must first be constructed. Linkage maps compare patterns of segregation for a group of markers in a population of related individuals, typically

an F_2, backcross, or a recombinant inbred line (RIL) population (Young, 2000). Because linked markers have lower rates of recombination, they typically segregate in more similar patterns than unlinked markers (Mohan et al., 1996). Segregation of parental alleles amongst these progenies can be analyzed using programs such as JoinMap (Stam, 1993) to arrange markers into linkage groups. With enough linked markers, groups of linked loci can be constructed into a map covering the genome of interest. Then, phenotypic data from the same population of interest can be collected and compared to the map. When phenotypic data for (a) trait(s) of interest is known, a logarithm of odds (LOD) score is calculated for each marker to identify potential QTL (Laird and Lang, 2011). Mapping QTL becomes more accurate and informative as linkage maps are saturated with additional polymorphic markers (Miles and Wayne, 2008). QTL identification in zoysiagrass has become more effective as molecular marker technology has become more efficient and accessible (Forster et al., 2004).

In 2009, Yaneshita et al. identified four genetic regions related to winter leaf color in zoysiagrass based on the linkage map of 115 RFLP loci identified in an interspecific *Z. japonica* x *Z. matrella* population. In addition, they found that winter leaf color was genetically controlled and that both *Z. japonica* and *Z. matrella* have functional genes that contribute to winter leaf color. Three QTL associated with soluble protein content, soluble sugar content, and superoxide dismutase activity in zoysiagrass under cold stress were observed by Ding et al. (2010). Guo et al. (2012) evaluated cold tolerance and green period (non-formant growth period) in a population of 96 zoysiagrass accessions using 254 SSR loci and 338 sequence-related amplified polymorphism (SRAP) loci. Of these markers three SSR loci and one SRAP locus were significantly associated with cold tolerance and three SSR loci and two SRAP loci were associated with green period, but no markers were associated with both cold tolerance

and green period simultaneously. In 2014, Guo et al. found more conclusive results for three QTL associated with salt tolerance traits in a *Z. japonica* population using a linkage map of 217 SRAP and 25 random amplified polymorphism DNA (RAPD) markers and covering 1211cM. Fall armyworm (FAW) resistance is also a trait of interest in zoysiagrass and was examined by Jessup et al. (2011) in a *Z. matrella* mapping population. Several loci of interest were found to be associated with FAW resistance, but no QTL were decisively determined. However, using high density linkage maps of 2,375 and 3,563 SNPs, respectively, that were developed using a genotyping-by-sequencing approach, Huang et al. (2016) identified six QTL associated with fall armyworm resistance in a Diamond x Cavalier *Z. matrella* mapping population. The advent of new sequencing technologies facilitates the creation of similar high density maps for the discovery of more genetic regions associated with traits of interest in zoysiagrass breeding.

Next generation sequencing is a powerful and efficient tool for genome sequencing and molecular marker discovery that is quickly becoming more cost-effective and accessible (Morozova et al., 2008). Sequencing protocols have been developed which allow for the simple, inexpensive sequencing of a wide range of species including those with complex genomes. Using adapter-tagged sequences from selected individuals, sequencing allows for the rapid detection of single nucleotide polymorphisms (SNPs), which are the most abundant molecular markers in a genome and useful for creating high density linkage maps (Elshire et al., 2011).

Sequencing protocols have been successfully applied for SNP discovery and QTL mapping in many crop species from wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (Poland et al., 2012; Liu et al., 2014), to soybean (*Glycine max*) (Sonah et al., 2013), cabbage

(*Brassica oleracea*) (Izzah et al., 2014), and even zoysiagrass (Huang et al., 2016). Most recently in zoysiagrass, whole genome shotgun sequencing and RNA-seq analysis were used to assemble reference genomes for the accessions *Z. japonica* 'Nagirizaki', *Z. matrella* 'Wakaba', and *Z. pacifica* 'Zanpa' (Tanaka et al., 2016). These reference genomes will be instrumental in genetic analysis of zoysiagrasses, allowing for more precise marker mapping, the development of physical maps, and the identification of genetic regions of interest associated with desirable traits.

Ultimately, the goal of identifying QTL in a breeding program is to use markers associated with the trait of interest to more efficiently select for individuals carrying superior alleles. This is especially true for traits that are complex (like tolerance to abiotic stresses) or difficult to phenotypically evaluate. Marker assisted selection (MAS) uses DNA markers associated with traits of interest to screen individuals for the presence of advantageous genes, eliminating the need for extensive phenotypic evaluation before selection. These genetic markers are generally not affected by environmental factors, eliminating the errors in selection that occur due to environmental variation in field testing. The identification of molecular markers that are associated (either through linkage or pleiotropy) with the trait(s) of interest is a useful approach to facilitate selection of complex traits, such as winter hardiness (Paterson et al. 1988; Stuber et al. 1992). Molecular markers associated with winter hardiness in zoysiagrass could allow for marker assisted selection of more winter hardy plants and eliminate some of the uncertainty in field selection due to variation in field conditions and temperature. Sixty-five years after Meyer's release, conventional breeding methods have not been able to create a cultivar that surpasses Meyer's winter hardiness. Marker assisted selection and molecular breeding techniques have the potential to overcome the restraints of conventional

methods and locate genomic areas associated with the elusive and complex trait that is winter hardiness.

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CHAPTER II.

A SNP-BASED HIGH DENSITY LINKAGE MAP OF ZOYSIAGRASS (*ZOYSIA JAPONICA*) AND ITS USE FOR THE IDENTIFICATION OF QTL ASSOCAITED WITH WINTER HARDINESS

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A SNP-based high density linkage map of zoysiagrass (*Zoysia japonica*) and its use for the identification of QTL associated with winter hardiness

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ABSTRACT

Zoysiagrasses (*Zoysia japonica* Steud. and *Zoysia matrella* (L.) Merr., 2n=4x=40) are warm-season turfgrasses well-adapted for the warm-humid and transitional climatic zones of the United States but are limited to warmer climates because of their relative lack of freezing tolerance compared to cool-season grasses. Molecular markers associated with this trait would be useful for effective selection of freezing tolerant zoysiagrass germplasm before field testing. A mapping population was developed from a cross of freeze-tolerant 'Meyer' (*Z. japonica*) with freeze-susceptible 'Victoria' (*Z. japonica*). In 2014, the 175 progenies of this cross and nine controls were evaluated in field trials in Laurel Springs, NC, and West Lafayette, IN. Winter survival data was taken in spring 2015 and 2016 and significant variation in winter injury was observed within the population, including eight lines that outperformed Meyer overall across both locations. Additionally, 112 SSR markers and 2,306 sequencing-derived SNPs were used with this population to construct the first SNP based high density *Z. japonica* map. The map covers 323 Mega basepairs (Mbp) and 1973.1 centimorgans (cM) as well as all 20 chromosomes in the zoysiagrass allotetraploid genome. This map was used in conjunction with winter injury data from six environments to identify one hundred seven putative quantitative trait loci (QTL) associated with zoysiagrass winter injury. Nine QTL with major effects ($R^2 \geq 10\%$) were observed across two or more of these environments. Two genomic regions of particular interest for maker assisted selection methods were identified at 62.44 - 89.69 cM on chromosome 12 and 0.56 - 21.91 cM on chromosome 18. These QTL and associated markers could be valuable in implementing marker assisted selection for winter hardiness in a zoysiagrass breeding program.

KEYWORDS: Genetic map, freeze tolerance, QTL, SNP, winter survival, zoysiagrass

INTRODUCTION

Zoysiagrasses (*Zoysia* spp. Willd.) are warm-season turfgrasses grown primarily in the warm-humid and transitional climatic zones of the United States as both ornamental and recreational turf. Zoysiagrass's fine texture, low growth habit, and general tolerance of many abiotic stresses such as drought, shade, and salinity make it desirable for home and commercial use (Li et al., 2009). These attributes also make zoysiagrass a generally low input turfgrass, requiring less maintenance and inputs than many other turfgrasses. However, one major factor limiting the widespread use of zoysiagrasses is a relative lack of winter hardiness, especially when compared to cool-season grasses. The release of the freeze tolerant cultivar, 'Meyer', in 1951, increased zoysiagrass's use and popularity in the transition zone (Grau and Radko, 1951). However, limited progress has been made in the development of new cold-tolerant cultivars since Meyer's release. Cultivars 'Chinese Common' and 'Zenith' have comparable winter hardiness to Meyer, (Patton and Reicher 2007), but improvements over this cultivar have not been released to date.

The limited progress in breeding for cold tolerance in zoysiagrass is in part due to the complexity of the trait, insufficient genetic knowledge of cold tolerance components, and lack of efficient selection criteria. Previous investigations in other crops (Bohnert et al., 1995, Lyons et al., 1979) have indicated that plant response to cold stress is a complex phenomenon, controlled by more than one gene and highly influenced by environmental variation. Marker assisted selection (MAS) could mitigate these issues by using DNA markers associated with the trait of interest to select individuals based on the presence of advantageous genes and without extensive phenotypic evaluation. These genetic markers are generally not affected by environmental factors, eliminating possible errors in selection that might occur due to

environmental variation in field testing. The identification of molecular markers that are associated (either through linkage or pleiotropy) with the trait(s) of interest is a useful approach to facilitate selection of complex traits, such as winter hardiness (Paterson et al. 1988; Stuber et al. 1992).

Molecular markers are powerful tools for a variety of genetic applications including the examination of genotypic variation between individuals in a population, the construction of linkage maps, and the identification and mapping of quantitative trait loci (QTL). DNA-based molecular markers including restriction length polymorphism (RFLP) (Yaneshita et al., 1999), amplified length polymorphism (AFLP) (Cai et al., 2004), microsatellites or simple sequence repeats (SSR) (Tsuruta et al., 2005; Cai et al., 2005; Ma et al., 2007; Li et al., 2009) and single nucleotide polymorphisms (SNP) (Huang et al., 2016) have been used to evaluate genetic diversity (Kimball et al., 2012), construct linkage maps (Yaneshita et al., 1999; Cai et al., 2004, 2005; Li et al., 2009) and identify QTL (Jessup et al., 2011; Guo et al., 2014; Huang et al., 2016) in zoysiagrass. Simple sequence repeats are generally easier to use than RFLPs or AFLPs and are highly variable, highly polymorphic, codominant, and abundant making them desirable and significant as genetic markers. In addition, SSRs are PCR-based and therefore easily detected and reproduced, as well as conducive for high-throughput sampling (Kalia et al., 2011). Single nucleotide polymorphisms (SNPs) are the most abundant molecular marker and useful for the creation of high-density linkage maps with the implementation of increasingly efficient and cost-effective next-generation sequencing techniques (Poland et al., 2012).

The first linkage map of zoysiagrass included 115 RFLPs covering 1506 cM in 22 linkage groups (Yaneshita et al. 1999). Other linkage maps of AFLPs (Cai et al., 2004), SSRs

(Cai et al., 2005; Li et al., 2009) have been created, and additional SSR markers have been developed and characterized for further genetic analysis of zoysiagrass (Tsuruta et al., 2005; Ma et al., 2007). Despite these advances in linkage mapping, the identification of QTL associated with traits of interest has been somewhat limited. A QTL for salt tolerance was identified using randomly amplified polymorphic DNA (RAPD) and sequence-related amplified polymorphism (SRAP) markers (Guo et al., 2014). Jessup et al. (2011) used SSR and AFLP markers to locate a QTL for fall armyworm resistance in zoysiagrass. Huang et al. (2016) further explored this fall armyworm resistance QTL using SNPs from restriction-site associated DNA sequencing (RADseq) for developing high-resolution linkage maps of two *Z. matrella* cultivars.

Next generation sequencing (NGS) is a powerful and efficient tool for genome sequencing and molecular marker discovery that is quickly becoming more cost-effective and accessible (Morozova et al., 2008). Sequencing protocols have been developed that allow for the simple, inexpensive sequencing of a wide range of species including those with complex genomes. By comparing adapter-tagged sequences from selected individuals, sequencing techniques allow for the rapid detection of SNPs and useful for creating high density linkage maps (Elshire et al., 2011). Although sequencing generates very large amounts of data, software is available for filtering large quantities of reads into high-quality SNPs, making analysis more manageable (Sonah et al., 2013; Melo et al. 2016). Because sequencing is simple, inexpensive, and effective for discovering large numbers of SNPs, the application of sequencing technology to turfgrasses would aid in the efforts to identify genomic regions that carry genes of interest. Zoysiagrass species are all allotetraploids (2n=4x=40) and have a relatively small genome size (334 Mb for *Z. japonica*) (Forbes 1952; Tanaka et al., 2016)

making next-generation sequencing techniques increasingly effective. Tanaka et al. (2016) used whole genome shotgun sequencing on Illumina HiSeq and MiSeq platforms to create reference genomes for *Z. japonica*, *Z. matrella*, and *Z. pacifica* (Goudswaard). These first reference genomes will greatly increase the efficacy of molecular markers in zoysiagrass breeding.

To date, few QTL have been successfully mapped in zoysiagrass. Further analysis and mapping of QTL associated with economically important traits, such as freeze tolerance, would greatly expedite breeding efforts through marker assisted selection techniques. The recent advances in DNA marker technology and the continued generation of zoysiagrass genetic information will expedite these efforts. The objectives of this study were to 1) develop sequence-derived SNPs to create a high density linkage map of the *Zoysia japonica* genome and 2) use the map with winter injury field data to identify QTL in zoysiagrass associated with this trait.

MATERIALS AND METHODS

Population Development

In 2013-2014 crosses were made between 'Meyer' (*Z. japonica*) and freeze susceptible cultivar 'Victoria' (*Z. japonica*). These parents were chosen based on lethal temperatures (LT_{50}s) reported by Patton and Reicher (2007) where Meyer was found to have the lowest LT_{50} (-11.5ºC) and Victoria to have the highest LT_{50} (-9.3ºC) of *Z. japonica* cultivars in controlled environment freeze tests. From these crosses, a pseudo-F_2 mapping population of 175 progeny lines was developed at the University of Georgia and maintained in the greenhouses at North Carolina State University (Raleigh, NC). Plants were propagated in 7.6 x 7.6 x 7.6 cm plastic

pots (Hummert International, Earth City, MO) filled with Fafard 4P potting mix (Conrad Fafard Inc., Agawam, MA) and allowed to completely fill the container.

Field Evaluation and Data Analysis

Progeny and parental lines along with controls Chinese Common, Diamond, Empire, JaMur, L1F, Zenith, and Zeon were planted in the summer of 2014 at the Upper Mountain Research Station (Laurel Springs, NC, 838 m elevation, 26.6°C average summer high temperature, -7.2°C average winter low temperature), and the William H. Daniel Turfgrass Research and Diagnostic Center (West Lafayette, IN, 187 m elevation, 28.3˚C average summer high temperature, -7.05ºC average winter low temperature). Entries were planted in 0.91m x 0.91m plots in a complete randomized block design with three replications. Because of the loss of some plots during the 2014-2015 winter, additional copies of the population were planted at both the North Carolina and Indiana locations in the summer of 2015 for a total of four complete copies of the population to obtain two years of evaluation for all genotypes (Table 2.1). At both locations, plots were mowed twice per week at a 6.35cm height, fertilized once per month at 0.23 kg ha^{-1} from May to October (for a total of 1.36 kg ha^{-1} per year), and irrigated to prevent drought stress.

