

## ABSTRACT

MALONEY, PETER VINCENT. New Advances in Fall Sown Oat Winter Hardness. (Under the direction of J. Paul Murphy.)

Fall sown oats (*Avena sativa* L.) are plagued by a poor ability to tolerate freezing temperatures. Of all the fall sown small grain cereals, oats have the poorest winter field survival. Advances in marker technology and mapping techniques have allowed for more efficient and accurate location of quantitative trait loci (QTL). With these new technologies, breeders can more accurately screen early segregating generations for winter hardiness component traits.

The objectives of this research were: (i) screen and validate new microsatellite or simple sequence repeat (SSR), single nucleotide polymorphism (SNP), and cleaved amplified polymorphic sequences (CAPS) markers; (ii) map new SSR, SNP, and CAPS markers to the Fulghum (winter tender) x Norline (winter hardy) recombinant inbred population (iii) screen the Fulghum x Norline population for QTL linked to winter hardiness component traits and (iv) develop an association mapping population to test for marker associations across a wider oat genetic base for winter hardiness component traits and validate marker use in marker assisted selection (MAS).

Selected primer pairs derived from oat, including 315 SSR primers, four SNP markers and one CAPS marker were tested on a panel of 11 oat lines. Two hundred fifty two of the 315 primers amplified products in oat, and 168 were polymorphic for at least one of the 11 oat lines tested. Markers supplied by Dr. Joseph Anderson, USDA-ARS (JAO) were screened and PIC scores were generated. Among the JAO primers, 106 were

co-dominant and 11 were dominant makers. Polymorphic information content (PIC) scores were generated for JAO primers with an average PIC score of 0.64 and an average of five alleles per primer pair.

Sixty-five new SSR markers, four SNP markers and one CAPS marker were added to the Fulghum x Norline linkage map. This brought the total number of markers mapped on the population to 101. The map contained 19 different linkage groups for a total distance of 326.9 cM. Four major QTL were identified for winter field survival. Norline contributed three of the QTL and Fulghum contributed one QTL for increased winter field survival. Most of the winter field survival QTLs were located around the TC7-17 translocation event characteristic of Norline. Other QTL were identified for crown freezing tolerance, photoperiod effect, vernalization effect, heading date, and plant height.

An association mapping population comprised of 63 fall-sown and spring-sown oats was selected for testing. Cultivars selected were released anywhere from 1775 to 1995 and consisted of two facultative, 25 spring sown and 36 fall sown type cultivars. The cultivars were chosen based on their lineage and significance to the oat breeding community. The basis of the research was done on 29 unlinked simple-sequence repeat markers. The population was phenotyped for crown freezing tolerance and winter field survival. An admixture model in Structure v3.2.1, was used for subpopulation analysis, where we showed eight sub populations. Tassel 2.1 was used to conduct all the association mapping techniques including kinship and the mixed linear model. Association mapping yielded six loci linked to traits of interest. The six loci found are readily available to be used in a marker assisted selection program.

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New Advances in Fall Sown Oat Winter Hardness

by  
Peter Vincent Maloney

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APPROVED BY:

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David Marshall

---

David P. Livingston

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Gina Brown-Guedira

---

J. Paul Murphy  
Chair of Advisory Committee

## **DEDICATION**

I would like to dedicate this work to my Parents. To my father for teaching me everything I know about agriculture and helping me focus my studies and to my mother for all the encouragement and support she has showed me throughout my advanced studies. Without both of them I would never have made it as far as I have.

## **BIOGRAPHY**

Peter Maloney was born in Indiana in 1984. He grew up on a farm in his home town of Brownsburg, IN. He graduated from Brownsburg Community High School in 2003 and proceeded to attend Purdue University in West Lafayette, IN. He graduated from Purdue in spring of 2007 with a major in Crop and Soil Science. During the summer of 2007 he began work for Dr. J. Paul Murphy in the small grains department at North Carolina State University. His dissertation research consisted of applying new advances for the improvement of winter hardy oat breeding. He plans to continue on to his PhD under the direction of Dr. J. Paul Murphy.

## ACKNOWLEDGEMENTS

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Lastly I would like to thank all my friends and family for all the help they gave me either in the classroom or out. Epicurus put it quite well, “It is not so much our friends' help that helps us, as the confidence of their help.” Without their support I surely would have never found the strength to continue through with my goals.

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**Chapter I**

**LITERATURE REVIEW**

## Oat Background

The annual US production of oat was about 78 million bushels in 2007 (FAO 2007). Oat production in the United States and around the world has declined over the past few decades due to the drop in on farm feed use. The United States production alone has been cut by over a third since 1990 (308 million bushels).

Oats were unknown in the Americas before Columbus. Two types of hexaploid cultivated oat were introduced to the Americas. The Spanish cultivars (*Avena byzantina* C. Koch) brought into the southern latitudes were of Mediterranean origin and the English cultivars (*A. sativa* L.) brought into the northern latitudes were of northern European origin. In the interim there has been widespread intermingling of the two germplasm pools in breeding programs. Contemporary cultivated hexaploid oats are generally classified as *A. sativa*, but Zhou et al. (1999) and Jellen and Beard (2000) illustrated their separate ancestry based on an interchromosomal translocation and molecular marker profiles.

*Avena sativa* is a hexaploid with 42 chromosomes ( $2n = 6x = 42$ ). Other species of oat occur naturally at the diploid, tetraploid and hexaploid level. Common cultivated oat has the A, C, and D genomes while the tetraploid species contain the A and D or A and C genomes (Hayasaki et al. 2000). Diploid species contain either the A or C genomes.

Oat is primarily used for livestock feed. Oat is fed to dairy cows, horses, mules, and turkeys. However feed oat utilization has declined since the 1950's (Buerstmayr et al., 2007) and was replaced by corn (*Zea mays* L.) in animal diets. Oat is also utilized in food products.

Oat consumption has been found to lower cholesterol and blood glucose levels (Wood et al., 2007; Nilsson et al., 2008), which has led to increased human consumption of oat over the last 20 years.

Most of today's U.S. cultivars are descendents of a limited number of introductions (Stanton, 1955). Important early cultivars and landraces include Red Rust Proof, Winter Turf, Victoria, Green Russian, Markton and Red Algerian. Red Algerian and Red Rust Proof (thought to be a selection from Red Algerian) were both introduced into the Mexico-California area before 1811. Red Algerian is thought to have originated in Algeria and Red Rust Proof from the Mediterranean region of Europe. Winter Turf was first introduced into Virginia from England and was cultivated by George Washington at Mount Vernon. Victoria was introduced into the US in 1927. It is thought to have originated from a cultivar grown for many years in Uruguay. Green Russian may have been introduced to Iowa or Minnesota in 1870 by Russian or some other European immigrant. Markton was a selection from an unnamed variety in Turkey. It was introduced into the US in 1904 (Stanton, 1955).

The domestication of oat is similar to many other cereal species such as wheat (*Triticum* spp.), barley (*Hordeum vulgare* L.), and rye (*Secale cereal* L.). Hexaploid oat is thought to have originated in the Fertile Crescent and likely has a common ancestor with wheat, rye and other wild weed species (Murphy and Hoffman, 1992). Hexaploid oat was likely domesticated in northern Europe having been transported there in an admixture of wheat or barley (Murphy and Hoffman, 1992).

## **Winter Hardiness**

Winter hardiness is a complex trait that encompasses tolerance to all adverse winter variables such as freezing temperatures, soil heaving, and reduction in vigor due to disease. The many factors influencing winter hardiness include, but are not limited to, heading date, vernalization response, photoperiod, and crown meristem freezing tolerance. Crown meristem freezing tolerance is the most important quantitative trait contributing to winter hardiness in oat (Marshall, 1965; Livingston et al., 2004).

Freezing damage occurs when water inside the plant cell becomes subject to ice formation. This can cause three different ice induced stresses on the plant: intracellular ice formation, adhesion and freeze dehydration. Intracellular ice formation is a sudden and destructive process of ice formation inside the plant cell. This can cause extreme damage to the cell resulting in death (Steponkus, 1984). In most cold hardy plants, however, as the temperature lowers the plant excludes water from its protoplast causing the water to freeze in the intracellular space (Steponkus, 1984). This causes a drop in the osmotic pressure outside the cell and water diffuses out of the cell causing an increase in the concentration of dissolved salts in the protoplast, which effectively stops ice formation (Steponkus, 1984; Olien and Smith, 1977).

Ice adhesion is caused by the competition for water in plants experiencing below freezing temperatures. With the drop in usable water, plant cells will compete with the growing ice formations for water. The competition for water can lead to the cells adhering to

the ice formations (Olien and Smith, 1977). This can cause distortion of the cell membrane and result in more internal damage to the cell, possibly involving plant death.

Freeze dehydration is caused by the lack of usable water inside a plant cell. As intracellular ice is formed, more water diffuses out of the cell changing the osmotic pressure and the pH inside the cell. This can have adverse effects on the cell wall and the other organelles inside the cell. As the pH changes, normal cell functions can be disrupted and cause cell damage (Steponkus, 1984). It can also cause cell death, depending on exposure and duration. Phenotypically this looks like a plant response to drought.

Crown freezing tolerance contributes the most to a plants ability to survive harsh winter climates. Crown freezing tolerance can be classified as the crown's ability to tolerate below freezing temperatures (Livingston, 1996). It has been hypothesized that crown freezing tolerance accounts for most of the winter hardiness in oat.

## **Genetics**

### ***QTL***

Quantitative Trait Loci "or QTL" are areas in the genome that contain alleles that are associated with a quantitative trait. QTL can consist of one or more genes that contribute to the expression of some phenotype. To find QTL one must usually have mapped markers on

the genome and find a correlation between the presence of a marker and a phenotypic response.

One of the most common sources of phenotypic and marker data is recombinant inbred lines (RIL). These are inbred lines derived from crossing two parents that differ phenotypically for the trait under investigation. Combining phenotypic data and marker data collected from a population of RIL can lead to the identification of QTL. The accuracy of mapping QTL is directly proportional to the population size, marker density and the quality of the phenotypic data collected. The larger the population the more recombinant events observed and the higher resolution the QTL map. Accurate tagging of QTL that control a low heritability trait such as freezing tolerance is particularly beneficial to the plant breeder because marker assisted selection enhances phenotypic selection.

One methodology utilized in mapping QTL is interval mapping. This method uses a highly complex number of statistical equations and a linkage map in order to find the location of QTL. A statistical t-test of a regression model can be used and does not require a linkage map. But the ability to detect QTL is smaller and the accuracy is greatly reduced (Falconer and Mackay, 1996). Nevertheless, the latter approach is necessary when polymorphic markers are identified among recombinant inbred populations (RIP), but cannot be incorporated into a linkage map.

## **Markers**

### *Microsatellite or SSR markers*

Simple Sequence Repeats (SSR) are Polymerase Chain Reaction (PCR) based markers. These are markers developed from simple repeats in the genome. Most repeats used are 2 to 5 base pairs in length and are found in tandem (thus their alternate name, Short Tandem Repeats (STR)). These markers are developed by locating a unique primer sequences that flank each side of the repeat. This primer plus genomic DNA undergoes the PCR amplification whereby a specific sequence of DNA is amplified. The size of the resulting fragment varies depending on the number of repeats that are present between the primers. A polymorphism is observed when the number of repeats present is different between the two parents of the RIP.