Plots were evaluated at both locations for percent green cover, winter injury, and turf quality from 2014-2016. Digital images were taken of each individual plot before and after winter dormancy to estimate percent green cover and winter injury. A portable light box was used to keep the light source, size, and orientation of each picture consistent. Pictures were analyzed using ImageJ-Fiji (Schindelin et al., 2012) which estimates percent cover by comparing the ratio of green pixels to total pixels using an HSB (hue-saturation-brightness) representation. Hues were set at 35-130, saturation at 1-255, and brightness at 1-255. Winter

injury of these plots was quantified by comparing total green coverage before winter dormancy to green coverage after winter recovery as: winter injury = 100 – [100 (coverage after winter dormancy/coverage before winter dormancy)] (Patton and Reicher, 2007). In July and October of each year, turf quality was evaluated visually on a scale of 1 to 9 according to the National Turfgrass Evaluation Program's (NTEP) guidelines (NTEP, 2012) where 1=poor quality, 5= minimum acceptable quality and 9=excellent quality.

In analyzing field data, the combination of each copy of the population and year was treated as an individual environment for a total of six environments (Table 2.1). Winter injury data was arcsine transformed for more homogeneous error variances (Steel et al., 1997). Data was analyzed using a mixed model procedure (PROC MIXED) in SAS 9.4 (SAS Institute; Cary, NC) to generate an analysis of variance (ANOVA) (method=type3) and least squared (LS) means. In this model, replications were considered random while the genotype, environment, and genotype within environment effects were fixed. Percent fall cover was treated as a covariate to account for the differences in establishment rates among plots. The interaction of genotype by replication within environment was used as an error term to account for the evaluation of plots over multiple years. After fitting the mixed model, LS means were outputted and then back-transformed for QTL analysis.

SSR Analysis

Total genomic DNA was extracted from the 175 progeny and 'Meyer' and 'Victoria' parents using a modified CTAB method (Afanador et al., 1993) and quantified on a Hoefer fluorometer (Hoefer Scientific Instruments, San Francisco, CA). Following quantification, each sample was diluted to 10 ng/ul and stored at -20ºC. The quality and purity of DNA from each sample was verified using a 1% agarose gel and ethidium bromide stain.

A total of 239 SSR primers were screened with parental DNA based on their previous success for creating SSR-based linkage maps in zoysiagrass (Guo et al., 2012; Li et al., 2009; Ma et al., 2007). One hundred and twenty-five of those were found to be polymorphic between the parents and were subsequently used for genotyping in the progeny. All PCR amplifications were performed according to Mulkey et al. (2013). SSRs were separated on 25-cm 12% v/v denaturing polyacrylamide gels (PAGE) (Wang et al., 2003) using a LI-COR 4300 DNA Analyzer Sequencer as detailed in Kimball et al. (2013).

Library Construction and Sequencing

A sequencing library was prepared according to the procedure detailed in Poland et al. (2012). Approximately 200ng of genomic DNA was digested with *PstI* (New England BioLabs, Inc.; Ipswich, MA) and *MspI* (New England BioLabs, Inc.; Ipswich, MA). The 175 progeny lines and three replications of 'Meyer' and 'Victoria' parents were multiplexed with 184 barcoded adapters with *PstI* restriction site overhangs. Additionally, a common-Y adapter compatible with the *MspI* restriction site was used to prevent dimerization of fragments and adapters. The 184-plex library was submitted the Genomic Sciences Laboratory at North Carolina State University for quality assurance, size selection, and sequencing. The library was sequenced on one lane of MiSeq 150 single read (SR) (Illumina, Inc.; San Diego, CA).

Sequence Analysis and SNP calling

Bioinformatics analysis was performed using the SNP Calling Reference Optional Pipeline (SNP-CROP) workflow v2.0 (Melo et al., 2016) to process raw sequencing data. Raw sequence reads were parsed based on intact barcodes and restriction enzyme cut sites. Reads were then subjected to basic quality filtering to remove low-quality sequences. Specifically, reads were trimmed based on a sequence of three contiguous bases with an average Phred score

Q≤30. Trimmed reads shorter than 32 base pairs (bp) were culled. Remaining reads were de-multiplexed into different genotypes according to their unique barcodes. The *Z. japonica* 'Nagirizaki' reference genome (ZJN_r1.1_pseudomol) (Tanaka et al., 2016) was downloaded from the Zoysia Genome Database and used as the reference sequence for alignment of processed reads with Burrows-Wheeler Aligner (BWA) v.0.7.15 (Li and Durbin, 2009).

The SNP-CROP workflow calls only potential bi-allelic SNPs by imposing a population-level allele frequency filter via the Alternative Allele Strength parameter (altStrength =0.96). To filter SNPs and call genotypes, several read depth based parameters were used as follows: mnHoDepth0=4, mnHoDepth1=20, mnHetDepth=3, mnAlleleRati0=0.25, mnAvgDepth=4 and mxAvgDepth=200. Finally, SNPs with less than 50% genotype calls were discarded.

Marker Mapping

All SNP reads and SSR primer sequences were aligned to the *Z. japonica* 'Nagirizaki' reference genome to create a high-density physical map. For the creation of a linkage map, SSR marker data was scored according to segregation type (Jansen, 2005) as outlined in JoinMap v.4.0 (van Ooijen and Voorrips, 2001). Chi-square tests for segregation distortion were performed for each locus at a threshold of P=0.05. Markers meeting these Mendelian segregation ratio requirements were used to construct a framework map based on all SSR markers and SNP markers with sufficient parental data. Initial linkage groups were formed with JoinMap 4.0 using the "cross-pollinator" (CP) population type and a LOD threshold of 4.0. Mapping distances were estimated with the Haldane's regression mapping function. The two parents were mapped separately then merged into one map. This framework map was compared to the physical map to estimate genetic distances of all markers for QTL analysis.

For QTL analysis, marker types were called for SNPs with missing parental data by imputing parental genotypes based on progeny segregation ratios.

QTL Analysis

Composite interval mapping (CIM) for quantitative trait loci analysis for winter injury was conducted using JMP Genomics 7 (SAS Institute; Cary, NC). Genotypes and marker types were re-scored as OneMap (Margarido et al., 2007) outcrossing non-delimited genotypes for analysis with JMP Genomics. First, single marker analysis was performed for winter injury for the genotypic main effect in each environment to identify areas of interest. The outcrossing add-in for JMP Genomics, which is designed specifically for outcrossing and pseudo-F_2 mapping populations, was implemented to design a genetic probability data set which determines conditional probabilities of each genotype determined by the cross prior to CIM analysis. In CIM, Haley-Knott regression was used to consider the winter injury trait with a simple regression model method (used because of missing marker data), a test window size of 10 cM, and a walking speed of 5 cM. CIM was conducted for each environment individually and with combined means from all environments for comparison of genomic regions of interest across all environments. Putative QTL were identified by logarithm-of-odds (LOD) scores at a significance level of p=0.05.

RESULTS

Field Testing

In field evaluations, genotype, environment, and genotype within environment effects were found to be significant (p>.0001) for winter injury (Table 2.2). Although there was missing data due to lines lost in winter 2014-2015, all environments were able to be used for QTL analysis. Progeny means fell outside the parental means in some of the environments where

milder pressure allowed for more separation of genotypes (Table 3.3). Of the progeny, eight individuals had less average winter injury than Meyer over all six environments.

Genotyping and Map Construction

After screening 239 SSR primers against the Meyer and Victoria parents, 125 primers were found to be polymorphic for the mapping population. Thirteen of these primers were excluded from mapping because of skewed segregation ratios. The sequences of these 112 primer pairs were successfully aligned to the *Z. japonica* 'Nagirizaki' reference genome to create a physical map.

A total of 29,794,721 reads were obtained with the MiSeq sequencing platform. Of these, 25,780,312 were usable reads. Thirteen progeny were excluded for SNP calling because of low read coverage. Before filtering, 39,120 SNPs were identified with an average read depth of 3.6 counts. These SNPs were filtered and culled to 2,306 high-quality SNPs with average read depth of 9.1 counts and less than 50% missing data. All SNP reads were successfully aligned to the *Z. japonica* 'Nagirizaki' reference genome. The resulting physical map of 2,306 SNPs and 112 SSRs covered 323 Mega base pairs (Mbp) (Figure 2.1). SNPs were present on all 20 chromosomes of the zoysiagrass genome with an average of 121 markers per chromosome and an average distance of 140 kbp between markers (Table 2.4).

To convert the physical distances in this map into genetic distances for QTL mapping, the 112 SSRs were used to create a linkage map in JoinMap 4.0 (Stam, 1993). Because the physical location and grouping of markers on chromosomes was already known, linkage groups containing markers on different chromosomes or out of order were discarded. Thirteen linkage groups were created using 64 of the SSR markers. Nine of these linkage groups were successfully merged, leaving three Meyer linkage groups and one Victoria linkage group. By

comparing the genetic distances of these markers with their physical distances, a conversion factor was created and the genetic distances of all markers were estimated. This genetic map covered 1973.1 cM with an average distance of 0.86 cM between markers (Table 2.4).

QTL Identification

Putative QTL for winter injury were identified using JMP Genomics 7 with the outcross linkage mapping add-in. Of the 2,306 SNPs, 372 were excluded from QTL analysis because of insufficient parental genotype information. The remaining 1,934 SNPs and 112 SSR markers were used to identify QTL in each environment. CIM analysis was completed with LS means from each environment individually as well as the LS means of all environments combined. A total of one hundred seven QTL were identified at a LOD threshold of 2.5 in the six individual environments, and an additional 24 QTL were identified in combined environment analysis (Table 2.5). Of these 131 total QTL, twenty-four QTL had major effects (R^2 >10%) (Table 2.6). Both Meyer and Victoria were found to have contributed alleles to these major QTL. There were twenty-one genomic regions of interest where QTL were detected in two or more environments with up to seven associated markers. Nine of these regions of interest, located on chromosomes 5, 7, 12, 13, 17, and 18, had a maximum proportion of variation explained by the QTL ranging from 10.0% to 20.5% (Table 2.7; Figure 2.2). QTL were identified on all chromosomes, but on chromosomes 3, 4, 6, 9, 10, 15, 16, and 19 no QTL were identified that were detected across multiple environments.

Two regions of particular interest were identified on chromosomes 12 and 18 and included QTL in both single and multiple environment with major effects (Figure 2.2). On chromosome 12, single environment analysis identified ten QTL between 62.44 – 89.69 cM (10248.8 – 14722.6 kbp) in environments NCA15, NCA16, INA15, and INA16, and an

additional five QTL were identified in combined environment analysis in this region. Of these, four QTL had major effects with a proportion of variation ranging from 10.22 – 14.37% (Table 2.6). Additionally, ten of fifteen these QTL fell at three specific locations at either 62.4 cM, 67.5 cM, and 87.4 cM (Table 2.7). On chromosome 18 between 0.56 – 21.91 cM (92.6 – 3596.6 kbp), thirteen QTL were identified in single environment analysis in environments NCA15, INA15, and INA16, and an additional three QTL were identified in combined environment analysis. Three of these QTL had major effects with a proportion of variation ranging from 11.96 – 13.27% (Table 2.6), and ten of these QTL were located in multiple environment at 4.1 cM, 7.4 cM, 12.6 cM, and 15.4 cM (Table 2.7). Markers associated with these QTL and located within these two regions of interest on chromosomes 12 and 18 may be particularly useful in marker assisted selection for winter hardy zoysiagrass breeding in the future.

DISCUSSION

High-density genetic maps are an integral tool for the identification of QTL associated with traits of interest. In zoysiagrass, although several linkage maps containing several hundred markers each have been constructed (Cai et al., 2004; Cai et al., 2005; Li et al., 2009; Huang et al., 2016), limited progress has been made in QTL identification with only a few examples to cite (Jessup et al., 2011; Guo et al., 2014; Huang et al., 2016). With the introduction of next generation sequencing, marker development as well as map density has dramatically increased which should lead to improved QTL analysis. Huang et al. (2016) created a high-density linkage map of SNPs for *Z. matrella* and examined QTL for fall armyworm resistance as well as performed comparative genomic analysis with members of the

Chloridoideae subfamily. Similar in depth analyses could be performed with a high density map of *Z. japonica*. In the present study, the first SNP-based *Z. japonica* map was constructed covering 323 Mbp of the zoysiagrass 334 Mbp genome (Tanaka et al., 2016). This map has dense marker coverage with both SNP and SSR markers present on every chromosome with an average distance of 140 kbp or 1.23 cM between markers (Table 2.4). Additionally, the SSRs in this map allow for transferability of this map to other zoysiagrass populations (Kalia et al., 2011; Harris-Schultz et al., 2012). For this study, an Illumina MiSeq platform was chosen because of equipment availability and time constraints. However, an Illumina HiSeq run may drastically improve the number of reads, read depth, and the number of high-quality SNPs, allowing for more accurate SNP calling (Liu et al., 2012; Griffin et al., 2011). The addition of more markers to this map would extend its coverage and increase its effectiveness in future QTL identification (Miles and Wayne, 2008).

Software choice is integral in optimal SNP calling depending on the structure of the population and the amount of genetic information available for the crop of interest (Clevenger et al., 2015). The efficacy of SNP calling is dramatically increased with the use of a reference genomes, and in species without a reference genome, *de novo* assembly of a reference is often required for SNP calling (Zhang et al., 2011). Although the SNP-CROP pipeline (Melo et al., 2016) allows for SNP calling without a reference genome by creating a mock reference genome, SNP calls in this study were increased from less than five hundred to 2,306 with the release and introduction of the *Z. japonica* 'Nagirizaki' reference genome (Tanaka et al., 2016). Because the SNP-CROP pipeline is specially designed for small population sizes, it has been reported to call as many as three times the SNPs as the TASSEL-GBS and the TASSEL-UNEAK pipelines (Melo et al., 2016). Upon the examination of the TASSEL-GBS v2 pipeline

with MiSeq reads in this study, 1,679 SNPs were called at an average read depth of 7.28 counts compared to 2,306 SNPs with an average read depth of 9.1 counts with the SNP-CROP pipeline. As previously documented by Clevenger et al (2011), these results indicate that selecting software and pipelines well-suited for the ploidy of the species, availability of genetic information for the species, and abundance of sequence reads is crucial for effective SNP calling.

For QTL mapping of winter hardiness and freeze tolerance traits, the evaluation of population over multiple locations and years is crucial to the identification of putative QTL. In zoysiagrass, many environmental factors such as cold acclimation (Patton and Reicher, 2007), low temperature tolerance (Dunn et al., 1999), and freeze tolerance (Patton and Reicher 2007, Hinton et al, 2012) influence winter hardiness but vary year to year and at different locations. Additionally, winter pressure extends beyond low temperatures and also varies based on the intensity, duration, and frequency of freeze events as well as snowfall and snow cover (Lewitt 1980; Blum 1988). This variability makes multi-environment analysis crucial. QTL for winter survival have been identified in other turfgrasses and similar crops like perennial ryegrass (*Lolium perenne* L.) (Yamada et al., 2004), barley (*Hordeum vulgare* L.) (Francia et al., 2003), lentil (*Lens cularis* Medik.) (Kahraman et al., 2002) and alfalfa (*Medicago sativa* L.) (Brouwer et al., 2000), but in these cases, QTL were only identified within single environments. In this study, one hundred seven QTL associated with winter injury were identified across six different environments. QTL were frequently identified in clusters with up to eight closely located markers significantly associated with winter injury in one or more environments. Although many of these QTL were identified in a single environment, nine QTL with major effects (R^2 values > 10%), including three on chromosome

12 and two on chromosome 18, were identified across multiple environments and are more likely to be genomic regions of interest contributing to winter hardiness in zoysiagrass (Boer et al., 2007; Verbyla et al., 2003). With further validation, these QTL may offer zoysiagrass breeders target regions for selection of more freeze tolerant germplasm.

Many QTL and genetic areas of interest associated with winter injury in zoysiagrass were identified in this study and could be used in the development of marker assisted selection strategies for the breeding of more winter hardy zoysiagrass cultivars. However, the complexity of the freeze tolerance trait requires further investigation of additional factors that may influence zoysiagrass's freeze response. There are numerous differences in *Z. japonica* cultivars for traits like recovery rates (Karcher et al., 2005), shade tolerance (Sladek et al., 2009), and establishment rates (Patton and Reicher, 2005). The colocation of putative QTL for these traits as well as traits like texture, color, and density with winter injury QTL identified in this study may help identify genomic regions controlling important morphological characteristics that influence freeze tolerance. Moreover, the metabolic processes influencing freeze tolerance in zoysiagrass are not fully understood, although studies in zoysiagrass (Patton et al., 2007a,b; Zhang et al., 2009), velvet bentgrass (*Agrostis canina* L.) (Espevig et al., 2012), and perennial ryegrass (Hoffman et al., 2010) have identified physiological changes that occur during cold acclimation and freezing. In this study, both Meyer and Victoria parents contributed alleles to markers associated with significant QTL for winter injury. Victoria is highly freeze-susceptible, and yet it contributed alleles that were influential in the winter hardiness of these progenies. It is common in hybrid populations for complex traits to exhibit transgressive segregation where the progeny phenotype is expressed at an extreme level compared to the parents (Tanksley, 1993). This type of complementary allele action in complex

traits has been observed for numerous traits including fruit shape in tomato (*Lycopersicon esculentum* Mill.) (deVincente and Tanksley, 1993), tiller angle and heading date in rice (*Oryza sativa* L.) (Xu et al., 1998; Yano et al., 1997), and palmitic acid and oleate content in soybean (*Glycine max* (L.) Merr.) (Wilcox et al., 1994; Alt et al., 2005). Further analysis of QTL associated with freeze tolerance components such as metabolic changes that occur during cold acclimation and freezing, and morphological characteristics that influence freeze tolerance, may help elucidate this complex trait and advance the ability to select for it.

CONCLUSIONS

Molecular breeding techniques could be instrumental in understanding zoysiagrass winter hardiness and producing new winter hardy zoysiagrass cultivars. In this study, the first SNP based high density *Z. japonica* map was used to identify one hundred seven putative QTL for winter injury in the six individual environments and twenty-one QTL for winter injury in multiple environments, nine of which had major effects (R^2 > 10%). Genomic regions of interest, particularly in the 62.44 - 89.69 cM region of chromosome 12 and the 0.56 - 21.91 cM region of chromosome 18 were identified and may be useful for marker assisted selection for winter hardiness in zoysiagrass. Once validated, these QTL and their associated markers can be used to incorporate winter hardiness into elite germplasm for the development of new zoysiagrass cultivars.

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Table 2.1 Environments for Meyer × Victoria mapping population evaluation from 2014-2016. After losses in the field in winter 2014-2015, an additional copy of the population was planted at both Laurel Spring, NC and West Lafayette, IN locations. Because of variability in winters and lines evaluated, each location × year combination was analyzed as an independent environment.

Environment Name	Environment Abbreviation	Location	Evaluation Winter	Planting Date	Lines Evaluated
NC, A, 2015	NCA15	Laurel Springs, NC	2014-2015	06/04/14	175
NC, A, 2016	NCA16	Laurel Springs, NC	2015-2016	06/04/14	147
NC, B, 2016	NCB16	Laurel Springs, NC	2015-2016	05/20/15	175
IN, A, 2015	INA15	West Lafayette, IN	2014-2015	6/26/2014	175
IN, A, 2015	INA16	West Lafayette, IN	2015-2016	6/26/2014	125
IN, B, 2016	INB16	West Lafayette, IN	2015-2016	06/04/2015	175

Table 2.2 Type 3 analysis of variance for winter injury for the 175 progeny in the Meyer × Victoria mapping population in six environments in Laurel Springs, NC and West Lafayette, IN from 2014 – 2016.

Source	DF	F-Value	Pr > F
Genotype	174	36.38	<0.0001
Environment	5	18.51	<0.0001
Genotype(Environment)	791	333.17	<0.0001
Rep(Environment)	12	7.75	<0.0001
Genotype*Rep(Environment)	1753	3.19	0.2688
Fall Cover	1	0.44	0.5764

Table 2.3 LS Means for parents, Meyer and Victoria, and progeny minimum, maximum, and average for percent winter injury in each environment as well as overall environments evaluated in the field from 2014-2016.

	NC, A, 2015	NC, A, 2016	NC, B, 2016	IN, A, 2015	IN, A, 2016	NC, B, 2016	Overall
Meyer	0.0	0.0	77.6	0.0	33.9	13.5	22.6
Victoria	100.0	--	72.2	100.0	--	90.1	95.4
Progeny Min	0.0	0.0	0.0	0.0	1.2	16.1	11.7
Progeny Max	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Progeny Average	57.3	60.3	51.5	78.2	48.8	52.0	64.8

Table 2.4 SNP and SSR marker distribution and coverage over all 20 zoysiagrass chromosomes, totaling 2,418 markers, 323 Mbp and 1973 cM.

Chromosome	Total Markers	Coverage (kbp)	Coverage (cM)	Markers per cM
1	160	18537.7	112.9	1.41
2	159	17411.3	106.1	1.50
3	83	7761.3	47.3	1.75
4	103	11036.2	67.2	1.53
5	223	19760.9	120.4	1.85
6	186	19286.2	117.5	1.58
7	189	76021.3	463.1	0.41
8	178	21053.2	128.3	1.39
9	57	8104.8	49.4	1.15
10	39	7951.7	48.4	0.81
11	119	10271.0	62.6	1.90
12	145	14676.8	89.4	1.62
13	98	11642.8	70.9	1.38
14	57	8693.7	53.0	1.08
15	56	7263.9	44.3	1.26
16	56	7879.6	48.0	1.17
17	82	10301.8	62.8	1.31
18	84	11016.9	67.1	1.25
19	200	19123.2	116.5	1.72
20	144	16076.9	97.9	1.47

Table 2.5 Distribution of identified QTL for zoysiagrass winter injury over six environments and combined environments. All QTL were detected at a LOD threshold of 2.5.

Chromosome	NCA15	NCA16	NCB16	INA15	INA16	INB16	Combined
1	-	2	-	2	-	-	1
2	-	2	2	1	2	1	-
3	-	-	-	-	1	-	-
4	1	-	-	-	-	1	-
5	2	1	2	3	-	1	2
6	1	1	1	2	1	-	-
7	4	3	4	1	-	-	3
8	-	1	-	2	1	-	2
9	1	1	-	-	-	-	-
10	1	-	-	-	-	1	-
11	-	-	-	3	2	-	-
12	1	3	-	4	2	-	5
13	-	3	-	-	4	1	2
14	1	-	-	-	2	-	2
15	-	-	-	-	2	-	1
16	-	1	-	-	-	-	-
17	1	1	2	-	-	-	-
18	3	-	-	5	4	-	4
19	1	-	-	2	2	2	1
20	2	1	-	-	2	-	1
Total QTL	**19**	**20**	**11**	**25**	**25**	**7**	**24**
Max% Var Explained	**20.5**	**13.2**	**8.9**	**9.1**	**14.4**	**8.7**	**10.0**
Associated Marker	**Ssr_17_2**	**Snp_05_104**	**Snp_17_34**	**Snp_12_133**	**Snp_12_90**	**Snp_05_79**	**Snp_07_64**
Chromosome	**17**	**05**	**17**	**12**	**12**	**05**	**07**

Table 2.6 Identified QTL for zoysiagrass winter injury with major effects (R^2 > 10%) in all six environments and combined environments.

Chromosome	Location (cM)	Flanking Marker(s)	Max % of variance explained				
			LOD	R^2 (%)	Meyer Add Eff	Victoria Add Eff	Envir
2	61.10-70.59	SSR_02_12, SNP_02_76	3.68	12.68	9.96	36.93	INA16
2	71.52	SNP_02_79	2.87	10.04	12.83	25.20	INA16
3	25.95-26.99	SSR_03_1, SNP_03_25	3.11	10.83	20.46	-6.87	INA16
5	74.02-76.29	SNP_05_104, SNP_05_109	4.48	13.19	-1.24	-203.06	NCA16
7	47.79	SNP_07_62, SNP_07_65	4.03	10.06	10.62	-5.32	Combined
9	39.04	SNP_09_36	3.68	10.94	-0.222	-287.83	NCA16
11	3.32-7.39	SNP_11_3, SNP_11_9	2.95	10.31	-9.00	-26.36	INA16
11	16.63	SNP_11_25, SNP_11_26	3.08	10.74	11.54	2.59	INA16
12	62.44-62.61	SNP_12_61, SSR_12_2	5.14	12.66	9.13	-9.03	NCA15
12	67.5	SNP_12_89, SNP_12_90	4.21	14.37	-14.11	-2.67	INA16
12	87.42-87.43	SSR_12_3, SNP_12_133	3.42	10.22	0.64	-11.79	NCA16
12	87.42-87.78	SNP_12_133 SSR_12_4	4.07	13.91	-8.35	-8.90	INA16
13	8.27-13.53	SNP_13_12, SNP_13_15	3.48	12.03	-3.21	53.23	INA16
13	62.95-64.74	SNP_13_71, SNP_13_75	3.61	10.78	17.04	13.08	NCA16
13	64.87	SSR_13_8	2.86	10.00	99.10	-174.06	INA16
15	5.57	SNP_15_22	3.02	10.53	1.55	-7209.00	INA16
16	19.23-24.00	SNP_16_17, SNP_16_19	3.35	10.01	14.37	-7.88	NCA16
17	2.21-5.68	SNP_17_1, SNP_17_3	3.54	10.56	-6.97	-33.81	NCA16
17	39.96-41.44	SNP_17_26 SSR_17_2	8.70	20.47	63.31	-982.59	NCA15
18	0.56-3.18	SNP_18_2, SNP_18_9	3.86	13.27	8.02	-3.04	INA16
18	7.42-10.15	SNP_18_23 SNP_18_25	3.61	12.45	-0.086	-18290.00	INA16
18	11.98-13.18	SNP_18_37, SNP_18_41	3.46	11.96	-14.22	18.98	INA16
19	82.33-82.86	SNP_19_119, SNP_19_121	2.88	10.07	2.31	18.86	INA16
20	70.29-71.63	SNP_20_101, SNP_20_102	3.23	11.21	-5.93	9799.33	INA16

Table 2.7 QTL for zoysiagrass winter injury detected in two or more environments. QTL with major effects ($R^2 \geq 10\%$) are highlighted in bold.

Chromosome	Location (cM)	Location (kbp)	Environments	Associated Markers	R^2 (%)	Parental Allele	LOD threshold
1	111.9	18362.0	INA15 Combined	Snp_01_153, Snp_01_154	8.4	Victoria	2.5
2	98.6	16185.9	NCB16 INB16	Snp_02_136	8.6	Victoria	2.5
5	54.9	8979.8	NCA15 INA15 INB16 Combined	Snp_05_76, Snp_05_77, Snp_05_78, Snp_05_79, Snp_05_81, Snp_05_82, Snp_05_83	8.8	Meyer	2.5
5	**76.3**	**12521.8**	**NCA15 NCA16**	**Snp_05_104, Snp_05_105, Snp_05_106, Snp_05_107 Snp_05_108, Snp_05_109**	**13.2**	**Victoria**	**2.5**
7	**47.8**	**7842.9**	**NCA16 Combined**	**Snp_07_62, Snp_07_64, Snp_07_65**	**10.1**	**Victoria**	**2.5**
7	73.9	12143.3	NCA15 NCB16	Snp_07_96, Snp_07_97	7.3	Meyer	2.5
8	14.5	2376.9	INA15 Combined	Snp_08_17	8.7	Victoria	2.5
11	3.3	545.1	INA15 INA16	Snp_11_3	9.7	Victoria	2.5
12	**62.4**	**10248.8**	**NCA15 INA15 Combined**	**Snp_12_61, Ssr_12_2**	**12.7**	**Meyer**	**2.5**
12	**67.5**	**11079.1**	**INA15 INA16 Combined**	**Snp_12_89, Snp_12_90**	**14.3**	**Victoria**	**2.5**
12	**87.4**	**14349.7**	**NCA16 INA15 INA16 Combined**	**Ssr_12_3, Snp_12_131, Snp_12_132, Snp_12_133**	**13.9**	**Meyer**	**2.5**

Table 2.7 cont'd QTL for zoysiagrass winter injury detected in two or more environments. QTL with major effects ($R^2 \geq 10\%$) are highlighted in bold.

Chromosome	Location (cM)	Location (kbp)	Environments	Associated Markers	R^2 (%)	Parental Allele	LOD threshold
13	**12.6**	**2073.3**	**INA16 Combined**	**SNP_13_14, SNP_13_15**	**12.0**	**Victoria**	**2.5**
13	68.8	11290.8	INA16 Combined	Snp_13_87	7.9	Victoria	2.5
14	5.51	904.6	INA16 Combined	SNP_14_7	8.0	Victoria	2.5
14	42.1	6912.9	NCA15 Combined	Ssr_14_7	7.5	Meyer	2.5
17	**41.4**	**6801.9**	**NCA15 NCB16**	**Ssr_17_2**	**20.5**	**Meyer**	**2.5**
18	4.1	680.7	NCA15 INA15 Combined	Snp_18_18	8.5	Victoria	2.5
18	**7.4**	**1217.3**	**INA15 INA16 Combined**	**Snp_18_23, Snp_18_25**	**12.5**	**Victoria**	**2.5**
18	**12.6**	**1965.9**	**INA15 INA16**	**Snp_18_37, Snp_18_39, Snp_18_40**	**12.0**	**Meyer**	**2.5**
18	15.4	2531.2	INA15 Combined	Snp_18_42, Snp_18_44	7.4	Victoria	2.5
20	16.4	2695.3	NCA15 INA15 Combined	Snp_20_27, Snp_20_28	9.4	Victoria	2.5

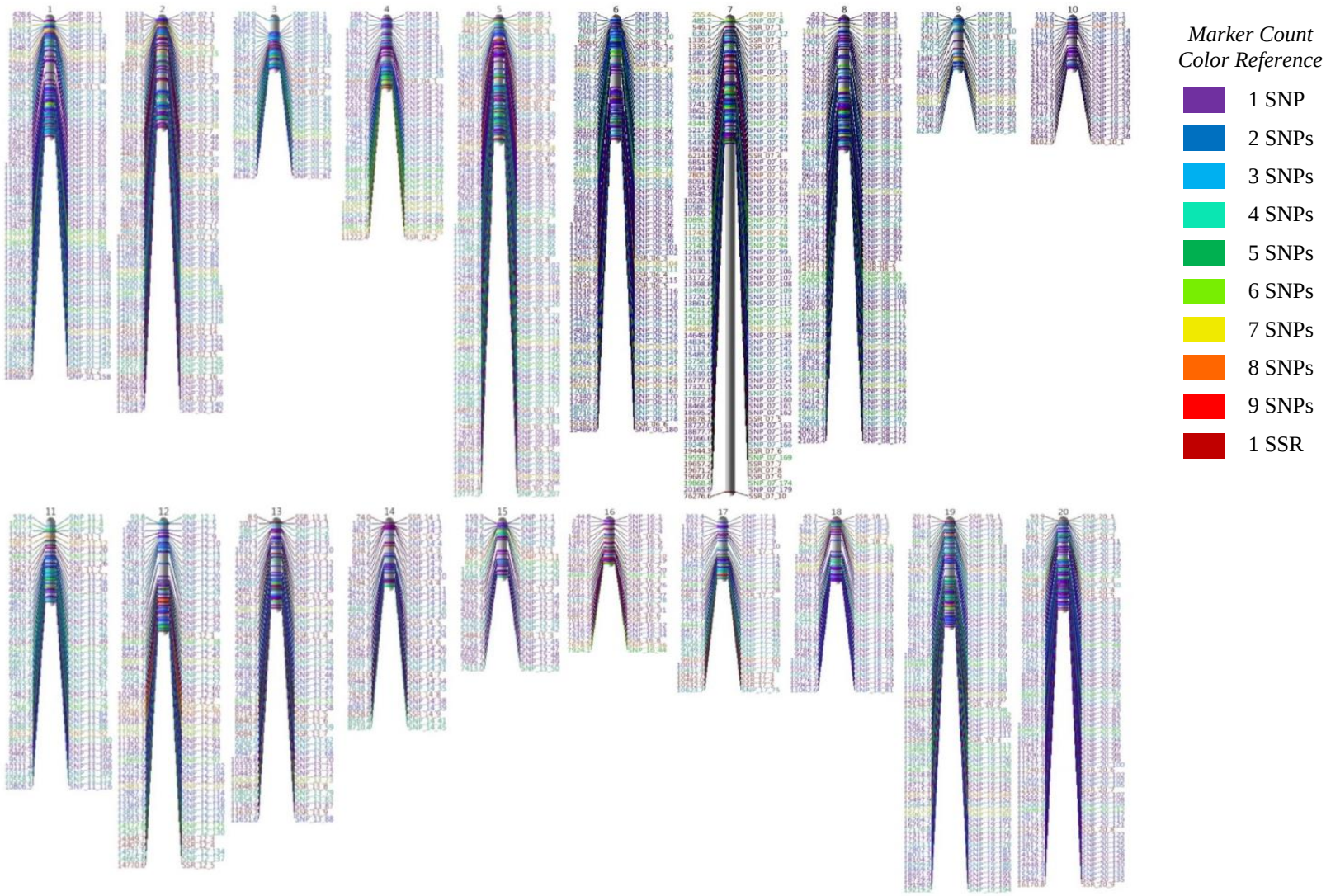

Figure 2.1 Physical map of 2,306 SNP markers and 112 SSR markers covering 323 Mbp over the 20 chromosomes of the zoysiagrass genome. Positions(kbp) with multiple markers are condensed to single position (color reference) for better visualization. This condensed map contains 1,024 markers.