### ***EST derived markers***

Expressed Sequence Tag (EST) derived markers are usually Microsatellite or Single Nucleotide Polymorphism (SNP) markers made from a cDNA library. mRNA is first collected from the plant or animal species of interest. Then, by use of reverse transcriptase the RNA produces a copy of DNA called cDNA. This cDNA approximates the sequence of the transposable DNA located in the host cell. The sequence of the cDNA can be utilized to develop markers. The drawback is that it is unknown where the DNA is located in the genome without having a sequenced genome, but it is a quick and cost effective way to recover markers. ESTs have been used to show the synteny between cereal crops like rice, wheat, and maize and match certain sequences to known gene functions (Ahn et al., 1993). Other plants like Arabidopsis have also been used to show similarity in sequences (Brautigam, 2005).

### ***Association Mapping***

Association mapping is a method of gene detection that utilizes linkage disequilibrium between markers and QTL to identify loci of interest to the researcher. In contrast to association mapping, Linkage mapping takes advantage of recombination events between two parents to locate loci of interest. Association mapping draws on the correlation between phenotypic information and genotypic information in a sample population resulting

from linkage disequilibrium. Linkage disequilibrium is identified by the non random correlation between alleles at a pair of markers. By using association mapping one can draw information from a larger germplasm base than a traditional QTL study. Through the use of association mapping, better marker trait associations can be identified and put directly into marker-assisted selection programs (Jannink et al., 2001). The down side to this approach is that association mapping is less powerful at detecting rare QTLs than in tradition linkage studies.

Association mapping is based on the historical and evolutionary recombination events at the population level (Nordborg and Tavaré, 2002; Risch and Merikangas, 1996). In contrast to standard linkage analysis, association mapping offers superior mapping resolution, a reduction in research time, and a greater number of alleles to work with (Yu and Buckler, 2006; Zhu et al., 2008). Association mapping has been gaining popularity since its debut in plants. It uses the statistical association between genotypes at a given locus and a phenotype. The strong correlation between the marker and the phenotype gives significant evidence to conclude that there is a close physical linkage between the marker and the QTL of interest.

Association mapping uses can be categorized into two main areas of research, candidate gene association mapping and genome-wide association (Zhu et al., 2008). Candidate gene association mapping connects polymorphisms in a select candidate gene to the phenotypic variation seen for specific traits, where as genome wide association mapping

scans through the whole genome to locate areas of association for complex traits (Risch and Merikangas, 1996).

Care must be exercised in Association mapping studies that population structure does not result in false associations not linked to causative loci. These associations can occur when the subpopulations show a variable phenotypic frequency (e.g., Lander and Schork, 1994). This will cause any marker allele at high frequency within a particular subpopulation to be associated with the phenotype (Ewens and Spielman 1995; Pritchard and Rosenberg 1999). Through the use of association mapping larger Type I and Type II error rates are seen. False positives, or Type I error, can arise from subpopulation structures that are not accounted for in the model (Kennedy et al. 1992). Type II error rates, or failure to identify positive results can be inflated because of three different factors: I) the decay of LD over time which causes a lower correlation of genotypes to phenotypes, II) presence of alleles at different frequencies in the sample population, and III) strict genome-wise significance threshold caused by multiple-testing problem (Carlson et al. 2004; Breseghello, F. and Sorrells, M.E. 2005).

## **Genetics of crown freezing tolerance**

### ***Previous Research***

Research was begun by David Wooten a PhD Graduate at NCSU - Crop Science Department. David used different marker systems to develop a partial linkage map in a targeted region in the Fulgum/Norline recombinant inbred population. The RIL used in the experiments were phenotyped and screened for QTL linked to winter hardiness component traits. He concluded there was a link between the barley marker HVM20 and the translocation event T7C-17. This translocation is known to have certain QTL associated with winter field survival and crown freezing tolerance (Santos et al., 2006; Wooten et al., 2007; Wooten et al., 2008; Wooten et al., 2009).

My research built upon Wooten's work to place more makers (mainly SSR markers) on the Fulghum x Norline linkage map, associate more QTL to the linkage groups, and develop an association mapping population to further the ability of marker assisted selection for winter hardiness.

### ***Objectives***

- 1. Place more SSR markers on the Fulghum / Norline linkage map.*

2. *Find additional markers linked to the winter hardiness component trait QTL in the Fulghum / Norline recombinant inbred population.*
3. *Search for additional winter hardiness component trait QTL in the Fulghum / Norline recombinant inbred population using the new linkage map enhanced with additional SSR markers.*
4. *Evaluate a selection of historically important winter oat cultivars for crown freezing tolerance using controlled environment and field evaluations.*
5. *Place markers on the association mapping population and screen for marker associations to winter hardiness component traits*

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## **Chapter II**

### **MARKER DEVELOPMENT AND QUANTITATIVE TRAIT LOCI IN A FALL-SOWN OAT RECOMBINANT INBRED POPULATION**

## **Abstract**

*Avena sativa* L., or common cultivated oat, has the poorest winter hardiness among the small grain cereals. Marker-assisted selection for improved winter survival in oat is difficult because the number of SSR markers available in this species is limited. The objectives of this research were to increase the number of SSR markers on the 'Fulghum' x 'Norline' recombinant inbred population genetic map, and to scan for QTL associated with winter hardiness component traits, including winter field survival, crown freezing tolerance, vernalization response, and heading date. SSR markers were developed from 'Kanota' and 'Ogle' genomic DNA libraries that were enriched for eight microsatellite motifs. New primers were evaluated for amplification, reproducibility, and polymorphism in 11 oat lines. SSRs showing high-quality polymorphism between Fulghum and Norline were subsequently examined in 128 recombinant inbred lines. Sixty-five new SSR, four SNP and one CAPS markers were added to the Fulghum x Norline linkage map. This brought the total number of markers mapped on the population to 101. Phenotypic data for winter hardiness component traits in the population were obtained in field and controlled chamber experiments. All previously mapped markers and new SSR markers were evaluated and QTL identified. Marker loci on linkage group FN3 accounted for multiple QTL associated with winter hardiness component traits. The addition of numerous new SSR markers to the Fulghum x Norline map in regions with winter hardiness component trait QTL will enhance marker assisted selection for these important traits.

## Introduction

Oat (*Avena sativa* L.) production in the United States dramatically declined during the latter part of the 20<sup>th</sup> century due to shifting farm practices (Murphy and Hoffman, 1992). Consequently, oat breeding and genetics research has not kept pace with other small grains, such as wheat (*Triticum aestivum*) or barley (*Hordeum vulgare* L.), due to reductions in the number of oat researchers and research funding. Nevertheless, oat research continues, and the utilization of new technologies is vital for more efficient genetic advance. Molecular marker research in oat was initiated in the 1980's and genetic maps were constructed based primarily on RFLP's (O'Donoghue et al., 1995; Hoffman et al., 2006; Portyanko et al., 2001). Mapping studies based on AFLP's were also reported (Jin et al., 2000). Other marker-based research in oats included the use of some wheat, barley and fescue (*Festuca arundinacea*) markers (Hu et al., 2007; Jensen et al., 2007), the development of new PCR based markers (Li et al., 2000; Song et al., 2005), and QTL analysis of important traits (Orr et al., 2007). Nevertheless, oat has lagged behind the other small grains in the development of PCR based markers and map construction. As a result, marker assisted selection has not become a routine procedure in oat cultivar development.

Microsatellite, or simple sequence repeat (SSR), markers are PCR based and use simple repeated sequences in the genetic code as bases for polymorphisms. Their distribution throughout the genome, variability in the number of repeats within a species, and ease of use made microsatellites desirable for a variety of applications including genetic map

construction, genetic diversity estimation, and marker-assisted selection (Roder et al., 1998; Pal et al., 2002; Song et al., 2005; Nersting et al., 2006; Yu and Herrmann, 2006; Becher, 2007; Fu et al., 2007). Relatively few SSR markers have been reported for oat and placed on genetic maps. Becher et al. (2007) used 326 expressed sequence tags (ESTs) to develop 216 new SSR markers, and mapped 51 of them in the ‘Kanota’ x ‘Ogle’ population. Jannink et al. (2005) used sequences available in GenBank to develop 32 new SSRs, and placed 20 loci from 16 markers on the ‘Ogle’ x ‘TAM-O301’ map. SSRs from related grass species have also been evaluated for amplification and marker development in oat (Hu et al., 2004).

Single nucleotide polymorphisms (SNPs) are the most common type of DNA-based markers and represent the smallest unit of genetic variation (Cho et al., 1999). SNP markers have been developed for a variety of crop species and were utilized for mapping and marker-assisted selection in barley, rye (*Secale cereale* L.), wheat, soybean (*Glycine max* L.), and grapevine (*Vitis vinifera* L.) (Kota et al., 2008; Varshney et al., 2007; Somers et al., 2003; Ha et al., 2007; Vezzulli et al., 2008). However, like other PCR-based markers, few SNPs have been developed and mapped in oat. Sequence information from PCR-based markers was used to develop SNP markers for the crown rust (caused by *Puccinia coronata f. sp. avenae*) resistance gene *Pc94* (Chen et al., 2007). SNP markers linked to short plant stature were reported by Tanhuanpaa et al. (2006).

Quantitative trait loci (QTL) or genes that are associated with winter hardiness component traits were described by Santos et al. (2006) and Wooten et al. (2009) in two recombinant inbred populations of fall-sown oats. Winter hardiness is a complex trait that

encompasses protection of the plant from freezing temperatures, soil heaving, and reduction in vigor due to disease. Wooten et al. (2009) mapped 23 RFLP markers based on their proximity to the chromosome 7C- Chromosome 17 (7C-17) translocation in the ‘Fulghum’ x ‘Norline’ recombinant inbred population, and 21 SSRs developed from oat genomic libraries or from sequences available in GenBank (Li et al., 2000; Jannink et al., 2005). SSR markers were randomly distributed throughout the linkage map, but the RFLP markers were selected for their association with known traits. Multiple QTL associated with winter hardiness component traits were identified, and most of the QTL were localized around the 7C-17 translocation event.

The objectives of this research were to increase the marker density on the ‘Norline’ x ‘Fulghum’ map with newly developed PCR based markers, and to examine marker-QTL associations for winter hardiness component traits in this marker enhanced mapping population.

## **Materials and Methods**

### ***Mapping Population***

One hundred twenty eight random F<sub>6</sub>-derived recombinant inbred lines from a cross between ‘Fulghum’ and ‘Norline’ were utilized. Norline is a winter hardy cultivar developed in Indiana (Patterson and Schafer, 1978), and contains the 7C-17 translocation (Jellen and Beard, 2000). Fulghum was developed by a single plant selection from the land race ‘Red Rustproof’ (Stanton, et al., 1926; Coffman, 1977). Fulghum is a winter-tender cultivar and it does not contain the 7C-17 translocation (Wooten et al., 2007).

### ***Molecular Markers***

#### ***DNA Extraction, Primer selection and PCR***

DNA was extracted from young fresh leaf tissue of the 128 RILs plus parents using the CTAB method (Stein et al., 2001). SSR primer pairs were selected from oat, wheat, and barley primers available in GrainGenes (<http://wheat.pw.usda.gov>), and from published genomic and EST-derived SSR markers for oat (Becher, 2007; Pal et al., 2002), wheat (Song et al., 2005; Gao et al., 2004; Somers et al., 2004; Yu et al., 2004; Pestsova et al., 2000;

Roder et al., 1998; Stephenson et al., 1998), tall fescue (Saha et al., 2004), and barley (Liu et al., 1996). “JAO” primers were provided by Dr. Joe Anderson, USDA-ARS, Purdue University.

SSR Primers were evaluated for amplification and marker quality using two panels. One panel contained six oat lines, ‘Kanota’ (Clav 839), ‘Ogle’ (Clav 9401) , ‘Wintok’ (Clav 3424), ‘Norline’ (Clav 6903), Fulghum (Clav 708), and ‘Rodgers’ (PI 593020), and was utilized for wheat, fescue, and barley primers. The second panel, utilized for the oat primers, contained one Kanota and one Ogle accession provided by Dr. Joe Anderson, USDA-ARS West Lafayette, IN, the Kanota and Ogle accessions from the first panel, Fulghum, Norline, Wintok, Rodgers, ‘Tam O-301’ (Clav 9198), PI220373 (*Avena sterilis* L.), and PI411744 (*Avena sterilis* L.). PCR amplifications were conducted to utilize both the LI-COR 4300 DNA Analyzer (LI-COR Biosciences, Lincoln, NE) and the ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) platforms. Forward primers were modified to include the M13 sequence (5'-cacgacgttgtaaaacgac-3') on the 5-prime end for universal fluorescent labeling (Rampling et al., 2001) with IRD700, IRD800 or labeled directly with NED, FAM, PET, or VIC. Primers were synthesized by MWG Operon (Huntsville, AL).

All PCR reactions contained 1X ThermoPol PCR buffer, 0.25mM dNTPs (New England Biolabs, Beverly, MA), 2X BSA, 0.5U Taq polymerase (New England Biolabs), and 60ng template DNA. Two PCR programs were used for amplification, depending on primer  $T_m$  and the clarity of the banding pattern. Cycling was carried out as written by Rampling et al. (2001) with an added final extension step of 10 min at 72°C, or as written by Becher

(2007), reducing the initial denaturing step to 5 min. Reactions for LI-COR analysis contained 0.4pmol forward primer, 1.5pmol reverse primer, and 1.5pmol M13-labeled primer in a 10µl final volume. PCR reactions for analysis on the ABI were carried out in a 12µl reaction volume with 1.5pmol of forward and 1.5pmol reverse primer.

PCR products were run on either 6.5% denaturing polyacrylamide gels in LI-COR 4300 DNA Analyzers (LI-COR Biosciences) at 48°C, 42W, 35mA, 1500V for 2.5 h, or on an ABI3130 Genetic Analyzer (Applied Biosystems) and analyzed according to the manufacturer's instructions. Fragment scoring was completed using AFLP Quantar 1.0 (KeyGene Products B.V., The Netherlands) or GeneMarker v1.5 (SoftGenetics, State College, PA). Primers that produced polymorphisms between the parents Norline and Fulghum were examined in the 128 F<sub>6:7</sub> RIL

A portion of the oat vernalization locus, *AsVRN1*, was amplified with primers PoidVRN1 and Asintron-rev (Preston and Kellogg, 2008) using 1X ThermoPol PCR buffer, 0.2mM dNTPs (New England Biolabs), 2X BSA, 0.5U Taq polymerase (New England Biolabs), 1.0pmol forward primer, 1.0pmol reverse primer and 60ng template DNA in a 15µl final volume. Cycling conditions consisted of a denaturing step at 95°C for 4 min, 17 cycles of 95°C for 30 s, 60°C for 30 s (-0.5°C per cycle), 72°C for 30 s, 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension step of 72°C for 5 min. PCR products were run on 1.5% agarose gels and visualized with ethidium bromide. Amplification products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH) following the manufacturer's instructions and directly sequenced by Genewiz (South Plainfield, NJ).

### *Band Recovery for Sequence Analysis*

PCR amplification was completed as written for the LI-COR 4300. Band recovery utilized the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences) according to the manufacturer's instructions with the following modifications. The grid-drawing step was eliminated. One 25mm spacer was cut with a notch and used as a spacer on the gel surface during the scan. After the scan was complete, the image was saved and imported into Adobe Photoshop v. 7.0 (Adobe Systems, Inc., San Jose, CA). The image size was adjusted to 25cm to match the gel size, and the resulting image was printed. The plate containing the gel was placed over the printed image, with the notch cut into the spacer exactly matching the notch shown on the printed image. Bands were excised while looking at the printed image underneath the gel. A second scan was completed to confirm that the fragments of interest were successfully excised.

The gel pieces were suspended in 50 $\mu$ l of water, incubated overnight at 4°C, and then incubated for 20 min at 95°C. Fragments were re-amplified in a 20 $\mu$ l reaction using 2 $\mu$ l of the gel solution as the DNA template, and using either the locus-specific primer or un-labeled M13 primer as the forward primer in the reaction. The PCR program consisted of an initial denaturing step of 95°C for 4 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension step of 72°C for 7 min. Successful re-amplification was verified on 1% agarose gels and PCR products were purified using the QIAQuick PCR Purification Kit (Qiagen, Valencia, CA). Sequencing was completed by MWG Operon

(Huntsville, AL) or Genewiz (South Plainfield, NJ). Sequences were evaluated using 4Peaks v.1.7 (Griekspoor and Groothuis, 2005). Sequence alignments were completed using ClustalW (Thompson et al., 1994).

### *CAPS Marker Development*

Oat-specific primers were designed using Primer3 software (Rozen and Skaletsky, 1998), and were synthesized by MWG Operon. Primers were tested for amplification using the PCR conditions for LI-COR analysis, reducing the final dNTP concentration to 0.2mM. Fragments were amplified in the oat lines Norline and Fulghum, and sequenced in both directions. Sequences were aligned using ClustalW and compared to identify single nucleotide polymorphisms (SNPs). The sequence surrounding each SNP was analyzed using NEBCutter V2.0 (New England Biolabs) to identify appropriate restriction sites for CAPS marker development. Amplification for the CAPS procedure was completed using oat specific primers in a 20 $\mu$ l reaction volume containing 1X ThermoPol PCR buffer, 0.2mM dNTPs (New England Biolabs), 1.0pmol forward primer, 1.0pmol reverse primer, 2X BSA, 0.5U Taq Polymerase (New England Biolabs) and 60ng genomic DNA. The following PCR program was used for amplification: initial denaturing step of 95°C for 4 min, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension step of 72°C for 7 min. A 7 $\mu$ l aliquot of the PCR reaction was digested for 3 h with 2U of restriction enzyme *MfeI* (New England Biolabs) and 1X appropriate buffer, based on the manufacturer's

instructions. Restriction digested PCR products were resolved by electrophoresis on 2.5% agarose gels and visualized with ethidium bromide.

### *SNP Marker Development for Luminex 100*

Oat ESTs were selected from the database at NCBI (<http://www.ncbi.nlm.nih.gov>). Primers were designed from these ESTs and used to amplify 400-600bp fragments in the oat lines Norline and Fulghum. PCR was carried out in 10 $\mu$ l reactions containing 1X ThermoPol PCR Buffer, 0.2mM dNTPs (New England Biolabs), 0.5pmol forward primer, 0.5pmol reverse primer, 2X BSA, and 0.6U Taq Polymerase (New England Biolabs). Cycling conditions were of 95°C for 4 min, 17 cycles of 95°C for 30 s, 60°C for 30 s (-0.5°C per cycle), 72°C for 30 s, 25 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, and a final extension step of 72°C for 5 min. Successful amplifications were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced by Genewiz Sequences were examined using 4Peaks (v.1.7), alignments were completed using ClustalW, and SNPs were identified by visual inspection.

Additional primers were generated and used to amplify a 150-200bp PCR product containing the SNP as written. A 1:10 dilution of the PCR product was incubated with 1U of shrimp alkaline phosphatase (USB, Cleveland, OH) and 1U of Exonuclease I (USB) for 1 h at 37°C, followed by 15 min at 75°C, prior to single base extension. Single base extension (SBE) probes were designed to be specific to each locus, terminate one nucleotide 5'-

upstream from the SNP, and contain a tag at the 5' end complementary to a ZipCode sequence that was coupled to a Luminex xMap microsphere (Luminex Corp., Austin, TX). SBE reactions were carried out in a 5µl total volume containing 1X PCR Buffer (Promega, Madison, WI), 3.0mM MgCl<sub>2</sub> (Promega), 6pmol SBE primer, 0.4µM biotin-ddNTP (Perkin-Elmer, Waltham, MA), 0.4µM ddNTP mix (Fermantas, Glen Burnie, MD), 0.192U Thermo Sequenase (Promega) and 2.5µl of cleaned PCR product. PCR cycling consisted of an initial denaturing step of 94°C for 1 min, followed by 79 cycles of 94°C for 15 s, 50°C for 15 s and 72°C for 20 s, and a final extension step of 72°C for 1 min. After cycling, SBE products were precipitated with 75% ethanol and dried overnight in the dark.

SNP genotyping was completed in a 50µl hybridization reaction containing dried PCR products, 1X TMAC buffer (3M tetramethylammonium chloride (Sigma, St. Louis, MO), 50mM Tris (pH 8.0), 4mM EDTA, and 0.1% Sarcosyl (Sigma)) and 3000 coupled microspheres (Luminex). Reactions were denatured at 90°C for 2 min, and then incubated at 54°C for 30 min. The microspheres were labeled with freshly made 0.04mg/µl streptavidin–R-phycoerythrin conjugate (Invitrogen, Carlsbad, CA) in 1X TMAC at 54°C for 30 min. Reactions were subsequently analyzed using the Luminex 100 flow cytometer (Luminex Corp., Austin, TX) with a setting of 100 beads per sample.

### ***Mapping Method***

The linkage map was constructed using JoinMap 3.0 (Kyzama B.V., The Netherlands). A logarithm of odds (LOD) score of 3.0 was used as a minimum threshold of

linkage. The mapping unit used was the Centimorgan (cM). A recombination fraction of 0.300 and the Kosombi mapping function were used to determine the order of the loci.

The genetic linkage maps created in JoinMap 3.0 (Van Ooijen and Voorrips, 2001) were imported into QTL Cartographer 2.5 (Basten et al., 1994) and used to locate QTL linked to winter hardiness component traits as well as plant height and heading date. An initial LD score of 2.5 was used in the Composite Interval Mapping process to locate the major effect QTL. The final locations, along with the QTL effects and variance, were obtained using Multiple Interval Mapping function of the program. Single factor analysis was done using the Proc GLM function in SAS v9.0 (SAS Institute, 2003)) was utilized to determine the significance of the unlinked makers.