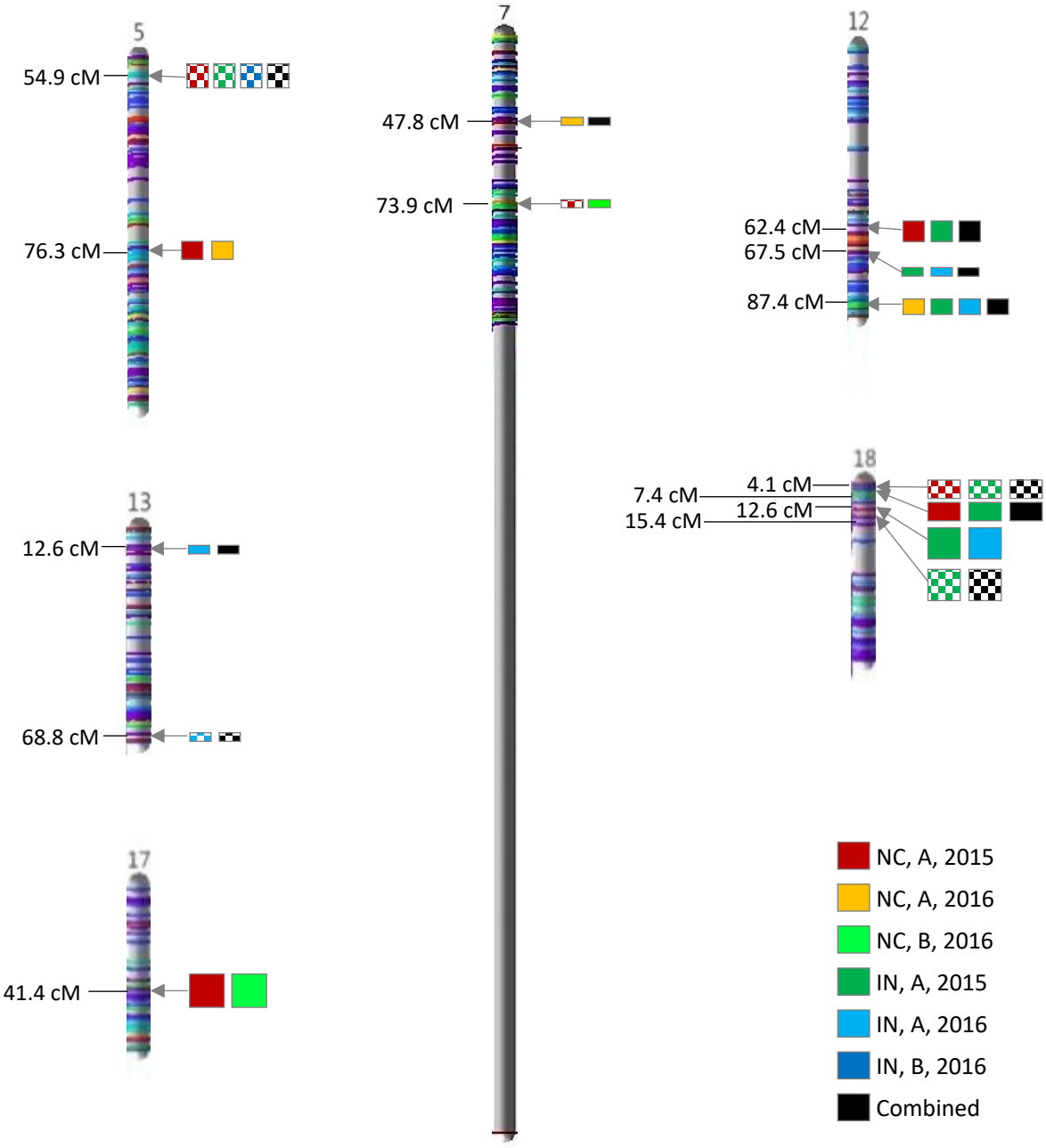

Figure 2.2 Nine QTL for zoysiagrass winter injury with major effects (R^2 ≥10) were identified across multiple environments on five different chromosomes. Solid filled blocks indicate QTL R^2 values ≥10%, and patterned fill blocks indicate QTL with R^2 values <10%. Size of blocks correspond with the physical distance the QTL covers on the chromosome. Markers associated with each QTL can be found in Table 2.7

CHAPTER III

CONTROLLED ENVIRONMENT FREEZE EVALUATIONS OF ZOYSIAGRASS (*ZOYSIA SPP.)* CULTIVARS

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Controlled Environment Freeze Evaluations of Zoysiagrass (*Zoysia spp.*) Cultivars

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List of abbreviations: LT_{50}, lethal temperature

ABSTRACT

Zoysiagrasses (*Zoysia* spp. Willd.) are warm season perennial turfgrasses primarily grown in the warm-humid and transitional climatic zones of the United States. They are geographically limited in their use because of a relative lack of winter hardiness. Winter hardiness is variable among zoysiagrass genotypes and cold acclimation significantly influences winter injury of zoysiagrasses. The physiological causes behind this variability are unknown. To investigate the relationship between cold acclimation and freeze tolerance in zoysiagrasses, selected cultivars with a reported range of freeze susceptibility, 'Meyer', 'JaMur', and 'Victoria', were chosen. Non-acclimated and cold acclimated plants were evaluated in four controlled freezing chambers reaching -8, -9, -10, and -11ºC. Results indicated that cold acclimation has a significant influence on the freeze tolerance of these cultivars. The interaction between cultivar and acclimation treatment was highly significant ($p=0.0037$). While cultivar was significant among cold acclimated plants ($p=0.0300$), it was not a significant effect among non-acclimated plants ($p>0.05$). Lethal temperatures (LT_{50}) calculated for all cultivar by acclimation treatment combinations using logistical regression modeling ranged from -8.47ºC for non-acclimated JaMur to -11.56ºC for cold acclimated Meyer. Overall, these results indicate that cold acclimated *Z. japonica* plants are able to better tolerate freezing stress, but in the transition zone, where late spring freezes frequently kill non-acclimated or deacclimated turfgrasses, *Z. japonica* varieties are particularly well-suited for freeze survival, even without acclimation.

KEYWORDS: Cold acclimation, freeze tolerance, LT_{50}, zoysiagrass

INTRODUCTION

Zoysiagrasses (*Zoysia* spp.) are warm season perennial turfgrasses primarily grown in the warm-humid and transitional climatic zones of the United States. They are geographically constrained to these regions because of their limited freeze tolerance and winter hardiness. Zoysiagrasses are popular for their low maintenance requirements as well as their slow growth habit, high shoot density, and tolerance to many abiotic stresses such as drought and shade. These attributes make zoysiagrass especially well-suited for home lawns and golf courses and creates a demand for more freeze tolerant cultivars that can be used in the colder climates of the Northern United States.

Varying levels of winter injury have been observed among zoysiagrass genotypes. The two most popular species of zoysiagrass in the U.S., *Zoysia japonica* Steud. and *Zoysia matrella* (L.) Merr., have significantly different levels of winter injury and spring green-up as first reported by Forbes and Ferguson (1947). In field and controlled environment freeze chamber studies, *Z. japonica* genotypes show better freeze tolerance than *Z. matrella* genotypes on average (Dunn et al., 1999; Patton and Reicher, 2007; Hinton et al., 2012). Patton and Reicher (2007) reported LT_{50}s of six *Z. japonica* cultivars and two experimental lines after cold acclimation and freezing in controlled environment chambers. Similarly, Hinton et al. (2012) used controlled environment freeze tests to estimate LT_{50}s of four *Z. japonica* cultivars collected from the field in winter (cold acclimated) and spring (deacclimated/non-acclimated). Reported LT_{50}s for *Z. japonica* commercial cultivars range from -9.5ºC to -11.5ºC for 'Victoria' and 'Meyer', respectively (Patton and Reicher, 2007). Additionally, LT_{50}s have been found to differ depending on the propagation methods of tested material and freeze testing protocol (Dunn, 1999; Patton and Reicher, 2007; Hinton, 2012). The physiological basis for

the range in winter hardiness observed in zoysiagrass freezing tests and the role of cold acclimation in freeze survivability is not fully understood (Patton et al. 2007a,b).

Cold acclimation refers to the natural physiological process that takes place in a plant when it is exposed to low but not freezing temperatures. Studies in zoysiagrass (Patton and Reicher, 2007; Hinton et al., 2012), as well as saltgrass (*Distichlis spicata* (L.) Greene) (Shahba et al., 2003), buffalograss (*Bouteloua dactyloides* (Nutt.) Engelm.) (Qian et al., 2001), bermudagrass (*Cynodon dactylon* (L.) Pers. × *C. transvaalensis* Burt-Davy) (Anderson et al., 1993; Anderson et al., 2003) and St. Augustinegrass (*Stenotaphrum secundatum* (Walt.) Kuntze) (Kimball et al. 2016) have indicated that warm-season grasses respond well to cold acclimation and suffer less injury from freezing than non-acclimated plants. Hinton et al., (2012) found that across nine different zoysiagrass cultivars with varying LT_{50}s, samples collected in the winter after cold acclimation were consistently more freeze tolerant in controlled environment freezing tests than non-acclimated samples collected in the spring.

Although field evaluation of cold tolerance provides the most realistic indication of how a cultivar will perform through a winter season, variable environmental conditions can make consistent and reproducible winter stress very difficult to attain (Anderson and Taliaferro, 2002). Controlled environment freeze tests may be a more cost-effective and efficient way to assess cold acclimation and freeze tolerance of turfgrass species and has generally corresponded well with field screenings (Anderson and Taliaferro, 2002; Qian et al., 2001; Dunne et al., 2016). This consistency demonstrates that plants most likely undergo similar physiological changes during cold acclimation, freezing, and deacclimation in temperature controlled chambers as in the field. In zoysiagrass, freezing chambers have been

successfully used to evaluate the low temperature tolerance of a variety of genotypes (Dunn et al., 1999; Patton and Reicher, 2007; Hinton et al., 2012).

The objectives of this study were (i) estimate LT_{50}s of three zoysiagrass cultivars, 'Meyer', 'JaMur', and 'Victoria' using controlled environment freezing tests, and (ii) compare the effects of cold acclimation treatments on freeze survival in these three cultivars.

MATERIALS AND METHODS

Plant materials and growth conditions

Commercial cultivars 'Meyer', 'JaMur', and 'Victoria' were selected for evaluation because of their reported range in freeze susceptibility (Table 3.1) (Patton and Reicher, 2007; Hinton et al., 2012). In the fall of 2015, cultivars were collected from research plots at the Lake Wheeler Turfgrass Field Laboratory (Raleigh, NC) and established in 24.5 cm x 50.8 cm plastic trays (Hummert International, Earth City, MO) in Fafard P3 potting mix (Conrad Fafard Inc., Agawam, MA) in the greenhouse. In spring 2016, plants were vegetatively propagated in 2.5-cm-diameter, 12-cm-deep Ray Leach cone-tainers (Stuewe & Sons, Inc., Corvallis, OR) in USGA grade sand using a single stolon or rhizome containing root, crown, and shoot material according to Patton and Reicher (2007). Plants were allowed to establish for at least six weeks in the greenhouse at 27±2°C.

Cold acclimation and freezing treatments

After establishment in the greenhouse, plants of each genotype were randomly arranged in a growth chamber and cold acclimated for four weeks. The growth chamber used a consistent light emitting diode (LED) and temperatures of 8/2°C day/night cycles with a 10-h photoperiod of 300 µmol $m^{-2}s^{-1}$ photosynthetically active radiation (Anderson et al.,

1993). Non-acclimated plants of each genotype remained in the greenhouse at 27°C during this four-week period. Immediately before placement in the freezer, non-acclimated plants were randomized with the cold acclimated plants.

Based on the capacity of the freeze chambers, plants were arranged in each rack in a randomized complete block design with two replications. Each experimental unit consisted of ten cone-tainers for each of the six genotype by acclimation treatment combinations. Based on reported LT_{50}s (Patton and Reicher, 2007; Hinton et al., 2012) and a pilot study in the spring of 2015, temperatures -8°C, -9°C, -10°C, and -11°C were selected for this study. Each rack was placed in a plastic bag to minimize desiccation during freezing (Kimball et al., 2016) and placed into modified commercial freezers (Price's Scientific Services, Inc., Durham, NC). In the freezers, plants were initially held at -3°C for 15 hours to remove latent heat from the soil. The temperature was then decreased at a rate of -1°C hr^{-1} until reaching the target temperature, which was maintained for 3 hours. Then, the temperature was increased at a rate of 2°C hr^{-1} to 3°C. All trays were subsequently removed from plastic bags and placed in a walk-in growth chamber at 3°C for 24 hours to thaw. Finally, plants were allowed to come to room temperature (22°C) overnight before being returned to the greenhouse. This experiment was repeated for a total of three runs. The second run of freeze tests was discarded because of a malfunction in the commercial freezers so only data from runs one and three were used in the analysis.

Plants were evaluated weekly for survival for four weeks after freezing. Survival data was taken on a binary scale of 0 (death) to 1 (survival) evaluating each individual plant for the presence of green tissue (Dunne et al., 2016).

Data Analysis

Survival data collected four weeks after freezing was evaluated with logistic regression analysis. This method uses binary data to determine the probability of survival against the temperature gradient and estimate LT_{50}s according to Dunne et al. (2016). The LOGISTIC procedure (param=ref) in SAS version 9.4 (SAS Institute; Cary, NC) with a stepwise selection method was used to generate several models to evaluate the relationships between cultivar, acclimation treatments, freezing temperatures and survival. The LT_{50} calculations were based on the logistic regression model where β_0 = the $\{Pi = (1+e)-(\beta_0 + \beta_1 X_1 + ...+ \beta_k X_{ki})\}$ intercept, β_1 = the estimate for the occurrence of the X_1 parameter for all estimates and parameters $\beta_k X_{ki}$ based on the effects reference and selection criteria (Dunne et al., 2016). These models generated maximum likelihood estimates that were used to calculate odds ratios, survival probabilities, and LT_{50}s.