### *Polymorphic Information Content*

Polymorphic information content (PIC) values for the “JAO” markers were calculated as described by Anderson et al. (1993):

$$PIC_i = 1 - \sum_{j=1}^k P_{ij}^2$$

where  $P_{ij}$  is the frequency of the  $j^{\text{th}}$  allele for the  $i^{\text{th}}$  marker in the 11 oat lines tested. Frequencies were summed over  $k$  alleles for the  $i^{\text{th}}$  marker. Dominant markers were analyzed using the same formula, assuming that each locus showed two alleles, present or absent. Dominant markers cannot have PIC scores above 0.5 by this method. More detailed

information for the “JAO” primer pairs, including expected fragment size, marker quality index, number of alleles, and PIC values, can be found in supplementary table 1.

### ***Phenotypic Evaluations***

Phenotypic evaluations on the 128 RILs of the Fulghum x Norline population were reported previously in Wooten et al. (2009). Briefly, crown freezing tolerance was evaluated at -10°C using a five replicate incomplete blocks within complete replications design in a controlled environment chamber. After three weeks of re-growth, recovery for each crown was visually measured on a scale of 0-10 (0=complete plant death, 10=no freezing damage). Winter field survival was evaluated using a randomized complete block design with five replications in each of three environments. The experiment was evaluated at the Upper Mountain Research Station (895m elevation) near Laurel Springs, NC in the 2003 and 2004 seasons, and at the Virginia Tech College of Agriculture and Life Sciences Kentland Research Farm (530m approximate elevation), near Blacksburg, VA in the 2004 season. Field survival was estimated for each plot after spring re-growth as the percent survival for the plots corrected for plot variation in germination.

Heading date was evaluated in a two replicate randomized complete block design at the Cunningham Research and Education Center, Kinston, NC during the 2003 and 2004 seasons. Heading date was recorded as the day of the year when 50% of panicles had emerged. Plant height was evaluated at the same location in the 2004 season. Plant height

was measured as the distance between the soil surface and the tip of the panicle of an average plant.

Photoperiod and vernalization responses were evaluated in a growth chamber experiment at the Southeastern Plant Environment Laboratory at North Carolina State University. A split-plot factorial design with three replications over time was used. Photoperiod was the whole plot factor, and vernalization and genotype were factorial sub-plot factors. The ‘vernalization effect’, ‘photoperiod effect’ and the ‘per se maturity alone’ data was all log transformed to normalize the error variances.

## Results

### *SSR markers*

Primer pairs derived from oat, included 41 AME SSRs from Becher (2007), one AM SSR from Pal et al. (2002), and 273 JAO primers from Dr. Joseph Anderson, USDA-ARS were selected for evaluation. Two hundred fifty-two of the 315 primers amplified products in oat, and 168 were polymorphic for at least one of the 11 oat lines tested. Among the JAO primers, 106 were co-dominant markers and 11 were dominant makers. JAO primers amplified an average of five alleles, with an average PIC score of 0.64. PIC values for the AME SSR primers were given in Becher (2007). Five primers pairs mapped to multiple loci; AME177, AME184, AME23, JAO233-258, JAO4818.

Of the 522 wheat genomic SSR primer pairs tested, 72 generated reproducible fragments in oat and were selected for additional study with Fulghum and Norline. Thirty primer pairs produced reproducible products in a sub-sample of the Fulghum x Norline population. Thirteen were polymorphic, and 12 produced dominant markers. Four wheat SSRs, *xcfd15*, *xcfd18*, *xcfd33*, and *xgwm391*, were placed on the Fulghum x Norline map.

Amplification products generated with wheat genomic SSR primer pairs mapping to known linkage groups were selected for band recovery and sequence analysis. Sequencing via band recovery generated 1865bp of sequence from Norline and 1851bp from Fulghum.

One co-dominant set of fragments, amplified with wheat primer *xcfd15*, contained an (AT)<sub>6</sub> repeat. Oat specific primers were designed to generate a cleaner PCR product. This marker mapped to linkage group FN3 and was designated *xncs15-3*. *Xcfd15* mapped to deletion bin C-1DS3-0.48 on the wheat Chinese Spring deletion map. To examine the possibility of transferability of other wheat SSR markers from this region to oat, additional primer pairs from Chinese Spring deletion bin C-1DS3-0.48 were tested for amplification. These primers did not yield any reproducible products.

The sequence of the amplification product generated with *xcfd18* primers contained three SNPs. The G/A polymorphism located at 147bp was used to create oat specific SNP primers and single-base extension primers for mapping using Luminex technology. This SNP, designated *xncs4-18*, mapped to linkage group FN7. A G/T polymorphism at 229bp occurred in a restriction enzyme recognition site. A CAPs marker, *xncs5-18C*, was produced by digesting PCR products amplified with oat-specific primers with *MfeI*. The restriction digest yielded two fragments in Fulghum (173bp and 103bp) and three fragments in Norline (121bp, 103bp, and 52bp). This marker mapped to linkage group FN7. The fragments from dominant markers *xgwm391* and *xcfd33* did not contain any SNPs or SSRs.

Fulghum and Norline sequences generated above were compared to sequences in GenBank using BLASTN. The sequences from *xgwm391* had a high level of sequence identity to GenBank sequence AY083682.1, described as *A. sativa* OP45 receptor kinase gene cluster (Cheng, D.W. unpublished). Other sequences did not return significant results.

Of 18 tall fescue primers, two did not amplify, and nine amplified non-polymorphic fragments. The remaining seven primer pairs amplified fragments that were polymorphic for at least one oat line in the testing panel, but were not useful for mapping in the Fulghum x Norline population.

### ***EST-derived SNP markers for oat***

Currently, there are about 7600 oat ESTs in Genbank and two approaches were used for selecting candidate ESTs. The oat SSR marker *xncs15-3*, derived from the wheat SSR *xcfd15*, mapped to linkage group FN3 in the Fulghum x Norline population in a region previously identified as an important QTL for winter hardiness (Wooten et al., 2009). *Xcfd15* is in bin C-1DS3-0.48 on the wheat Chinese Spring deletion map. In the first approach, 53 wheat ESTs from this deletion bin were used to identify oat ESTs for marker development through similarity searches. Nineteen oat ESTs were identified and primers were designed for 12 of them. In the second approach, 156 sequences associated with cold acclimation and sub-zero acclimation in wheat (Herman et al., 2006) were used to select candidate oat ESTs. Thirty-nine oat ESTs were identified and primers were designed for 20 of them.

Primer pairs were designed to amplify single 400-600bp fragments and were tested for amplification in Fulghum and Norline. Of these primer sets, 16 amplified single bands, five amplified multiple products, and 11 did not amplify any product. Single fragments were

directly sequenced to look for SNPs. Sequencing products were aligned with the EST from which they were derived to verify amplification of the expected product. A total of 6994bp were sequenced from Norline and 5948bp were sequenced from Fulghum. Sequencing yielded two SNPs and one single base pair deletion polymorphism from three separate PCR products. SNPs were validated in multiple reactions, and have been designated *xncs1-CN819400*, *xncs2-CN815942*, and *xncs3-CN820496*.

The SNP *xncs1-CN819400* is a G/T polymorphism and maps on the Fulghum x Norline map to linkage group FN7 (Figure 2.1). This SNP was identified through sequence similarity between the wheat EST BE442851, described as wheat seedling root normalized cDNA from *T. aestivum*, and the oat EST CN819400. EST BE442851 maps in deletion bin C-1DS3-0.48 on the wheat Chinese Spring deletion map. *Xncs2-CN815942* is a 1bp deletion polymorphism at the end of a small poly-C region, where an additional 'C' base is present in Norline. Oat EST CN815942 was identified through similarity searches with the sequence HF17H18r-s-at, described as 2e-20 cyclophilin-A-1 *T. aestivum* (Herman et al., 2006). This SNP cannot be placed on the Fulghum x Norline map with the markers currently available. *Xncs3-CN820496* maps to linkage group UNK2 and is a T/C polymorphism. Primers for this SNP were designed using oat EST CN820496, which had sequence similarity to contig 7394\_at in Herman et al. (2006), described as 6e-97 beta expansion protein B2 from meadow fescue.

## *AsVRN1*

A portion of intron one from *AsVRN1* was amplified using primers from Preston and Kellogg (2008). A large size polymorphism was observed between the parents, with a 393bp fragment being amplified from Norline and a 960bp fragment being amplified from Fulghum. Sequencing confirmed a 567bp insertion in Fulghum. The 960bp fragment was amplified from other lines in the oat panel, including Kanota, Wintok, TAM-301, Rodgers and *A. sterilis* PI220373. This polymorphism, labeled here as VRN1, mapped in the ‘Fulghum’ x ‘Norline’ population to linkage group FN16-23 (Figure 1).

## *Linkage Map*

One hundred and one polymorphic loci were screened on the population. The resulting linkage map contained 73 different loci on 19 linkage groups that were located on up to 17 different chromosomes (Figure 1). Thirty seven loci were unlinked. The total length of the map was 326.94 cM. Comparatively, the Kanota x Ogle map contains 29 linkage groups and has a total length of 1890 cM (Wight et al., 2003), and the TAM-O301 x Ogle linkage map contains 34 linkage groups and covers 2049.2 cM. Linkage groups were assigned in accordance with previously named linkage groups (O’Donoghue et al., 1995; Wight et al., 2003; Wooten et al., 2009). Five linkage groups (UNK1-5) containing two to four loci were not associated with any previously named groups. Linkage group FN3 included the T7C-17 translocation event, and was the largest linkage group with 15 loci. The

breakpoint according to the nullisomic line for TC<sup>17</sup> (Wooten et al., 2009) was between the *xcfd15* and *AM270S\_1* loci.

The segregation ratio for 64 markers did not differ significantly from the expected 1:1 ratio. Thirty seven markers, particularly those linked to the translocation breakpoint, showed segregation distortion.

### ***Quantitative Trait Loci***

QTL were identified for all 7 traits phenotyped in this population (Table 2.1). Two main effect QTLs, around 17 cM and 30 cM on linkage group FN3, for both winter field survival and crown freezing tolerance, showed very similar R<sup>2</sup> values to those reported by Wooten et al. (2009) (Table 1). A reduction in the number of significant QTL for certain traits was observed. The major QTL for Crown Freezing Tolerance, CFT1, on FN3 explained over 46% of the variation. Three minor QTL on linkage groups FN3, FN16\_23, and FN22 were also associated with crown freezing tolerance. The combination of three QTL accounted for the 18% of the variation in this trait.

Other QTL were found for Per se Maturity alone, Vernalization effect, Photoperiod effect, Heading date and Plant height. These QTL accounted for 11.6%, 21.7%, 10.4%, 3.9 and 13.1% of the variation explained respectively. QTL were located across FN1, FN3, FN22, FN 24, FN49, FN16\_23, and FN9/21+46\_31+40/42.

## **Discussion**

The addition of 89 SSR, three SNP, and one CAPS locus significantly increased the size of the Norline x Fulghum genetic linkage map in comparison to Wooten et al. (2009). Wooten et al. (2009) selected RFLP markers based on their proximity to the 7C-17 translocation, or their association with vernalization or photoperiod effects. In this study, markers were chosen based on their ability to show polymorphism between the same two parental cultivars. This resulted in more extensive genome coverage and the addition of linkage groups.

JAO primer pairs amplified one to 15 alleles with a mean allele number of five. PIC scores ranged from 0.15 to 0.98, with a mean score of 0.64. The mean number of alleles and PIC scores compare well with the mean of other studies in other small grain cereals (Peng et al., 2005; Hayden et al., 2006; Becher et al., 2007; Chabane et al., 2008).

Research has shown that SSR markers from one grass species can be utilized in related species (Saha et al., 2004; Zhang et al., 2006). To expand the number of markers available for oat, SSR primers from wheat and tall fescue were evaluated for their utility in oat research. After primer screening and validation, only 30 primer pairs amplified consistent fragments in multiple oat lines. This constituted 5.7% of the SSRs evaluated, and was a much lower percentage than other reports of transferability of SSRs across grass species (Hu et al., 2007; Saha et al., 2004; Zhang et al., 2005). A majority of the observed fragments were dominant markers and may not represent true SSRs. Sequences of a subset

of six of these fragments revealed an SSR in only one of them. This suggested that dominant polymorphisms may have been generated by differences in the priming region of one or both primers. Additional fragment sequencing needs to be completed in order to determine if these are true SSR markers. Sequencing these six fragments identified three SNPs, two of which were developed into oat markers. Overall, evaluating SSRs from wheat and tall fescue yielded few markers for the Fulghum x Norline genetic map.

EST databases have been informative for the development of SNP markers for a variety of crop plants including wheat (Somers et al., 2003), barley (Kota et al., 2003), and almond (*Prunus dulcis* L.) (Wu et al., 2008). Sequence information from PCR-based markers was used to develop SNP markers for the oat crown rust resistance gene *Pc94* (Chen et al., 2007). A previous study in sunflower (*Helianthus sp.*) used ESTs with candidate functions in traits to develop SNP markers (Lai et al., 2005). A similar targeted approach was utilized to develop additional SNP markers for oats, focusing on ESTs with candidate functions in cold and sub-zero acclimation processes.

Of 32 primer pairs designed from oat ESTs, half of those amplified single products for direct sequencing. The majority of the sequences for Fulghum and Norline did not contain any polymorphisms. Two SNPs and one single base deletion were identified in three separate PCR fragments. This translated to one SNP every 1983 bases in Fulghum, which is a much lower rate of polymorphism than has been reported in wheat (Somers et al., 2003). Two of these new markers were placed on the Fulghum x Norline map. Designing the SNP markers using candidate sequences did not result in markers that mapped to regions with

known oat winter hardiness QTLs. This approach may be more successful as ESTs are added to the oat database and further research yields additional information about the oat genome.

Amplification of *AsVRN1* intron one generated a large, unexpected size polymorphism between Fulghum and Norline. Sequencing confirmed a 567bp insertion in Fulghum. This polymorphism was consistent, segregated in the population, and mapped to FN16-23. This differs from previous results of amplification in this region where a 395bp fragment was amplified from both Fulghum and Norline (Preston and Kellogg, 2008), and may represent variation among Fulghum accessions. This polymorphism was associated with a minor QTL for vernalization effect. It should be noted that even though a different sized polymorphism was found, the same trait association was identified.

Thirty seven markers exhibited segregation distortion. This is a relatively common occurrence in oats (Portyanko et al., 2001; Wright et al., 2003), and distortion was especially noticeable for markers associated with the translocation event. This was expected because previous research has shown the translocation was inherited in 67.5 percent of the RIL's in the Fulghum x Norline population (Wooten et al., 2007). The cause of this distortion is unknown, but is likely related to the translocation *per se*. (Santos et al., 2006; Wooten et al., 2007). Recombination could be reduced due to the translocation event, causing a distortion in the segregation ratios. Thus, a larger population would be required to more accurately determine the distance between loci. It is hard to conclude what is causing the other segregation distortion in the marker data but this is a common problem seen in other oat linkage map studies. Most of the new SSR markers in the FN3 linkage group mapped to

areas on the distal ends of the linkage group; however *xefd15*, *xncs15-3*, and *AME107* mapped to the translocation event.

### ***QTL***

Winter hardiness component QTL are commonly found linked to one another in clusters. (Kobayashi et al., 2005; Pan et al., 1994; Storlie et al., 1998; Sutka et al., 1999; Toth et al., 2003). Large amounts of QTL clustering were observed around the markers CDO1319, AME23a, AME184b, and JAO4250 in this study. It is possible that the QTL in these regions are linked together in clusters of genes controlling like traits. Santos et al. (2006) found the region around the T7C-17 translocation event associated with higher winter field survival and crown freezing tolerance. Crown freezing tolerance and winter field survival QTL were shown in Wooten et al. (2009) and in this study to cluster in the region associated with the T7C-17 translocation. It could be hypothesized that during the translocation event an adaptive gene cluster was created on the linkage group FN3. Two QTL linked to AM270s\_1 and CDO1319 accounted for around 59 percent of the variation in crown freezing tolerance, and two QTL near UMN433 and CDO1319 accounted for 34 percent of the winter field survival variation in this study. Similar values were reported by Wooten et al. (2009). These loci are not on the translocation event, but in close proximity to the event. Fewer QTL were found for winter field survival, crown freezing tolerance, plant

height, and heading date traits but the variation explained by the QTL was similar. The decrease in QTL can be attributed to the more densely populated linkage map.

The QTL for vernalization and winter field survival in the area around the translocation event were linked in repulsion. This was consistent with findings from Wooten et al. (2009) and Santos et al. (2006). It was hypothesized by Wooten et al. (2009) that the reason for the larger winter field survival effect caused by the translocation event in the Fulghum x Wintok population, was because the vernalization genes were linked in coupling instead of repulsion, unlike in the Fulghum x Norline population. Wintok and Norline have been used for many years as winter hardy checks in the uniform winter oat nursery. Wintok was a line developed from a cross between ‘Winter Fulghum’ and ‘Hairy Culberson’ in the 1940s. Wintok contains T7C-17, as does Norline. It is noteworthy that the QTL associated with AME 184b and AME23a were associated with many of the winter hardiness component trait. This suggests regions of gene clustering located around these marker loci.

The location of two major effect QTLs for the translocation event T7C-17 on linkage group FN3 is believed to identify two different areas associated with the translocation event. It is known that this translocation is a swapping of the distal ends of chromosomes 7C and 17 (Wooten et al., 2007), and the mapping population segregates for this trait. The fact that all the lines do not possess the translocation event causes a distortion when calculating the linkage map. The two main effect QTL for the translocation are associated with previously reported areas of the translocated section of 17 (Wooten et al., 2009) but have been separated with the addition of new markers with different recombination frequencies.

In the previous study by Wooten et al. (2009), more difficult and expensive restriction fragment length polymorphism (RFLP) markers were used because few SSR and SNP markers had been developed for oat. The increase in number and density of SSRs and SNPs on the Fulghum x Norline population in this study should translate into a more productive marker assisted selection program for winter hardiness component traits. For QTL like WFS2, WFS4, CFT2, and CFT3, which were associated with RFLP markers, SSR have been linked to these areas within 15 cM of each QTL. These additional SSR markers can substitute for the previous available RFLP markers for easier and faster marker assisted selection.

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Table 2.1: Component of winter hardiness traits, QTL name, Type of Effect, Linkage Group where QTL is located, associated linkage group, associated marker positions and effects in an oat population of 128RIL developed from a cross of Fulghum and Norline.

Trait	QTL	Type	Linkage Group	Marker	Position	Effect on Fulghum allele	R <sup>2</sup> (%)
Winter Field Survival	WFS1	A	FN3	UMN433	15.1609	-7.1117	18.7
	WFS2	A	FN3	CDO1319	29.1632	-5.9299	15.6
	WFS3	A	FN16_23C	AME184b	2.01	5.258	7.6
	WFS4	A	FN22	CDO504	11.6386	-5.1133	6.8
Crown Freezing Tolerance	CFT1	A	FN3	AM270s_1	21.5328	-1.2703	46.5
	CFT2	A	FN3	CDO1319	30.1632	-0.4274	12.3
	CFT3	A	FN16_23B	CDO1326	19.959	-0.3469	1.3
	CFT4	A	FN22	CDO504	0.01	-0.4616	4.4
Per se Maturity Alone	PER1	A	FN9/21+46_31+40/42	AME23a	0.01	0.4733	7
	PER2	A	FN16_23C	AME184b	0.01	0.4226	4.6
Vernalization Effect	VERN1	A	FN3	JAO4250	4.5658	0.0624	2.9
	VERN2	A	FN9/21+46_31+40/42	AME23	0.01	0.0989	9.7
	VERN3	A	FN16_23B	VRN_1	0.01	-0.0587	4
	VERN4	A	UNK_3	JAO4234	0.01	0.077	5.1
Photoperiod effect	PHOTO1	A	FN9/21+46_31+40/42	AME23a	4.01	0.058	10.4
Heading Date	HD1	A	FN1	JAO8631	6.029	4.1074	3.9
Plant Height	PH1	A	FN24	UMN220a	32.5448	8.9439	13.1

Table 2.2: Marker designations, number of alleles per locus and PIC Scores generated from a panel of 11 oat cultivars.