In the first logistic regression model evaluated each cultivar by acclimation treatment combination, the stepwise selection method excluded the interaction of cultivar by temperature, acclimation treatment by temperature, and cultivar by temperature by acclimation treatment from the model (Table 3.2). Non-acclimated Victoria was used as a reference variable for the generation of the logistic regression model of cultivar by acclimation treatment because it had the lowest reported LT_{50}. Another model was generated to evaluate acclimation treatments while ignoring the effects of cultivars. In this model, the stepwise selection method dropped the temperature by acclimation treatment effect as well. In this model, the non-acclimated treatment was considered a reference variable (Table 3.3). To further examine the role of cultivar, logistic regression models were generated for each acclimation separately. In

both of these models, Victoria was the reference variable. The no selection method was used for these models in order to examine all possible effects (Table 3.4, Table 3.5). In all four of these models, temperature was treated as a continuous variable to account for variability in the commercial freezers, and treatment and cultivar were independent variables used to estimate LT_{50}s. Maximum likelihood estimates in these models were used to calculate odds ratios and probabilities of survival curve according to the formulas:

Log_Odds = Intercept Estimate + Survival Estimate +

(Temperature Estimate * x Temperature),

Odds = exp(Log_Odds),

Survival Probability = Odds/(1+Odds).

According to these formulas, LT_{50} occurs at 50% survival probability and LT_{50} =

(0 – Intercept Estimate – Survival Estimate)/Temperature Estimate.

RESULTS

Treatment, temperature and the interaction between them had significant type III effects based on the Wald x^2 in the logistic regression model (Tables 3.2, 3.3). In the logistic regression model that evaluated each cultivar by acclimation treatment combination, cultivar was not significant ($p = 0.3959$) (Table 3.2). In the second model were cultivar was excluded, acclimation treatment and temperature were both significant ($p<0.0001$). In the two models where acclimation treatments were evaluated individually, cultivar was significant among cold acclimated plants ($p=0.0300$) (Table 3.4), but not significant ($p>0.05$) among non-acclimated plants (Table 3.5).

Maximum likelihood estimates generated from the logistic models were used to estimate the survivability of each cultivar, acclimation treatment, or cultivar by acclimation treatment combination in relation to the reference variable. In the model ignoring cultivars and modeling acclimation treatments alone, cold acclimated plants were 2.3 times more likely to survive than non-acclimated plants (Table 3.6) and the cold acclimation treatment lowered the 50% probability of survival of plants overall by 1.35ºC (Figure 3.1). The maximum likelihood estimates in the cultivar by acclimation treatment model were used to calculate LT_{50} values for each cultivar by acclimation treatment combination (Table 3.7, Figure 3.2). Cold acclimated Meyer had the lowest LT50 at -11.56°C, and non-acclimated JaMur had the highest LT_{50} at -8.5°C (Table 3.8). Cold acclimated Meyer and cold acclimated JaMur were significantly different from cold acclimated Victoria, but none of the non-acclimated cultivars were significantly different from each other (Table 3.8). Because non-acclimated Victoria was used as a reference variable, all estimates show differences in freezing tolerance in relation to non-acclimated Victoria. Fisher's LSD showed cultivar within acclimation treatments predicted means to be significantly different ($p < 0.05$).

DISCUSSION

In this study, acclimation treatment had a significant effect on survival of zoysiagrass plants after freezing. Plants that were cold acclimated were 2.3 times more likely to survive a freeze event than non-acclimated plants. Similar studies in zoysiagrass (Hinton et al., 2012), St. Augustinegrass (Kimball et al., 2016), bermudagrass (Anderson et al., 2003) buffalograss (Qian et al., 2001) and saltgrass (Shahba et al., 2003) have also reported that plants demonstrated better freeze tolerance when subjected to cold acclimation. This difference in

survivability between acclimated and non-acclimated plants indicates that there are physiological changes taking place in the plant during cold acclimation that influence freeze tolerance. Patton et al., (2007) observed that specific carbohydrates, proline, and certain proteins in cold acclimated zoysaigrass were correlated with freeze tolerance in thirteen genotypes. A proteomic response to cold acclimation was also observed in velvet bentgrass (*Agrotosis canina*) (Espevig et al., 2012) where cold acclimated plants were more freeze tolerant and showed thirteen different protein spots that were differentially expressed in acclimated versus non-acclimated plants. In this study, the cold acclimation treatment altered Meyer, JaMur, and Victoria's LT_{50}s by -2.66ºC, -2.98ºC, and -1.50ºC, respectively. Further analysis of metabolic processes that occur during cold acclimation and how they differ between these cultivars, particularly Meyer and Victoria, may explain why there is a divergence in freeze tolerance among *Z. japonica* genotypes.

The cultivar by acclimation treatment interaction was significant in this study ($p=0.0037$), and the estimated LT_{50}s of cultivars were significantly different ($p<0.05$) depending on the acclimation treatment they were subjected to. For cold acclimated plants, the cultivar rankings expected based on reported LT_{50}s (Patton and Reicher 2007; Hinton et al., 2012) were upheld. Within both acclimation treatments, Meyer was the most freeze tolerant cultivar with LT_{50}s of -11.56ºC and -8.89ºC for cold acclimated and non-acclimated treatments, respectively. Meyer's superior freeze tolerance has been documented in both field tests and controlled environment freeze tests (Forbes and Ferguson 1947; Dunn et al., 1999; Patton and Riecher 2007; Hinton et al., 2012), and this study confirms its low temperature survivability. Patton and Reicher (2007) reported cold-acclimated Meyer's LT_{50} as -11.5±0.8ºC. With similar cold acclimation procedures and freezing temperatures in this study,

Meyer's LT_{50} is comparable to the LT_{50} reported by Patton and Riecher (2007) at -11.56ºC. Cold acclimated JaMur's LT_{50} value was lower than previously reported by Hinton et al., (2012). This discrepancy may have been a result of JaMur's hearty growth habit in the cone-tainers in the greenhouse before acclimation and freezing treatments giving the better-established plants an advantage in the freeze tests. Likewise, cold-acclimated Victoria had an LT_{50} 1.0ºC lower than previously reported in Patton and Reicher (2007). The reported LT_{50}s for these cold acclimated cultivars are commensurate with previously documented lethal temperatures, and the freeze tolerance rankings were upheld. Within the non-acclimated plants, there were no significant differences between cultivars. The LT_{50} rankings of cultivars were not maintained, and non-acclimated Victoria was similar to other cultivars. This uniformity in freeze tolerance of non-acclimated cultivars suggests that the physiological changes that occur during cold acclimation may be the separating factor between freeze tolerances of zoysiagrass cultivars. These physiological changes could be further investigated by testing levels of specific cell components such as proteins and lipids before and after acclimation treatments in different cultivar. However, even in non-acclimated plants, estimated LT_{50}s are lower than reported LT_{50}s of other warm-season grasses like bermudagrass and St. Augustinegrass (Anderson et al., 2002; Kimball et al., 2016), making zoysiagrass an optimal turfgrass choice for the variable winters of the transition zone.

CONCLUSIONS

In this evaluation of acclimation treatments, cold acclimation had a positive effect on *Zoysia japonica* cultivars' freeze tolerance, indicating that cold acclimation events are integral to winter survivability. LT_{50}s calculated with logical regression modeling for the six

cultivar by acclimation treatment combinations ranged from -11.56°C to -10.30°C for cold acclimated plans and from -8.89°C to -8.47°C for non-acclimated plants. When these acclimation treatments were evaluated individually, cultivar was only significant among cold acclimated plants ($p=0.0300$), suggesting there is a difference in freeze tolerance between cultivars only if plants have undergone cold acclimation. In the transition zone, where late spring freezes frequently kill non-acclimated or deacclimated turfgrasses, *Z. japonica* varieties are particularly well-suited for freeze survival, even without acclimation. A further evaluation of the physiological and proteomic changes that occur in zoysiagrasses during cold acclimation may shed light on phenotypic traits useful in the breeding of more cold tolerant varieties of zoysiagrass and other warm-season grasses.

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Table 3.1. Selected zoysiagrass genotypes for evaluation in controlled environment cold acclimation and freeze testing.

Genotype	Species	Source	Acclim. LT_{50} (ºC)	Non-Acclim. LT_{50} (ºC)
Meyer	*Z. japonica*	USDA&USGA (Grau, 1951)	-11.5‡	n/a
JaMur	*Z. japonica*	Bladerunner Farms (Douget 2002)	-10.2†	-4.6†
Victoria	*Z. japonica*	University of California (Gibeault et al., 1994)	-9.5‡	n/a

† Hinton et al., 2012

‡ Patton and Reicher. 2007

Table 3.2. Type III analysis of effects for stepwise logistic regression model used to estimate LT_{50} values of cultivar by acclimation treatment combinations in freeze tests of zoysiagrass cultivars under controlled environmental conditions. Fitness this model shows high concordance and predictability with the data collected confidence interval displacement diagnostic of 0.89.

Effect	DF	Wald Chi-Square	Pr > ChiSq
Acclimation Treatment	1	127.2155	<0.0001
Temperature	1	215.0433	<0.0001
Cultivar	2	1.8534	0.3959
Cultivar*Acclimation Treatment	2	11.2150	0.0037

Table 3.3. Type III analysis of stepwise selection logistic regression model evaluating the effect of acclimation treatment with cultivars pooled in freeze tests of zoysiagrass cultivars under controlled environmental conditions. This model allows for the assessment of the effect of cold acclimation on freeze tolerance. Fitness of this model shows high concordance and predictability with the data collected confidence interval displacement diagnostic of 0.88.

Effect	DF	Wald Chi-Square	Pr > ChiSq
Acclimation Treatment	1	125.4565	<0.0001
Temperature	1	216.1628	<0.0001

Table 3.4. Type III analysis of logistic regression model evaluating the effect of cultivar with the cold acclimated treatment alone. This model allows for the assessment of the effect of cultivar choice for plants that have undergone cold acclimation. Fitness of this model shows high concordance and predictability with the data collected confidence interval displacement diagnostic of 0.84.

Effect	DF	Wald Chi-Square	Pr > ChiSq
Entry	2	7.0127	0.0300
Temp	1	28.4094	<0.0001
Temp*Entry	2	5.3530	0.0688

Table 3.5. Type III analysis of logistic regression model evaluating the effect of cultivar with the non-acclimated treatment alone. This model allows for the assessment of the effect of cultivar choice for plants that are not cold acclimated or have deacclimated. Fitness of this model shows high concordance and predictability with the data collected confidence interval displacement diagnostic of 0.87.

Effect	DF	Wald Chi-Square	Pr > ChiSq
Entry	2	0.1986	0.9055
Temp	1	44.3364	<0.0001
Temp*Entry	2	0.3296	0.8481

Table 3.6. Maximum likelihood estimates used to quantify the effect of acclimation treatment on freeze tolerance of all plants in this study, disregarding cultivar, at temperatures -8ºC to -11ºC in freeze tests of zoysiagrass cultivars under controlled environmental conditions.

Parameter	Estimate†	Wald Chi-Square	Pr > ChiSq
Intercept	8.2760	199.7112	<0.0001
Cold Acclimation Treatment	2.2822	125.4565	<0.0001
Temperature	0.9511	216.1628	<0.0001

† The non-acclimated treatment was used as the reference variable for the estimation of the logistic regression model. Temperature was treated as a continuous variable.

Table 3.7. Maximum likelihood estimates used to calculate survival probabilities and LT_{50} values for each cultivar by entry combination for the temperature range -8ºC to -11ºC in freeze tests of zoysiagrass cultivars under controlled environmental conditions.

Parameter	Estimate†	Wald Chi-Square	Pr > ChiSq
Intercept	8.5368	184.8072	<0.0001
Meyer Acclimated	2.7645	63.3162	<0.0001
JaMur Acclimated	2.6516	60.2016	<0.0001
Victoria Acclimated	1.5113	23.7094	<0.0001
Meyer Non-Acclimated	0.1322	0.1981	0.6562
JaMur Non-Acclimated	-0.2679	0.8017	0.3706
Temperature	0.9755	215.0433	<0.0001

† Victoria Non-Acclimated was used as the reference variable for the estimation of the logistic regression model. Temperature was treated as a continuous variable.

Table 3.8. Estimated LT_{50} values for cultivar by acclimation treatment combinations in freeze tests of zoysiagrass cultivars under controlled environmental conditions based on maximum likelihood estimates. Significant differences between cultivar by acclimation treatment combinations assessed using Fisher's LSD.

Cultivar	Cold Acclimated LT_{50} (ºC)	Non-Acclimated LTL_{50} (ºC)
Meyer	-11.56a[†]	-8.89c
JaMur	-11.48a	-8.47c
Victoria	-10.30b	-8.75c

[†] Within and across columns and rows, values with the same letters are similar according to Fisher's protected LSD ($\alpha = 0.05$)

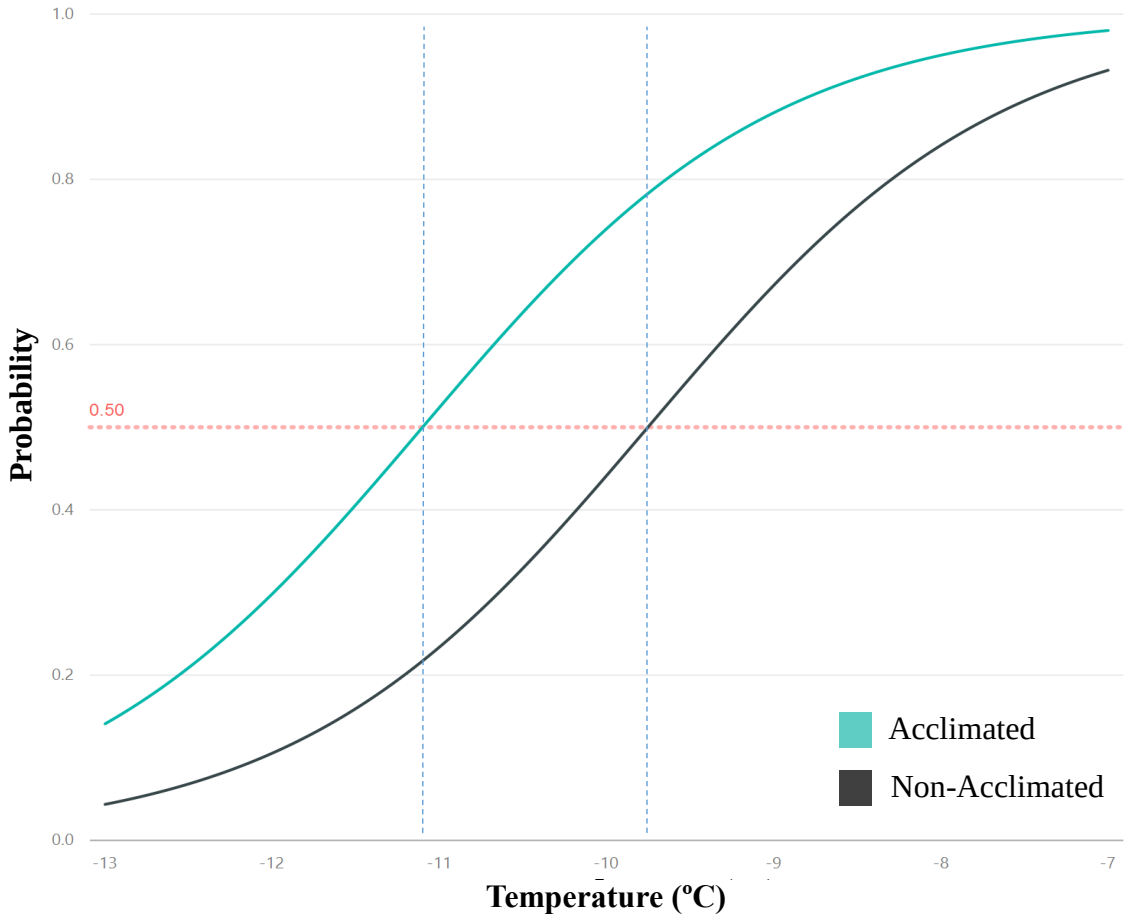

Figure 3.1. Logistic regression of predicted probabilities for survival of zoysiagrass survival across temperature for acclimation treatments in controlled environment acclimation and freeze tests. In this regression, survival probability = Odds/(1+Odds) using maximum likelihood estimates generated by model. Lethal temperature (LT_{50}) values occur at probability 0.50. In this logistic regression model, the odds ratio estimate for acclimated versus non-acclimated treatments is 9.80 with 95% confidence of 6.63 – 14.70, and the odds ratio estimate for temperature is 2.59 with 95% confidence interval of 2.29 – 2.95.