Marker	n=	PIC	Marker	n=	PIC	Marker	n=	PIC
jao_1080	8	0.84	jao_3758	7	0.81	jao_5203	2	0.17
jao_1130-196	8	0.83	jao_3787-245r	5	0.72	jao_5240-158	2	0.39
jao_1130-218	9	0.83	jao_3787	5	0.75	jao_5240-294	4	0.73
jao_1153	13	0.91	jao_3791	13	0.88	jao_5240	12	0.86
jao_131-315	5	0.73	jao_3823	5	0.73	jao_5243-134	4	0.72
jao_131	7	0.81	jao_3903	8	0.83	jao_5243-140	3	0.67
jao_1372	2	0.50	jao_3939	5	0.64	jao_5247-123	6	0.80
jao_138	4	0.75	jao_3963	7	0.84	jao_573-143	4	0.69
jao_1745	2	0.48	jao_3969-186	12	0.87	jao_5841	4	0.52
jao_176	4	0.70	jao_4002	8	0.86	jao_5871-294	3	0.61
jao_1819	3	0.57	jao_4039	2	0.46	jao_6126	1	0.40
jao_2029	3	0.54	jao_4042	8	0.83	jao_6454	3	0.64
jao_2033	3	0.48	jao_4111	4	0.69	jao_654-141	6	0.77
jao_2091	7	0.71	jao_4149	6	0.71	jao_6895-193	6	0.71
jao_2095	2	0.46	jao_4172-254	2	0.15	jao_6895-312	2	0.32
jao_2141	13	0.89	jao_4234	9	0.84	jao_7042	2	0.49
jao_2280-200	3	0.64	jao_4243	10	0.88	jao_713	3	0.57
jao_2292	4	0.66	jao_4249	2	0.50	jao_7286	2	0.22
jao_233-258	7	0.83	jao_4250	9	0.82	jao_734	3	0.44
jao_2400	2	0.18	jao_4259	5	0.76	jao_761-286	5	0.68
jao_2448	4	0.75	jao_4285	8	0.84	jao_7778	1	0.17
jao_2555	3	0.63	jao_4323	9	0.80	jao_7900	11	0.87
jao_2884	11	0.91	jao_4369	5	0.71	jao_7940	9	0.82
jao_3054	2	0.50	jao_4424-153	2	0.34	jao_8093	11	0.89
jao_3404	2	0.46	jao_4434	1	0.46	jao_8351	10	0.84
jao_3516	5	0.70	jao_4636	7	0.98	jao_8357	2	0.49
jao_353	3	0.63	jao_4646	4	0.65	jao_8480	1	0.17
jao_3540	8	0.79	jao_4715	4	0.72	jao_8491	7	0.81
jao_3557	6	0.69	jao_4750	3	0.65	jao_8631	8	0.77
jao_3558	7	0.80	jao_4768-123	1	0.46	jao_8671	2	0.40
jao_3570	3	0.58	jao_4768-199	1	0.28	jao_8717	6	0.78
jao_3589-185r	9	0.80	jao_4818	9	0.83	jao_8780	4	0.73
jao_3657	15	0.89	jao_4877	2	0.30	jao_8951	6	0.76
jao_3676	11	0.86	jao_4979	2	0.49	jao_898-141	3	0.61
jao_3687	2	0.34	jao_5046-110	2	0.41	jao_898-148	1	0.17
jao_3693	9	0.84	jao_5154-203	6	0.81	jao_9079	4	0.64

Table 2.2 (continued)

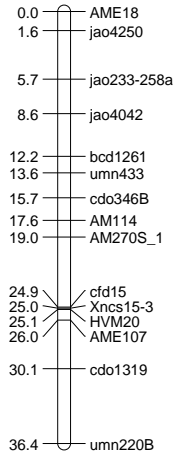
jao_3707	10	0.80	jao_5195-195	0.47	jao_968	1	0.30
jao_3714	2	0.49	jao_5108-100	0.30			
jao_3733-299r	5	0.69	jao_5195	0.71			
					<b>Average</b>	<b>5</b>	<b>0.64</b>

Figure 2.1: Molecular marker linkage map from 'Fulghum' X 'Norline' using 128 F<sub>6</sub> derived recombinant inbred lines. Markers include RFLPs, SSRs, and SNPs and distances are listed by cM positions. Linkage groups are named according to previously identified linkage groups. Five groups are unnamed.

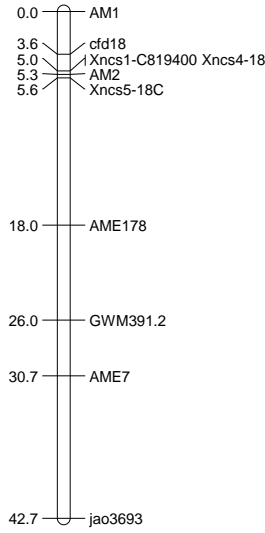
**FN1**



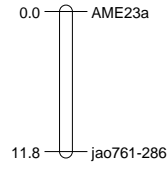
**FN3**



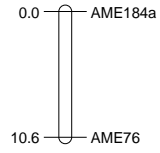
**FN7**



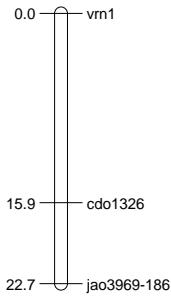
**FN9/21+46\_31+40/42**



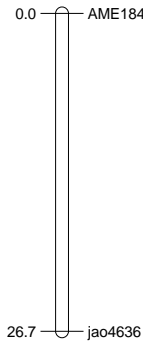
**FN16\_23A**



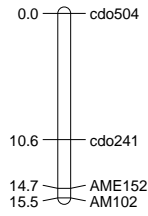
**FN16\_23B**



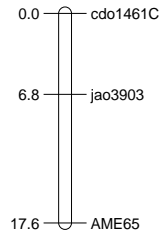
**FN16\_23C**



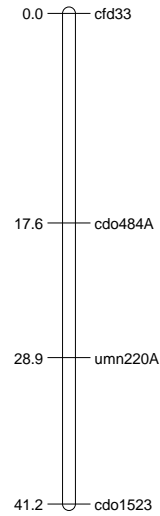
**FN22**



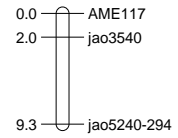
**FN22\_48**



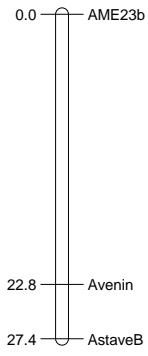
**FN24**



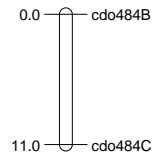
**FN24\_26\_34**



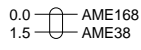
**FN42**



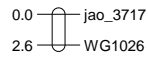
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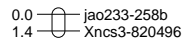
**FN49**



**UNK\_1**



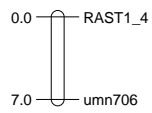
**UNK\_2**



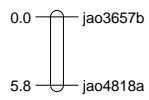
**UNK\_3**



**UNK\_4**



**UNK\_5**



### **Chapter III**

## **ASSOCIATION MAPPING OF CROWN FREEZING TOLERANCE IN OAT**

## **Abstract**

Association mapping is a method used to test the association between molecular markers and quantitative trait loci (QTL). Association mapping uses linkage disequilibrium to detect gene effects. This study utilized association mapping techniques to discover novel genes for crown freezing tolerance using 63 different cultivars of oat. Lines were phenotyped for crown freezing tolerance using an incomplete block design with three replications. Population structure and association analysis was done using 29 simple sequence repeat (SSR) markers. Marker trait association used 135 SSR loci in the mixed linear model (MLM) option in TASSEL 2.1. The subpopulation structure and kinship matrix were used as covariates in the MLM. A total of nine loci were associated with crown freezing tolerance. Some of the associated markers show linkage to winter hardiness component trait QTL in previous studies. These results confirm the existence of the QTL and further strengthen their possible use in marker assisted breeding efforts.

### *Abbreviations*

*QTL* Quantitative Trait Loci; *SSR*, Simple Sequence Repeat, *CFT*, Crown Freezing tolerance; *PCR*, Polymerase chain reaction *MAS*, Marker Assisted Breeding; *MLM*, Mixed Linear Model.

## Introduction

Winter hardiness, or the ability of a plant to remain viable and dormant during winter, is a complex trait involving diverse components such as vernalization response, cold stress, and crown freezing tolerances. Oat (*Avena sativa* L.) is the least winter hardy of the small grains and fall sown production only extends as far north as approximately the 2.8°C isotherm which includes regions of Missouri, Illinois, Indiana, Ohio and Pennsylvania (Marshall and Sorrells, 1992). Progress through selection for winter hardiness is difficult due to the quantitative genetic control and infrequent test winters. Nevertheless, marker assisted selection for winter hardiness traits has become feasible because of developments in QTL mapping, PCR based markers, and advanced computer software (Hu et al., 2007; Tanhuanpää et al., 2008; Tinker et al., 2009; Wooten et al., 2007; Wooten et al., 2008; Wooten et al., 2009; Maloney et al., in review). These recent studies make the testing and location of winter hardiness component trait QTL easier and more applicable for marker assisted selection.

Typical mapping studies in oats have utilized linkage mapping in bi-parental populations of recombinant inbred lines (O'Donoghue et al., 1995; Hoffman et al., 2006, Portyanko et al., 2001; Wooten et al., 2009). These mapping approaches gave useful information on the genome structure and QTL information for which the populations were developed, but were limited to the differences between the two parental lines at the QTL of interest. Association mapping approaches have been investigated in

several plant species because of the potential advantages gained by sampling unrelated individuals in the population, a higher mapping resolution, and a greater number of possible alleles at marker loci because of the larger portion of the genome sampled (Flint-Garcia et al., 2003; Buckler and Thornsberry, 2002). In addition, association mapping can prove useful for validating QTL previously found in linkage analysis studies (Jefferies et al., 2000; Skøt et al., 2007). This allows for better validation of certain loci that may have application in a marker assisted selection program (Jannink et al., 2001, Breseghello and Sorrells, 2006; Gupta et al., 2005).

The association mapping approach was first developed to identify markers linked to human diseases, due to inherent limitations in family structure (Corder et al., 1994; Kerem et al., 1989). Association mapping draws on the correlation between phenotypic and genotypic information in a sample population based on linkage disequilibrium (LD) (Zondervan and Cardon et al., 2004). Only recently has association mapping been applied to plants due to the concerns that population structure caused distortion in the results (Flint-Garcia et al., 2003). It has been utilized in wheat (*Triticum aestivum* L.) to map both kernel size and milling quality (Breseghello and Sorrells, 2006), and *Stagonospora nodorum* blotch resistance (Tommasini et al., 2007). Other association studies have been reported in maize (*Zea mays* L.) (Remington et al., 2001; Liu et al., 2003; Flint-Garcia et al., 2005), *Arabidopsis* (Aranzana et al., 2005) and conifers (*Pinus sp.*) (Gonzalez-Martinez et al., 2007).

Association mapping approaches can be subject to greater type I and type II error rates. False positives, or type I errors, can arise from subpopulation structures that are not accounted for in the model, whereby a marker allele associated with just a single subpopulation may be positively associated with a trait seen in that subpopulation alone. Type II error rates, or false negatives, can also be inflated because of i) the decay of LD over time, ii) the presence of alleles at different frequencies in the sample population, and iii) a strict genome-wide significance threshold caused by multiple-testing problems (Carlson et al., 2004; Breseghello and Sorrells, 2006).

Recent studies have shown multiple QTL for winter hardiness component traits using the biparental populations of Fulghum x Norline and Kanota x Ogle (Wooten et al., 2008; Wooten et al., 2009; Maloney et al., in review). Building on this research, the objectives of this study are: i) to confirm that markers linked to QTL for crown freezing tolerance in the biparental population between ‘Fulghum’ and ‘Norline’ are associated with one another in the association population, ii) to locate new marker trait associations for crown freezing tolerance, and iii) to trace the origin of the crown freezing tolerance significant alleles to a specific ancestral cultivar(s) in the United States cultivated oat gene pool.

## **Materials and Methods**

### *Plant material*

Sixty three contemporary and historically important cultivars of oat, released between 1775 and 1995, were evaluated in this study (Table 3.1). The 25 spring-sown, 36 fall-sown and two facultative cultivars represented North American introductions and a broad sample of cultivars prominent in oat pedigrees during the past century. Two cultivars, Winter Turf and Fulghum, were represented by two accessions due to the variation between the different accessions. Seed of the cultivars was obtained from the United States National Plant Germplasm System (<http://www.ars-grin.gov/npgs/>, verified October 3<sup>rd</sup>, 2008).

### *DNA extraction, Marker selection, and PCR*

DNA was extracted from young fresh leaf using the CTAB method (Stein et al., 2001). Tissue from three plants was collected and bulked for the extraction process. Twenty-nine markers were chosen based on the Fulghum x Norline linkage map reported by Maloney et al. (in review). SSR marker loci were selected based on linkage group, and all linkage groups reported by Maloney et al. (in review) were represented. Three

primer pairs were used based on their proximity to winter hardiness component trait QTL (Maloney et al., in review). Sequences for the SSR primer pairs came from published genomic and EST-derived SSR markers for oat (Becher, 2007; Pal et al., 2002), wheat (Guyomarc'h et al., 2002), and barley (*Hordeum vulgare* L.) (Liu et al., 1996) and are available through GrainGenes (<http://wheat.pw.usda.gov>). “JAO” primers were provided by Dr. Joe Anderson, USDA-ARS, West Lafayette, IN.

Forward primers were modified to include the M13 sequence (5'-caccgacgttgtaaacgac-3') on the 5-prime end for universal fluorescent labeling (Rampling et al., 2001). Primers were synthesized by MWG Operon (Huntsville, AL). All PCR reactions contained 1X ThermoPol PCR buffer, 0.25mM dNTPs (New England Biolabs, Beverly, MA), 0.4pmol forward primer, 1.5pmol reverse primer, 1.5pmol M13-labeled primer, 2X BSA, 0.5U Taq polymerase (New England Biolabs), and 60ng template DNA in a 10µl final volume. Two PCR programs were used for amplification, depending on primer T<sub>m</sub> and the clarity of the banding pattern. Cycling was carried out as written by Rampling et al. (2001) with an added final extension step of 10 min at 72°C, or as written by Becher (2007), reducing the initial denaturing step to 5 min. PCR products were run using 6.5% denaturing polyacrylamide gels in LI-COR 4300 DNA Analyzers (LI-COR Biosciences, Lincoln, NE) at 48°C, 42W, 35mA, 1500V for 2.5 h and analyzed according to the manufacturer's instructions. Fragment scoring was completed using AFLP Quantar 1.0 (KeyGene Products B.V., The Netherlands).

## *Phenotypic data*

### *Crown freezing tolerance*

The experimental design was an incomplete block with three replications over time. Each replicate consisted of eight incomplete blocks with eight random cultivars in each block. A random line was selected to fill the last remaining spot in each replication, but it was not included in the analysis. Each replicate was grown for five weeks at the Southeastern Plant Environment Laboratory at North Carolina State University. The chamber was illuminated for 12 hours each day with the combination of T-12, 1500 ma, cool-white fluorescent and 100 W incandescent lamps that produced  $650 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Daytime temperature was set at 13°C and nighttime temperature was set at 10°C. Ten seeds from each cultivar were planted 1.5 cm deep in 10 adjacent 20 cm long nursery tubes held in racks of 80. Plants were grown in a Fafard 2 Mix (Conrad Fafard, Inc., Agawam, MA) and were lightly watered each day with a complete nutrient solution. After the fifth week (about the five leaf stage), plants were transferred to a hardening chamber held at a constant 2°C with a 12 hour photoperiod. Plants were watered once a day. A complete nutrient solution was provided three times per week.

After three weeks of hardening the plants were transferred to sponges for sub-zero acclimation and crown freezing periods. One plant from each of eight cultivars was placed in a cold, slightly moist sponge. Each cultivar in a single rack was represented in

each of the sponges. Ten plants per cultivar were evaluated totaling ten sponges per incomplete block. Roots and crowns were trimmed to 5cm in length. Sponges were placed in a plastic bag and sprinkled with ice chips to prevent super cooling. The bags were sealed and placed on steel plumbing flanges to provide thermal and structural stability. The units were placed randomly throughout a freezer. The freezer temperature was set at  $-3^{\circ}\text{C}$  for 48 hours for sub-zero acclimation. After the 48 hour sub-zero acclimation period, the temperature was reduced by  $1^{\circ}\text{C}$  per hour until reaching a final temperature of  $-9^{\circ}\text{C}$ . Temperature was held for three hours before rising at a rate of  $2^{\circ}\text{C}$  per hour to a final temperature of  $2^{\circ}\text{C}$ .

Following the freezing cycle, the sponges thawed for 24 hours. The crowns were removed and the roots were trimmed to decrease the chance of molding. Crowns from each set of ten sponges were planted in flats of Fafard 2 Mix in rows of ten, one row per cultivar. The flats were returned to the growth chambers at the Southeastern Plant Environment Laboratory. The temperature and light regimes were the same as described above for the five week growth period. After 3 weeks of recovery, the plants were removed from the flats, washed and visually rated. Ratings were recorded crowns on a zero to ten scale. Zero indicated complete crown death and ten indicated no freezing damage. Values one to nine indicated an increasing amount of both healthy root and leaf tissue. Leaf tip necrosis, tiller number, root mass and re-growth were taken into account during visual ratings for crown scores. This scale is based on Wooten et al. (2007), where both the roots and the crowns were scored together. The distribution of the data

was tested using the univariate procedure in SAS v9.1 (SAS Institute, Cary NC). The Shapiro-Wilk test statistic was used to determine the significance of normality (Shapiro and Wilk, 1965). The data were found to be normally distributed and no transformation was preformed. The phenotypic data were analyzed using the PROC Mixed statement in the SAS v9.1.

### ***Genotypic and Subpopulation Analysis***

Twenty-nine primer pairs were screened on the 63 cultivars. Each marker band was scored as a separate marker locus where alleles were score as either 1 (present) or 0 (absent). Because rare alleles can lead to distortion in the association analysis (Lewis 2002), loci with an allele present at a frequency of less than 0.1 were discarded (Breseghello and Sorrells, 2006).

Subpopulation structure was analyzed using the model-based Bayesian cluster software STRUCTURE 2.3.1 (Pritchard et al., 2000). The parameter set chosen to test the 63 cultivars and 135 alleles used a burn-in period of 20000 and a run length of 20000. The parameter set used an admixture model where markers were considered to have some correlation. The number of subpopulations tested ranged from 1 to 15.

### *Association mapping*

The association analysis utilized TASSEL 2.1 (<http://www.maizegenetics.net>) with a mixed linear model as described in Yu et al. (2006). The model used the subpopulation structure estimated using STRUCTURE 2.3.1 and the kinship matrix was constructed using TASSEL 2.1. Locus significance was determined by p-value and QTL effects were determined by  $r^2$  value.

## Results

### *Phenotypic and Genotypic Data*

Twenty-nine primer pairs, residing on all the linkage groups reported by Maloney et al., (in review), yielded 285 loci with an average of 4.7 loci per primer pair. After discarding marker loci where an allele was present at a frequency less than 0.1, 135 loci identified by the 29 primer pairs, were used for the analysis of population structure and marker trait association.

The mean crown freezing tolerance was 3.09 with a range of 6.98 to 0.38 (Table 3.2). The crown freezing tolerance data was normally distributed with a W-statistic = 0.97 and corresponding p-value of 0.002.

### *Subpopulation analysis*

Population structure was assessed using a clustering method to test the hypothesis of 1 to 15 subpopulations present in the association mapping population (K=1 – K=15). Selection of the subpopulation size was based on the average logarithm of the probability of data likelihood (Ln P(D)). The Ln P(d) for K=1 to K=15 groups was -4407.6, -4282.5, -4179, -4080.1, -4039.4, -3993.3, -3962.6, -3910.4, -3925.1, -4268, -4387.3, -4116.9, -

4166.7, -4411.4, -4870.9, -4480, and -4413. A subpopulation size of  $K=8$  was selected based on the lowest  $\ln P(D)$ . It was not possible to show the origin-related basis for all of the subpopulations. Certain subpopulations were classified into categories of fall sown versus spring sown oats and some were classified by common ancestry. Subpopulation K1 was comprised mainly of cultivars classified as spring sown oats, while subpopulation K6 was comprised mainly of cultivars classified as fall sown oats (Figure 3.1). Subpopulation K3 contained cultivars closely related to the introduction 'Red Algerian'.

### *Association mapping*

Nine candidate loci were found to be associated with crown freezing tolerance. An  $\alpha$ -value of 0.05 was used as the critical value for analysis. Loci were located in the regions around AM102, HVM20, JAO4636, AM114, AME177, AM1, JAO4234, AME178, and xncs15-3 on linkage groups FN22, FN3, FN16\_23C, FN3, FN24\_26\_34, FN7, UNK\_3, FN7, and FN3 respectively (Maloney et al., in review). The  $r^2$  values for these loci ranged from 0.026 to 0.131 for the crown freezing tolerance trait. The largest association, with a p value of  $5.13 \times 10^{-4}$  and an  $r^2$  value of 0.131 for crown freezing tolerance, was located proximal to AM102 with an allele size of 220 base pairs. According to Maloney et al. (in review), this marker locus was linked to the crown freezing tolerance QTL, CFT4, on linkage group FN22. Each of these primer pairs

amplified multiple alleles across the population. The allele sizes for the marker loci, AM102, HVM20, JAO4636, AM114, AME177, AM1, JAO4234, AME178, and xncs15-3, are most highly associated with the crown freezing tolerance, are 220, 143, 277, 260, 189, 219, 260, 182, and 233 base pairs in length respectively (Table 3.3). The marker loci AM102, HVM20, JAO4636, AM114, JAO4234, and xncs15-3 were found match to regions where QTL for winter hardiness component traits were found by Maloney et al. (in review).

## **Discussion**

### *Genotypic analysis*

The association mapping population contained older progenitor lines such as, Red Rustproof, Red Algerian, Winter Turf, Green Russian, Cliff, and RL800, current lines thought to contain winter hardiness QTL such as Rodgers, Coker 716, Dallas, Simpson, Ozark, and Brooks, and other lines commonly used in oat breeding programs. This restricted the cultivars selected for the study, yet even with this restriction a relatively high level of polymorphism was observed. This study averaged 4.7 alleles per primer pair and with a high of eight alleles per primer pair. These results were similar to the polymorphic levels observed in other association mapping studies using hexaploid wheat (Stachel et al. 2000; Breseghello and Sorrells, 2006).

The sample size of this population is larger than some past studies (Tenaillon 2001; Zhu et al., 2003). A larger sample size permitted more of the oat genome to be taken into account when trying to trace the origins of the increased crown freezing tolerance. The use of larger sample sizes can increase detection and quantification of alleles that are present at a lower frequency in the population. Since most oats show very little resistance to freezing temperatures, it was thought wise to increase the size of the sample population.