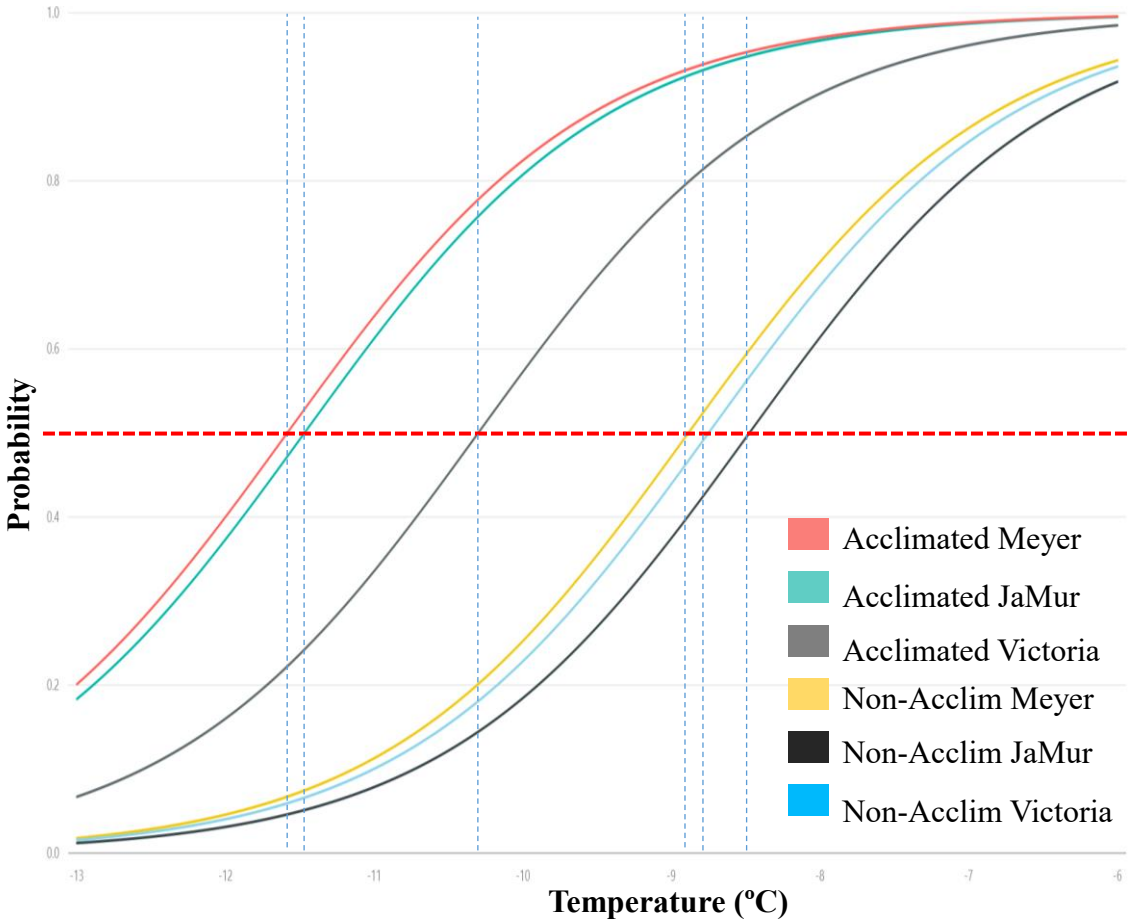

Figure 3.2. Logistic regression of predicted probabilities for survival each zoysiagrass cultivar by acclimation treatment combination across temperature in controlled environment acclimation and freeze tests. In this regression, survival probability = Odds/(1+Odds) using maximum likelihood estimates generated by model. Lethal temperature (LT_{50}) values occur at probability 0.50 (Table 2.6).

APPENDICES

Appendix 1. SAS code for analysis of field data in Chapter II. (A SNP-Based High Density Linkage Map of Zoysiagrass (*Zoysia Japonica*) and its use for the identification of QTL associated with winter Hardiness).

```
PROC MIXED DATA = ZOYSIA.MAPPING  METHOD = TYPE3;
CLASS GENO ENVIR REP;
MODEL ARC_INJURY = GENO ENVIR GENO(ENVIR);
RANDOM REP(ENVIR) GENO*REP(ENVIR) FALL_COVER;
LSMEANS GENO GENO(ENVIR);
RUN;

PROC MIXED DATA = ZOYSIA.EST METHOD = TYPE3;
CLASS GENO REP ENVIR FALL_COVER FALL_TQ SPRING_TQ;
MODEL TRAIT = GENO ENVIR GENO*ENVIR;
RANDOM REP(ENVIR);
LSMEANS GENO GENO*ENVIR;
RUN;
```

Appendix 2. SAS code for estimating LT_{50}s in Chapter III. (insert chapter III title here)

```
PROC LOGISTIC DATA=ZOYSIA.FREEZE. OUT=ZOYSIA.TEST
PLOTS(ONLY)=(EFFECT(CLBAND SHOWOBS) ODDSRATIO);
CLASS ENTRY TREATMENT (PARAM=REF);
MODEL SURVIVAL(EVENT="1") = ENTRY TREATMENT TEMP
ENTRY*TREATMENT ENTRY*TEMP ENTRY*TREATMENT*TEMP /
SELECTION=STEPWISE CLODDS=PL;
RUN;

PROC TRANSPOSE DATA=ZOYSIA.TEST OUT=ZOYSIA.TEST;
COPY INTERCEPT TEMP;
VAR  ENTRYMAC ENTRYMNA ENTRYJNA ENTRYJAC ENTRYVAC;
RUN;

DATA ZOYSIA.TEST;
SET ZOYSIA.TEST (DROP= _LABEL_);
IF INTERCEPT = . THEN INTERCEPT = 8.5368;
IF TEMPERATURE = . THEN TEMPERATURE = 0.9755;
RUN;

DATA ZOYSIA.TEST;
INPUT INTERCEPT TEMP _NAME_$ SURVIVAL;
DATALINES;
8.5368 0.9755 ENTRYVNA 1.0;
RUN;

DATA ZOYSIA.TEST;
SET ZOYSIA.TEST;
DO TEMP = -13 TO -6 BY 0.001; OUTPUT;
END;
RUN;

DATA ZOYSIA.TEST;
SET ZOYSIA.TEST;
LOG_ODDS = INTERCEPT + SURVIVAL + (TEMP*TEMPERATURE);
ODDS = EXP(LOG_ODDS);
PROBABILITY = ODDS / (1+ODDS);
RUN;
```

Appendix 3. Full list of zoysiagrass primers used for the identification of SSRs which were used in linkage mapping and QTL analysis in Chapter II.

Primer	SSR	Chromosome	Forward and reverse primer sequences with m-13 tail (5'-3')	Source
ZB08B08_F ZB08B08_R	SSR_01_1	1	CACGACGTTGTAAAACGACAAAATCAAGGAATTAAGAAGCA CCATTTCTTTATCGCACG	Cai et al., 2005
ZB03J03_F ZB03J03_R	SSR_01_2	1	CACGACGTTGTAAAACGACGGCTTTATAGCGAAGTTTGA CTACCCTGACACAAGAAAGC	Cai et al., 2005
ZB03M21_F ZB03M21_R	SSR_02_1	2	CACGACGTTGTAAAACGACAAATTCAACAGGATGCTGAT GGCTTGCTCGAGAGATAGTA	Cai et al., 2005
ZB06E05_F ZB06E05_R	SSR_02_10	2	CACGACGTTGTAAAACGACCAGATCCAAACCCATCAGT ATTTTGCTTGAAACGGATC	Cai et al., 2005
ZA02E14_F ZA02E14_R	SSR_02_11	2	CACGACGTTGTAAAACGACAACAGAGGAAAGGATGGC TCTTTTGCTCGGTCAGTG	Cai et al., 2005
ZB07E21_F ZB07E21_R	SSR_02_12	2	CACGACGTTGTAAAACGACAGATTGGTCTCTGGGTAGCT GCTAGTGGATGAACACATGA	Cai et al., 2005
ZA03K14_F ZA03K14_R	SSR_02_13	2	CACGACGTTGTAAAACGACAGGACCAGTGAACATAGTGG TGATTGAACAGAAAATTCACC	Cai et al., 2005
ZB03C21_F ZB03C21_R	SSR_02_14	2	CACGACGTTGTAAAACGACGCAGAGTGATTCACAGGC GGCTCCTAGTTTGTTGGAG	Cai et al., 2005
ZA03H16_F ZA03H16_R	SSR_02_15	2	CACGACGTTGTAAAACGACAACGAAATCTCGTCCTATAACA AAGAGCAAGCTCTTTGTCTG	Cai et al., 2005
ZB08E05_F ZB08E05_R	SSR_02_16	2	CACGACGTTGTAAAACGACACTACGTCAGCAACAGCAC AGCCAGCAGCAGAATATG	Cai et al., 2005
ZB10K17_F ZB10K17_R	SSR_02_17	2	CACGACGTTGTAAAACGACCAGTTGGGCTAGAAGAGATG GACAAATTACTCGAGAACCG	Cai et al., 2005
ZB01K19_F ZB01K19_R	SSR_02_2	2	CACGACGTTGTAAAACGACAATCGAATCAGGGAAAGG CCAGCAGTCTTCCATCAG	Cai et al., 2005
ZB07A22_F ZB07A22_R	SSR_02_3	2	CACGACGTTGTAAAACGACGGTCGACCTCACCCTAGG TACAACCTGCAGATCGATC	Cai et al., 2005
b04e21_F b04e21_R	SSR_02_4	2	CACGACGTTGTAAAACGACACTCCCTCAATCCAATCC GCGCTTACTAGCTTTCTGAG	Li et al., 2009
b01p03_F b01p03_R	SSR_02_5	2	CACGACGTTGTAAAACGACAAGTACTACTGCACTCGGGA CACATGCATGATAAAAGCC	Li et al., 2009
b06d18a_F	SSR_02_6	2	CACGACGTTGTAAAACGACATCACGGATGAACAATAATTG	Li et al., 2009

b06d18a_R			GTACGTACGTTGGCTCTACTG	
ZB01N24_F ZB01N24_R	SSR_02_7	2	CACGACGTTGTAAAACGACTTTAAGGTTTTGGAAATACACC TGAAATGATTCCTTCTTGCT	Cai et al., 2005
ZB09H08_F ZB09H08_R	SSR_02_8	2	CACGACGTTGTAAAACGACAAACTGACCGGTTTTGATC ACTTGGGACAAACAAATTTG	Cai et al., 2005
ZB03G06_F ZB03G06_R	SSR_02_9	2	CACGACGTTGTAAAACGACTCAAATCCTGATGCAATGTA AAACACAACAGGCATGCT	Cai et al., 2005
ZB02A24_F ZB02A24_R	SSR_03_1	3	CACGACGTTGTAAAACGACGATTCAGCATCAGAATCCAT CATGTCCCTAATCCACCTTA	Cai et al., 2005
ZB03D04_F ZB03D04_R	SSR_03_2	3	CACGACGTTGTAAAACGACGTGACGGGTGAGCTGTAG GGCATTGATACCTGGAGTTA	Cai et al., 2005
ZB03B08_F ZB03B08_R	SSR_04_1	4	CACGACGTTGTAAAACGACCTTCAATCTCATAGCCCTTG ACAGGGGAAAGTATTGGAAT	Cai et al., 2005
b07i05_F b07i05_R	SSR_04_2	4	CACGACGTTGTAAAACGACATGGAATTAATCTTCTCCCTG GTTGCCTCTTTCATACTTGTG	Li et al., 2009
b03l03_F b03l03_R	SSR_05_1	5	CACGACGTTGTAAAACGACATAGCTAGCTGCCTTGAGG CAAGTATGCATTTCTGCTCA	Li et al., 2009
ZB02P16_F ZB02P16_R	SSR_05_10	5	CACGACGTTGTAAAACGACAGGTGAGTGCCGTCGTAG CTTGAGGATCACGTACGG	Cai et al., 2005
ZB10M09_F ZB10M09_R	SSR_05_11	5	CACGACGTTGTAAAACGACCAGCATAGGAAAGGAAGCTA AGTGTAAGCCGTTGCTTG	Cai et al., 2005
ZB02N19_F ZB02N19_R	SSR_05_12	5	CACGACGTTGTAAAACGACGCGTCACGCTCTTTACAT GGAGTCTGCATGATTTGC	Cai et al., 2005
b06p10a_F b06p10a_R	SSR_05_13	5	CACGACGTTGTAAAACGACTATTGCAATCTTGGTCTCCT AGGGAAGAACATGCACATAC	Li et al., 2009
ZB09F24_F ZB09F24_R	SSR_05_2	5	CACGACGTTGTAAAACGACTTATCTCCGAAGCATTGAAT GCGTATGTAAGTTCGTTAAACA	Cai et al., 2005
ZA03O20_F ZA03O20_R	SSR_05_3	5	CACGACGTTGTAAAACGACCAGTGGATGATGCGTAGAC GACTACTATCATGCAGGTTGC	Cai et al., 2005
b03o03a_F b03o03a_R	SSR_05_4	5	CACGACGTTGTAAAACGACTGCAACAGGTAGCTGGTAG GCATCAGCAGGTAGATTCTC	Li et al., 2009
ZA01M20_F ZA01M20_R	SSR_05_5	5	CACGACGTTGTAAAACGACCCATGTTCACTTTCCTCATT GAATGGATTGGGTCTAAACA	Cai et al., 2005
ZA01C15_F ZA01C15_R	SSR_05_6	5	CACGACGTTGTAAAACGACAAACCAATTAAGTCATTTGCA CAATGAATAGAAGTCTGTCGG	Cai et al., 2005

ZB09O10_F ZB09O10_R	SSR_05_7	5	CACGACGTTGTAAAACGACCCAAGTTCCCATCTTTGTAA ATAGCCAGGAGGCAAGAC	Cai et al., 2005
ZA02F16_F ZA02F16_R	SSR_05_8	5	CACGACGTTGTAAAACGACCCAACTGCCTAATCTAGTGG AGTCGAAAGGATATACCAAATG	Cai et al., 2005
ZB09D12_F ZB09D12_R	SSR_05_9	5	CACGACGTTGTAAAACGACAGAATGGGTGAGGACTGAG CTCGGAAAGGTGTAGACTTG	Cai et al., 2005
ZB07J16_F ZB07J16_R	SSR_06_1	6	CACGACGTTGTAAAACGACAGAATATGCTGAAATCATCACA ACAGTTGTTTGAAGATCATTCA	Cai et al., 2005
ZB08F21_F ZB08F21_R	SSR_06_2	6	CACGACGTTGTAAAACGACATCAGGGAGCAGGAAGAC GCGAGTCATGAAGACGAC	Cai et al., 2005
c03j01_F c03j01_R	SSR_06_3	6	CACGACGTTGTAAAACGACTCGTCGACGATGTAGTACG GACGAATATCGATGACCTGT	Li et al., 2009
b03i20_F b03i20_R	SSR_06_4	6	CACGACGTTGTAAAACGACAACTCAGCACAACATGATCA CACTGCTAATCTTGCACTGA	Li et al., 2009
b03i14_F b03i14_R	SSR_06_5	6	CACGACGTTGTAAAACGACTGTGTCTCCCAGAGATGG AGGAGAGCTGTCGTTCTGT	Li et al., 2009
b03c24_F b03c24_R	SSR_06_6	6	CACGACGTTGTAAAACGACGACTGCTGGATATGCCTG CTACGACCTTCTCTCGTCTG	Li et al., 2009
ZC01D05_F ZC01D05_R	SSR_07_1	7	CACGACGTTGTAAAACGACTTATCTCGGACTTCATGCTT CTTCTGCTGCTGAATGCT	Cai et al., 2005
b03f18_F b03f18_R	SSR_07_10	7	CACGACGTTGTAAAACGACTTCTGTTCCCCATCAATG CGATCGAGACATGATTGAG	Li et al., 2009
ZB01F23_F ZB01F23_R	SSR_07_2	7	CACGACGTTGTAAAACGACTCTGCCAGTCATATATGGGT ATCCAGCATGAAGCTGATAT	Cai et al., 2005
ZB07H12_F ZB07H12_R	SSR_07_3	7	CACGACGTTGTAAAACGACATATCCAGCATGAAGCTGAT TGCCAGTCATATATGGGTCT	Cai et al., 2005
ZD02G09_F ZD02G09_R	SSR_07_4	7	CACGACGTTGTAAAACGACCCCTTGTTTAATGAGAGACG CACTATGGGTGATCAGCAC	Cai et al., 2005
ZB03J15_F ZB03J15_R	SSR_07_5	7	CACGACGTTGTAAAACGACTCGGAGGAATTAGTGGAAC CTGAGTTGCTAGCGTGGT	Cai et al., 2005
b04n13_F b04n13_R	SSR_07_6	7	CACGACGTTGTAAAACGACCTGCGCAGTTCTCTCTAATT GACACCGACTTCTTCACG	Li et al., 2009
ZB07D23_F ZB07D23_R	SSR_07_7	7	CACGACGTTGTAAAACGACTCGGATGATGTTGTACGTC CTAGAGCAACCGTGCAAGT	Cai et al., 2005
ZB08I05b_F ZB08I05b_R	SSR_07_8	7	CACGACGTTGTAAAACGACAACCGGCCAATTAGCTAC GTCCTTGATTCCCCTGTC	Cai et al., 2005

b04c05_F b04c05_R	SSR_07_9	7	CACGACGTTGTAAAACGACCCTCCTTTTCCAATCGAT CCTCTCCCTTCCTTCTCTT	Li et al., 2009
b09o08_F b09o08_R	SSR_08_1	8	CACGACGTTGTAAAACGACATCTGGATCAGCTTTCAAGA AAAGGTCGCTAGATTGATCA	Li et al., 2009
ZB09K11_F ZB09K11_R	SSR_08_2	8	CACGACGTTGTAAAACGACCATAGCAGTCTCAAGATGCA TAACTTGCTCTACCTTTTCCTC	Cai et al., 2005
ZB06D19_F ZB06D19_R	SSR_08_3	8	CACGACGTTGTAAAACGACCCATAGGCTCGTAGAAGAAA AATCATGGCATTCTGAGATT	Cai et al., 2005
ZB02D07_F ZB02D07_R	SSR_09_1	9	CACGACGTTGTAAAACGACAGTAGCTGTGTATGCTTCTGG CTACAGCTCATCATGGGC	Cai et al., 2005
b06a14_F b06a14_R	SSR_10_1	10	CACGACGTTGTAAAACGACACTCGTTTTCTGCACGATAT GAAATATATCGTACGTGCTGG	Li et al., 2009
ZA01P11_F ZA01P11_R	SSR_11_1	11	CACGACGTTGTAAAACGACTCCGCTACCAAGTAATCACT TGCGCTTTCTAGAGATCTTC	Cai et al., 2005
ZB01C11_F ZB01C11_R	SSR_11_2	11	CACGACGTTGTAAAACGACCCTTCTGACTCTACGGCTC CCCAACCTCTTCTTCCTAGT	Cai et al., 2005
ZB10G01_F ZB10G01_R	SSR_12_1	12	CACGACGTTGTAAAACGACCTAATCATCACAAAGCCATTC CTCACAAGGAACCTAACTGC	Cai et al., 2005
ZB08I08_F ZB08I08_R	SSR_12_2	12	CACGACGTTGTAAAACGACTAAATAATTCCGAGTGTTCTGA TCGCTGTTTACATCTCTTCTC	Cai et al., 2005
ZB02I21_F ZB02I21_R	SSR_12_3	12	CACGACGTTGTAAAACGACGAAGAGCTTTTGTCGAAGAA GAAATAAAGAGGAGCAGCAA	Cai et al., 2005
ZB07P19_F ZB07P19_R	SSR_12_4	12	CACGACGTTGTAAAACGACGGACTTCTGTTGGATCTGAA CCTCCTCTTGGCTCAGTT	Cai et al., 2005
ZC01P08b_F ZC01P08b_R	SSR_12_5	12	CACGACGTTGTAAAACGACAAGATCGATTCAGCATTCAT TTTGAGAATTATGACATGATGG	Cai et al., 2005
b02g18_F b02g18_R	SSR_13_1	13	CACGACGTTGTAAAACGACGAAGCTCGACAATCTGGAT TCCAAATATGGCATTGCT	Li et al., 2009
ZB06J05_F ZB06J05_R	SSR_13_2	13	CACGACGTTGTAAAACGACGGACTAAAGTTTGCTGTGATG TAAGACTCTCATTGCAAAAGG	Cai et al., 2005
ZB07C10_F ZB07C10_R	SSR_13_3	13	CACGACGTTGTAAAACGACCTAGACCGCGAGATCCAC CTCCTTCTCCGCTAGCAC	Cai et al., 2005
ZB01C23a_F ZB01C23a_R	SSR_13_4	13	CACGACGTTGTAAAACGACCAAGAGGAGTTTGGGCTC TCAGTCCCTCAAGGAAATTA	Cai et al., 2005
ZB03D16_F ZB03D16_R	SSR_13_5	13	CACGACGTTGTAAAACGACGCAAAACATGAATTTGGC ACAGTGCCTTACCTTCCC	Cai et al., 2005

ZB07A09_F ZB07A09_R	SSR_13_6	13	CACGACGTTGTAAAACGACAGGATTCTACCCTGGTAAACA CAGTTACAATTGGAGACAACTC	Cai et al., 2005
ZB07A06_F ZB07A06_R	SSR_13_7	13	CACGACGTTGTAAAACGACGGAATTTATAATCCAAGCCC ATCTCGCTGTCTCTCAGCTA	Cai et al., 2005
ZB03N17_F ZB03N17_R	SSR_13_8	13	CACGACGTTGTAAAACGACTTACTTGTGCGGTTTTACG TTCCTTTGGCAGAAACAG	Cai et al., 2005
a02o06_F a02o06_R	SSR_13_9	13	CACGACGTTGTAAAACGACGATTGAAGCTCATGTCTATGTG GCAATAAACAACTCTCTTCCTC	Li et al., 2009
ZT-A108_F ZT-A108_R	SSR_14_1	14	CACGACGTTGTAAAACGACTAGCACTTGCACTACCAGC TTTGTTCACTTGTTGCTTTG	Cai et al., 2005
ZB06J11_F ZB06J11_R	SSR_14_2	14	CACGACGTTGTAAAACGACTGATCTCTACTTTAACCGCTG TGAGCTCTAGACGGAGGATA	Cai et al., 2005
b03h23_F b03h23_R	SSR_14_3	14	CACGACGTTGTAAAACGACAAGACCATTGTAGGCTCAAA CCCTGGCCTTAAACAGTT	Li et al., 2009
b01c12_F b01c12_R	SSR_14_4	14	CACGACGTTGTAAAACGACGGAACAGACTTGCCTTCTC CTCATCAACGACTCTGCC	Li et al., 2009
b08o01_F b08o01_R	SSR_14_5	14	CACGACGTTGTAAAACGACCTTCTCTACCCCTAAAACAGC CGGGTGAAAGGATAGCTAG	Li et al., 2009
ZB02K23_F ZB02K23_R	SSR_14_6	14	CACGACGTTGTAAAACGACTCTGCTCTCAGTTGCTCAC TATTTTGCTTCTCTTTCCTAGC	Cai et al., 2005
ZB08I05a_F ZB08I05a_R	SSR_14_7	14	CACGACGTTGTAAAACGACTCTTGTGACCTGGGTCTC AGAAAATGTAGCTAATTGGCC	Cai et al., 2005
b08j05b_F b08j05b_R	SSR_14_8	14	CACGACGTTGTAAAACGACTGACCGTTGTAGCCTAGC GAACAAGAACGGGTAGGG	Li et al., 2009
ZA02O09_F ZA02O09_R	SSR_14_9	14	CACGACGTTGTAAAACGACAGTCAAAATGCATACGGAGT CTGCCATGCTAAATAAAATACA	Cai et al., 2005
b01d10_F b01d10_R	SSR_15_1	15	CACGACGTTGTAAAACGACATCCTTGCCTCTGATGATC GGTGACAACGTAACCGTCT	Li et al., 2009
ZB04I23_F ZB04I23_R	SSR_15_2	15	CACGACGTTGTAAAACGACTTTCATCAGCAGTAGTGTGG TTGAGGGCAGATAAGTAGGA	Cai et al., 2005
ZB01B08_F ZB01B08_R	SSR_15_3	15	CACGACGTTGTAAAACGACGGAAGACGACAATAGTTTGC AGTCGCAAATACTAGTGGGA	Cai et al., 2005
ZB07O09b_F ZB07O09b_R	SSR_16_1	16	CACGACGTTGTAAAACGACTAGGGTTTTTCCCCACTTCC GTCGGCGTCCGCCATT	Cai et al., 2005
ZC01P20_F ZC01P20_R	SSR_16_2	16	CACGACGTTGTAAAACGACAGTTCTGAGGAGAAGGGAAG GGTACGTCAACATCTGCTG	Cai et al., 2005

ZB09K08_F ZB09K08_R	SSR_16_3	16	CACGACGTTGTAAAACGACCCCTAATAAGTTGCCTTGC CGTCACTGTGAGGGAGAG	Cai et al., 2005
ZB01B12_F ZB01B12_R	SSR_16_4	16	CACGACGTTGTAAAACGACGCTAGTGTTTGTTGATGACTTG AACTTGAGCGTGCTATGC	Cai et al., 2005
ZC01D15_F ZC01D15_R	SSR_16_5	16	CACGACGTTGTAAAACGACGAAGCACTCGAACAATCTTC GTGGGGTGTAGGGAGATC	Cai et al., 2005
b02g17_F b02g17_R	SSR_16_6	16	CACGACGTTGTAAAACGACTCAACGAGAGGAGAGCAG CACATCCATGTGCTTCCT	Li et al., 2009
ZB04C09_F ZB04C09_R	SSR_16_7	16	CACGACGTTGTAAAACGACATCTCTGGTGCCTTCCTC ATCCCTATTTATACTCCGTGTG	Cai et al., 2005
ZB01C08_F ZB01C08_R	SSR_16_8	16	CACGACGTTGTAAAACGACCTAGCTAGCTCGTCGTAAGC TTGCTTGGTTCCAAATAAGT	Cai et al., 2005
c03l24_F c03l24_R	SSR_17_1	17	CACGACGTTGTAAAACGACCAGCCAGCACATAGGATC GAGTGAGACCTGCACGAG	Li et al., 2009
b09j13_F b09j13_R	SSR_17_2	17	CACGACGTTGTAAAACGACCAGCAACATCAGACAAACC GAGAATACCTTTCTGCCTTTC	Li et al., 2009
ZB01N17_F ZB01N17_R	SSR_17_3	17	CACGACGTTGTAAAACGACGTGAGGAGGTGACTGGACT GTTTGATTCCGAGCGAAC	Cai et al., 2005
ZB07C02_F ZB07C02_R	SSR_17_4	17	CACGACGTTGTAAAACGACGCCCAAACGGTATATACTTG GACGCTGATATTGCGATC	Cai et al., 2005
ZB06E20_F ZB06E20_R	SSR_17_5	17	CACGACGTTGTAAAACGACTCCTAGGGAGAGGATACATTT CATCCATTGGCGTATGTAG	Cai et al., 2005
ZB03F11_F ZB03F11_R	SSR_18_1	18	CACGACGTTGTAAAACGACTTGGAACCTGTTCCATTATC TGCTGTCTTTAGAAATTGTTTG	Cai et al., 2005
ZC01B12_F ZC01B12_R	SSR_18_2	18	CACGACGTTGTAAAACGACTCTACATTGATCGACAGCAG TTTGGTATTGTGAGGAGGAC	Cai et al., 2005
c01g15_F c01g15_R	SSR_19_1	19	CACGACGTTGTAAAACGACATCGACCGTAACCTCCTC CACTTGTGACTTGTCAGCC	Li et al., 2009
ZB01C06_F ZB01C06_R	SSR_19_2	19	CACGACGTTGTAAAACGACATAAAGATACGAGTGGAATTGG GCACAAAGAAGCTAGACCC	Cai et al., 2005
b06m04_F b06m04_R	SSR_19_3	19	CACGACGTTGTAAAACGACCTCGCAGCCAAGAAACT CATACATTGCATTATTGTGTAATG	Li et al., 2009
b01n07_F b01n07_R	SSR_20_1	20	CACGACGTTGTAAAACGACTGGAACCAGAGACTATCCTATC ACACATGGTGATGGCTTC	Li et al., 2009
ZB03J14_F ZB03J14_R	SSR_20_2	20	CACGACGTTGTAAAACGACGGCGTACAAATCAGCATC CCATTTGGTTTTTTTAGTGC	Cai et al., 2005

ZB04F12_F ZB04F12_R	SSR_20_3	20	CACGACGTTGTAAAACGACTGTGCCGTACTGATAATTAATC TAGGAAACATGGACTGAGCT	Cai et al., 2005
ZB06O03_F ZB06O03_R	SSR_20_4	20	CACGACGTTGTAAAACGACCAAAGTACATCCATCATCCC GTTCTCAGCTGAGATCCGT	Cai et al., 2005
ZA01O15_F ZA01O15_R	SSR_20_5	20	CACGACGTTGTAAAACGACGCTTGATGCTCCACTCAC AACAGGAGGAATTTCAATCTC	Cai et al., 2005
b02c06_F b02c06_R	SSR_20_6	20	CACGACGTTGTAAAACGACCAGTTGCTGCTAAGGATTCT CCTCGCTATGAGTGGTCTAC	Li et al., 2009
ZB01B13_F ZB01B13_R	SSR_20_7	20	CACGACGTTGTAAAACGACGCATATCAGTGAAAAGGAGC TTATGCTCGCACAAGAGTC	Cai et al., 2005
ZB02L18_F ZB02L18_R	SSR_20_8	20	CACGACGTTGTAAAACGACACTCTGGAGACCCTCTGG TGACTTGTCTGGCTTTCC	Cai et al., 2005
a01c06_F a01c06_R	SSR_20_9	20	CACGACGTTGTAAAACGACTTGCCGTAGTATATTGGTATTG GCATTATATGACGAGAAATGG	Li et al., 2009
ZA03F03_F ZA03F03_R	SSR_N_1	n/a	CACGACGTTGTAAAACGACATCAAGGTAACAAGATCACGA GAGAAGGACGTAACGTAACAA	Cai et al., 2005
ZB01D04_F ZB01D04_R	SSR_N_2	n/a	CACGACGTTGTAAAACGACAGTAGTGTTTGGAATCTTCCG ACAGTAGCTTGTTTCCTCTCTG	Cai et al., 2005
ZB01D05_F ZB01D05_R	SSR_N_3	n/a	CACGACGTTGTAAAACGACTCGATCTGAGCTATTTTAACG TAACCGCAATACCTGTTTCT	Cai et al., 2005
ZB03B05_F ZB03B05_R	SSR_N_4	n/a	CACGACGTTGTAAAACGACGAGAGGCTTCTTGACAAGG GTACCAGACCGAAGGCTAC	Cai et al., 2005

Appendix 4. Full list of quantitative trait loci (QTL) associated with zoysiagrass winter injury found in CIM mapping analysis as described in Chapter II.

Chrom	Associated Markers	Environment	Max R^2	Location (kbp)	Location (cM)	LOD Threshold
1	SNP_01_17, SNP_01_18, SNP_01_19, SNP_01_20, SNP_01_21	NCA16	7.67	1846.5 – 1870.8	11.25-11.40	2.5
1	SNP_01_40, SNP_01_41, SNP_01_43, SNP_01_45, SNP_01_46, SNP_01_47, SNP_01_49, SNP_01_50, SNP_01_52, SNP_01_53	NCA16	8.47	3082.8 - 4263.3	18.78-25.97	2.5
1	SNP_01_134, SNP_01_135, SNP_01_138, SNP_01_139	INA15	9.03	17107.1-17184.8	104.22-104.69	2.5
1	SNP_01_153, SNP_01_154	Combined	7.49	18362.0	111.86	2.5
1	SNP_01_153, SNP_01_154	INA15	8.41	18362.0	111.86	2.5
2	SNP_02_2, SSR_02_1	NCA16	8.22	153.3-153.7	0.93-0.94	2.5
2	SNP_02_46, SSR_02_8	NCA16	8.46	4081.0-4481.0	24.86-27.30	2.5
2	SNP_02_72	NCB16	6.82	9670.9	58.92	2.5
2	SSR_02_12, SNP_02_76	INA16	12.68	10029.4-11586.3	61.10-70.59	2.5
2	SNP_02_79	INA16	10.04	11738.8	71.52	2.5
2	SNP_02_132	INA15	6.86	16073.1	97.92	2.5
2	SNP_02_136	INB16	6.38	16185.9	98.61	2.5
2	SNP_02_136, SNP_02_137, SNP_02_139, SNP_02_140, SNP_02_141, SNP_02_142	NCB16	8.46	16185.9-17564.7	98.61-107.01	2.5
3	SSR_03_1, SNP_03_25	INA16	10.83	4259.3-4429.5	25.95-26.99	2.5
4	SNP_04_49, SNP_04_50	INB16	6.65	8889.7-8892.0	54.16-54.17	2.5
4	SNP_04_74, SNP_04_75,	NCA15	6.85	10211.3-10280.1	62.21-62.63	2.5

	SNP_04_76, SNP_04_77					
5	SNP_05_26, SNP_05_27	NCB16	7.67	1708.4-1760.3	10.41-10.72	2.5
5	SSR_05_5	INA15	8.85	4385.6	26.72	2.5
5	SSR_05_6, SNP_05_76, SNP_05_77, SNP_05_78, SNP_05_79, SNP_05_80, SNP_05_81, SNP_05_82, SNP_05_83	INB16	8.44	4926.5-9339.9	30.01-56.90	2.5
5	SNP_05_68, SNP_05_69	INA15	6.59	5390.6-5619.5	32.84-34-23	2.5
5	SNP_05_76	NCA15	7.07	8979.8	54.71	2.5
5	SNP_05_76	Combined	6.63	8979.8	54.71	2.5
5	SNP_05_76, SNP_05_77, SNP_05_78, SNP_05_79, SNP_05_81, SNP_05_82, SNP_05_83, SSR_05_7	INA15	8.83	8979.8-9591.3	54.71-58.43	2.5
5	SNP_05_104, SNP_05_105, SNP_05_106, SNP_05_107, SNP_05_108, SNP_05_109	NCA16	13.19	12149.3-12521.9	74.02-76.29	2.5
5	SNP_05_108, SNP_05_109	NCA15	6.49	12521.8	76.29	2.5
5	SNP_05_122, SSR_05_9	NCB16	6.48	13359.9-13382.0	81.39-81.53	2.5
5	SNP_05_164	Combined	6.68	16167.8	98.50	2.5
6	SNP_06_6, SNP_06_7	NCA16	9.89	516.8-546.8	3.15-3.33	2.5
6	SNP_06_9, SNP_06_10	NCB16	7.58	760.7-867.1	4.63-5.28	2.5
6	SSR_06_1	INA15	6.92	998.5	6.08	2.5
6	SNP_06_15, SNP_06_16	INA16	9.72	1391.2	8.48	2.5
6	SNP_06_64	NCA15	6.67	4535.2	27.63	2.5
6	SNP_06_146	INA15	6.37	16286.1	99.22	2.5
7	SSR_07_3, SNP_07_16, SNP_07_17,	NCA15	6.54	1339.3-2138.5	8.16-13.03	2.5

	SNP_07_18					
7	SNP_07_38	NCB16	6.53	3741.6	22.80	2.5
7	SNP_07_84, SNP_07_85, SNP_07_87	NCB16	7.33	11751.9-11779.2	31.59-71.76	2.5
7	SNP_07_62, SNP_07_64, SNP_07_65	NCA16	8.41	7843.9	47.79	2.5
7	SNP_07_62, SNP_07_64, SNP_07_65	Combined	10.06	7843.9	47.79	2.5
7	SNP_07_76	INA15	6.39	10890.3	66.35	2.5
7	SNP_07_89	NCA16	7.70	11779.1	71.76	2.5
7	SNP_07_96, SNP_07_97	NCB16	7.23	12143.3	73.98	2.5
7	SNP_07_96, SNP_07_97	NCA15	6.77	12143.3	73.98	2.5
7	SNP_07_98, SNP_07_100, SNP_07_101	Combined	8.47	12143.3-12330.1	73.98-75.12	2.5
7	SNP_07_137	NCB16	7.99	14554.6	88.67	2.5
7	SNP_07_139, SNP_07_142, SNP_07_144, SNP_07_146	NCA15	7.57	14834.2-15758.4	90.37-96.00	2.5
7	SNP_07_168, SSR_07_6	NCA15	9.87	19358.1-19444.3	117.93-118.46	2.5
7	SNP_07_173, SSR_07_8	Combined	7.12	19668.6-19671.3	119.82-119.84	2.5
7	SSR_07_9	NCA16	8.01	19686.9	119.94	2.5
8	SNP_08_9	INA16	8.95	1134.3	6.91	2.5
8	SNP_08_11, SNP_08_12, SNP_08_13, SNP_08_14, SNP_08_15, SNP_08_16, SNP_08_17	INA15	8.65	1338.0-2377.0	8.15-14.48	2.5
8	SNP_08_17	Combined	6.65	2376.9	14.48	2.5
8	SNP_08_50, SNP_08_51, SNP_08_52, SNP_08_55, SNP_08_56, SNP_08_59	NCA16	8.96	7699.9-8820.9	46.91-53.74	2.5
8	SSR_08_3	Combined	6.68	14773.1	90.00	2.5
8	SNP_08_145, SNP_08_146	INA15	6.62	18470.4-18570.2	112.53-113.13	2.5
9	SNP_09_36	NCA16	10.94	6407.4	39.04	2.5

9	SNP_09_42	NCA15	7.04	6614.5	40.30	2.5
10	SNP_10_6	INB16	6.91	839.4	5.11	2.5
10	SNP_10_12, SNP_10_13	NCA15	6.58	865.5-886.0	5.27-5.40	2.5
11	SNP_11_3	INA15	6.70	545.1	3.32	2.5
11	SNP_11_3, SNP_11_5, SNP_11_9	INA16	10.31	545.1-1212.8	3.32-7.39	2.5
11	SNP_11_25, Snp_11_26	INA16	10.74	2728.9	16.63	2.5
11	SNP_11_70	INA15	7.20	7217.6	43.97	2.5
11	SNP_11_112, SNP_11_113	INA15	8.82	10558.0	64.32	2.5
12	SNP_12_61, SSR_12_2	NCA15	12.66	10248.8-10277.1	62.44-62.61	2.5
12	SNP_12_61, SSR_12_2	INA15	8.02	10248.8-10277.1	62.44-62.61	2.5
12	SNP_12_61, SSR_12_2	Combined	8.76	10248.8-10277.1	62.44-62.61	2.5
12	SNP_12_67	INA15	6.42	10629.9	64.76	2.5
12	SNP_12_69, SNP_12_70, SNP_12_71, SNP_12_72	NCA16	8.20	10668.1-10740.1	64.99-65.43	2.5
12	SNP_12_89, SNP_12_90	INA15	7.23	11079.1	67.50	2.5
12	SNP_12_89, SNP_12_90	INA16	14.37	11079.1	67.50	2.5
12	SNP_12_89, SNP_12_90	Combined	8.06	11079.1	67.50	2.5
12	SNP_12_92, SNP_12_93, SNP_12_94	Combined	8.73	11146.8-11356.2	67.91-69.18	2.5
12	SNP_12_99, SNP_12_100	Combined	7.52	11754.3	71.61	2.5
12	SSR_12_3, SNP_12_131, SNP_12_132, SNP_12_133	NCA16	10.22	14349.7-14350.7	87.42-87.43	2.5
12	SSR_12_3, SNP_12_131, SNP_12_132, SNP_12_133	INA15	9.11	14349.7-14350.7	87.42-87.43	2.5
12	SNP_12_133	Combined	8.36	14350.6	87.43	2.5
12	SNP_12_133, SSR_12_4	INA16	13.91	14350.6-14407.9	87.42-87.78	2.5
12	SNP_12_140	NCA16	8.13	14722.6	89.69	2.5
13	SNP_13_11	INA16	9.26	1031.7	6.29	2.5

13	SNP_13_12, SNP_13_14, SNP_13_15	INA16	12.03	1356.9-2220.6	8.27-13.53	2.5
13	SNP_13_14, SNP_13_15	Combined	7.55	2073.3-2220.6	12.63-13.53	2.5
13	SNP_13_17	INA16	9.61	2608.2	15.89	2.5
13	SNP_13_45	NCA16	8.80	6012.3	36.63	2.5
13	SNP_13_61, SSR_13_7	INB16	8.27	8910.4-9084.8	54.28-55.35	2.5
13	SNP_13_71, SNP_13_73, SNP_13_74, SNP_13_75	NCA16	10.78	10333.0-10626.5	62.95-64.74	2.5
13	SSR_13_8	INA16	10.00	10648.8	64.87	2.5
13	SNP_13_87	NCA16	7.96	11290.8	68.79	2.5
13	SNP_13_87, SSR_13_9	Combined	7.89	11290.8-11639.7	68.79-70.91	2.5
14	SNP_14_26	INA16	9.04	6142.7	37.42	2.5
14	SNP_14_33, SSR_14_7	NCA15	7.40	6800.8-6913.0	41.43-42.12	2.5
14	SNP_14_7	INA16	9.12	6912.9	42.12	2.5
14	SNP_14_7	Combined	6.73	6912.9	42.12	2.5
14	SSR_14_7, SNP_14_34, SNP_14_35, SNP_14_36	Combined	7.71	6912.9-7638.2	42.12-46.53	2.5
15	SNP_15_1, SNP_15_2	Combined	7.75	159.2-174.6	0.97-1.06	2.5
15	SNP_15_22	INA16	10.53	914.2	5.57	2.5
15	SNP_15_24	INA16	9.21	964.0	5.87	2.5
16	SNP_16_17, SNP_16_19	NCA16	10.01	3157.0-3939.8	19.23-24.00	2.5
17	SNP_17_1, SNP_17_2, SNP_17_3	NCA16	10.56	363.3-932.6	2.21-5.68	2.5
17	SNP_17_26, SSR_17_2	NCA15	20.47	6560.0-6802.0	39.96-41.44	2.5
17	SSR_17_2, SNP_17_29, SNP_17_30, SNP_17_32, SNP_17_33, SNP_17_34, SNP_17_35, SNP_17_38, SNP_17_39, SNP_17_40, SNP_17_41, SNP_17_42,	NCB16	8.88	6801.9-8472.3	41.44-51.61	2.5

	SNP_17_43					
17	SNP_17_48, SNP_17_52, SNP_17_53	NCB16	7.54	8769.5-9149.6	53.43-55.74	2.5
18	SNP_18_2, SNP_18_3, SNP_18_5, SNP_18_7, SNP_18_8, SNP_18_9	INA16	13.27	92.6-522.6	0.56-3.18	2.5
18	SNP_18_9	INA15	7.28	522.5	3.18	2.5
18	SNP_18_15, SNP_18_16, SNP_18_17, SNP_18_18	NCA15	8.48	679.3-680.8	4.13-4.15	2.5
18	SNP_18_18, SNP_18_19	INA15	7.08	680.7-711.9	4.15-4.34	2.5
18	SNP_18_18	Combined	6.53	680.7	4.15	2.5
18	SNP_18_22	Combined	7.51	942.0	5.74	2.5
18	SNP_18_22, SNP_18_23, SNP_18_25	INA15	6.75	942.0-1666.5	5.74-10.15	2.5
18	SNP_18_23, SNP_18_25	INA16	12.45	1217.3-1666.5	7.42-10.15	2.5
18	SNP_18_32, SNP_18_33, SNP_18_35	INA16	9.98	1894.8-1904.7	11.54-11.60	2.5
18	SNP_18_37, SNP_18_38, SNP_18_39	INA15	6.48	1965.9-2069.7	11.98-12.61	2.5
18	SNP_18_37, SNP_18_38, SNP_18_39, SNP_18_40, SNP_18_41	INA16	11.96	1965.9-2164.2	11.98-13.18	2.5
18	SNP_18_42, SNP_18_44	INA15	7.40	2531.2-3596.6	15.42-21.91	2.5
18	SNP_18_42, SNP_18_44	Combined	6.59	2531.2-3567.2	15.42-21.91	2.5
18	SNP_18_63	NCA15	7.16	8744.9	53.28	2.5
18	SNP_18_64	Combined	6.54	8949.7	54.52	2.5
18	SNP_18_69, SNP_18_70, SNP_18_71	NCA15	8.65	9985.7-10139.6	60.84-61.77	2.5
19	SSR_19_2	NCA15	6.42	487.2	2.97	2.5
19	SNP_19_40, SNP_19_41	Combined	9.30	3423.9-3622.5	20.86-22.07	2.5
19	SNP_19_41	INA15	7.67	3622.4	22.07	2.5
19	SNP_19_59	INA15	7.16	7315.5	44.57	2.5

19	SNP_19_87, SNP_19_88	INB16	7.27	11426.4-11602.5	69.61-70.68	2.5
19	SNP_19_119, SNP_19_120, SNP_19_121	INA16	10.07	13513.6-13600.9	82.33-82.86	2.5
19	SNP_19_179, SNP_19_180	INA16	9.32	17690.2	107.77	2.5
19	SNP_19_188	INB16	7.79	18299.4	111.48	2.5
20	SNP_20_1	NCA15	6.57	100.5	0.61	2.5
20	SNP_20_27	NCA15	6.46	2695.3	16.42	2.5
20	SNP_20_27, SNP_20_28	INA16	9.39	2695.3-2704.5	16.42-16.48	2.5
20	SNP_20_27, SNP_20_28	Combined	7.15	2675.3-2704.5	16.42-16.48	2.5
20	SSR_20_3	NCA16	9.35	2708.6	16.50	2.5
20	SNP_20_101, SSR_20_6, SNP_20_102	INA16	11.21	11537.4-11757.7	70.29-71.63	2.5