## *Association Mapping*

A mixed linear model (MLM) was used to avoid false-positive and false-negatives that can arise when conducting an association analysis. MLM reduces both false-positive and false-negative rates through the addition of population structure and kinship as covariates in the model. The MLM detected significant association with crown freezing tolerance at marker loci AM102, HVM20, JAO4636, AM114, AME177, AM1, JAO4234, AME178, and xncs15-3. The marker loci AM102, HVM20, JAO4636, AM114, JAO4234, and xncs15-3 were found to be linked to winter hardiness component trait QTL in the previous study by Maloney et al. (in review). These markers were found linked to QTL CFT4 (AM102), CFT1 (AM114), and CFT2 (HVM20 and xncs15-3) in the biparental population between the parents of Fulghum and 'Norline' (Maloney et al., in review). The markers JAO4636 and JAO4234 were not linked to crown freezing tolerance QTL but were linked to other winter hardiness component traits. The marker JAO4636 was linked to a winter field survival QTL (WFS3) and per se maturity QTL (MAT2). The marker JAO4234 was found linked to vernalization effect QTL (VERN4).

The major crown freezing tolerance loci, CFT1 and CFT2, associated with the T7C-17 translocation event in Maloney et al. (in review) were found on the linkage group FN3. The T7C-17 was shown to be significantly associated with winter hardiness in Santos et al. (2006), but it can cause the general estimates on the importance of certain marker loci to be underestimated. The reason for this is because the translocation event

occurs at low frequency in fall sown oats, and spring sown oats do not exhibit crown freezing tolerance traits (Zhou et al., 1999; Jellen and Beard, 2000). It is thought that an adaptive gene cluster is created during the translocation event, but in the absence of the T7C-17 translocation and therefore the absence of the adaptive gene cluster, the marker loci become less significant.

### ***Origins of the Crown Freezing Tolerance Trait***

It is unknown where the QTLs for increased crown freezing tolerance originated in oat germplasm. The significant marker loci, AM102, HVM20, JAO4636, AM114, AME177, AM1, JAO4234, AME178, and xncs15-3, were used in an attempt was made to show the origin of the increased crown freezing tolerance trait.

These data showed which progenitor lines contributed marker alleles associated with crown freezing tolerance and by default the QTL per se. Though all the progenitor lines contained at least one of the associated alleles found in this study, ‘Winter Turf’ and Fulghum together contained alleles at seven of the significant loci (Table 3.1). Winter Turf contributed alleles at the AM102, AME177, AM1, JAO4234 and xncs15-3 marker loci and Fulghum contributed alleles at the AM102, JAO4636, AME177, AM1, JAO4234, and xncs15-3 marker loci. However, the two Fulghum accessions and the two Winter Turf accessions differed in their allele content, underscoring the variation in the progenitor oat landraces. Both Fulghum and/or Winter Turf can be found in the

pedigrees of the top 15 cultivars tested except for ‘Cliff’. However, Cliff did contain significant alleles at the AM102, AME177, AME178, and xncs15-3 marker loci. Cliff was an introduction from Mexico with unknown ancestry.

Two Winter Turf and Fulghum accessions were used in the study because of the large amount of variability observed from accession to accession. Winter Turf contained alleles for five out of the nine associated marker loci and Fulghum seven out of the nine associated marker loci. Both Winter Turf accessions have alleles at the AM102, AM177, and xncs15-3 marker loci but Winter Turf CIav1234 also contained an allele at the JAO4234 marker locus while Winter Turf CIav 1570 contained an allele at the AME178 marker locus. Fulghum was not considered a winter hardy line but it contained the most alleles out of any other cultivar tested. Fulghum CIav 3228 contained alleles at only three of the associated marker loci, AME177, AM1, and JAO4234 while Fulghum CIav 1204 contained alleles at five of the seven associated marker loci, AM102, JAO4636, JAO4234, AME178, and xncs15-3. Fulghum was used as the winter tender check during this study (CIav 3228) and during previous winter hardiness studies (CIav 708) (Maloney et al., In Review; Wooten et al., 2009), yet it still exhibited a significantly higher level of crown freezing tolerance than most of the lines tested. Fulghum is a fall sown oat, so it contains higher levels of crown freezing tolerance than spring sown or facultative type lines. Red Rustproof, from which Fulghum was derived (Stanton, 1955), contained an allele at only one of the nine significant loci which was present in only one of the Fulghum accessions (CIav 1204) test.

All of the lines tested had an allele at least one of the significant loci for crown freezing tolerance. The lack of expression of the increased crown freezing tolerance in some of the more winter tender lines could result from the lack of linkage between the specific marker alleles and the crown freezing tolerance QTL, and/or by the additional presence of inferior QTL for crown freezing tolerance. Because no genome sequence exists for oat, it is impossible to tell how close the QTL and alleles are to each other, but more importantly, it is not possible to show how many other QTL are present in these lines that negatively impact crown freezing tolerance.

The origin of winter hardiness in the oat population cannot be established with absolute certainty. However, it is believed that the cultivars Winter Turf and Fulghum have contributed to the higher level of winter hardiness seen in contemporary cultivars. The fact that these two lines exhibited more significant marker loci than another progenitor line tested, coupled with the fact that they occur in most of the winter hardy pedigrees found today leads to the conclusion that they have played a major role in crown freezing tolerance.

### ***Marker Assisted Selection***

Using a mapping procedure that uses a larger genetic base for the association of markers and traits facilitates better validation of marker loci for their use in MAS. Including the lines that were suspected to be extremely winter tender in the study

illustrated the importance of each of these loci in the current fall sown oat population. Further research will be needed to show the precise location of the traits in relation to the marker loci described, including the need for an *Avena sativa* consensus map and more PCR based markers.

With the information provided by this study, along with recent linkage mapping studies by Maloney et al. (in review) and Wooten et al. (2009), we were able to show that these loci are important in today's fall sown oat breeding programs. We feel confident in the use of AM102-220bp, HVM20-143bp, JAO4636-277bp, and AM114-260bp as markers for MAS.

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Table 3.1: Cultivars used for the mapping population, the CIav identification number, the cultivars growth habit, and the scores for the four marker loci with corresponding allele sizes in base pairs (bp).

Cultivar	CIav	Growth Habit	AM102 220bp	HVM20 143bp	JAO4636 277bp	AM114 260bp	AME177 189bp	AM1 219bp	JAO4234 260bp	AME178 182bp	xncs15-3 233bp
Arlington	4657	F	--	--	✓	--	✓	--	--	--	✓
Aurora	831	S	--	--	--	--	--	--	--	--	✓
Beltsville Selection 279	7662	S	--	--	--	--	✓	--	--	--	--
Bond	2733	F	--	--	--	--	✓	✓	--	--	✓
Brooks	9260	F	✓	✓	--	--	✓	--	--	✓	✓
Coker 242	7513	W	✓	--	--	--	✓	--	--	✓	✓
Coker 716	2771	W	✓	✓	--	--	--	--	✓	✓	✓
Carolee	8311	S	--	--	--	--	✓	--	--	--	✓
Ceirch Du Bach	518	W	✓	--	--	--	✓	--	--	--	✓
Checota	4629	S	✓	✓	--	--	--	--	--	--	✓
Cliff	7927	S	✓	--	--	--	✓	--	--	✓	✓
Clinton	9418	S	--	--	--	--	✓	✓	--	--	✓
Corbit	9266	S	--	--	--	--	✓	--	--	--	✓
Coronado	8260	S	✓	--	--	✓	✓	--	--	✓	✓

Table 3.1 (continued)

Culberson	273	S	✓	--	--	--	--	--	--	--	--
Dallas	PI 596631	W	✓	--	✓	✓	✓	✓	--	--	✓
Dawn	8029	S	--	--	--	--	✓	--	--	--	--
Delair	4653	S	--	--	--	--	--	✓	✓	✓	✓
Desoto	3923	W	--	--	--	--	--	--	--	✓	✓
Excel	5849	W	--	--	✓	--	--	--	--	--	✓
Florida 167	4320	S	--	--	--	--	✓	✓	--	--	--
Florida 500	8023	S	✓	--	✓	✓	✓	--	✓	--	✓
Floriland	6588	S	--	--	--	--	✓	--	✓	--	✓
Forkedeer	3170	W	✓	--	--	--	✓	--	✓	--	✓
Fulghum	3228	W	--	--	--	--	✓	✓	✓	--	--
Fulghum (2)	1204	W	✓	--	✓	--	--	--	✓	✓	✓
Fulgrain	4831	W	--	--	--	--	--	--	--	✓	✓
Fulgrain Strain 3	3697	S	--	--	--	--	--	✓	✓	--	✓
Golden Rain	2194	S	--	--	✓	--	✓	✓	--	--	✓
Green Russian	1978	S	--	--	--	--	✓	--	--	--	✓
Houston	7912	S	--	--	--	✓	✓	✓	--	✓	--
Kanota	2223	S	✓	--	--	--	✓	✓	✓	✓	✓
Landhafer	3522	W	--	--	--	--	--	--	--	--	✓
Lee	2042	S	✓	--	✓	--	✓	--	--	✓	✓
Madison	9404	W	✓	--	--	✓	✓	--	--	✓	✓
Markton	2053	S	--	--	--	--	✓	--	--	✓	--
Morota	2344	S	--	--	--	--	✓	--	--	--	✓

Table 3.1 (continued)

Norline	6903	W	✓	--	--	--	✓	--	--	--	✓
Norton	2501	S	✓	✓	--	--	✓	--	--	--	--
Ogle	9401	S	--	--	--	--	✓	--	--	--	✓
Osage	3991	W	--	--	--	--	✓	✓	--	✓	--
Ozark	9421	S	✓	✓	--	--	✓	--	✓	--	✓
Pioneer	3427	W	✓	--	--	--	✓	✓	--	--	✓
Quincy Grey	4078	W	--	--	--	--	--	--	--	--	✓
Red Algerian	2033	W	--	--	--	--	--	--	--	--	✓
Red Rustproof	1845	W	--	--	--	--	--	--	--	--	✓
Richland	787	S	--	--	--	--	✓	--	--	✓	--
R1800	4023	S	✓	--	--	--	✓	--	--	--	✓
Rodgers	PI 593020	W	✓	✓	--	✓	--	✓	✓	--	✓
Sante Fe	7051	S	--	--	--	--	✓	✓	--	✓	✓
Simpson	PI 494755	W	✓	✓	--	--	--	--	✓	✓	✓
Sioux	8172	S	--	--	--	--	--	--	--	✓	--
Suregrain	7155	S	✓	--	--	✓	✓	--	--	✓	✓
Tabor	1777	S	--	--	--	--	✓	✓	--	--	✓
Tift	3526	S	--	--	--	--	✓	--	✓	--	--
Trispernia	5100	W	--	--	--	--	--	--	--	--	✓
Verde	4312	S	--	--	--	--	✓	✓	--	--	--
Victorgrain	4238	W	✓	--	--	--	✓	--	--	✓	✓
Victoria	2401	S	--	--	✓	--	--	--	--	✓	--
Windsor	9140	S	✓	✓	--	--	--	--	✓	✓	--

Table 3.1 (continued)

Winter Fulghum	2498	W	✓	--	--	--	--	--	✓	--	--
Winter Turf	1234	S	✓	--	--	--	✓	--	✓	--	✓
Winter Turf (2)	1570	S	✓	--	--	--	✓	--	--	✓	✓

†F denotes a facultative growth habit, S denotes a spring sown oat growth habit, and W denotes a fall sown oat growth habit

‡✓ Denotes the presences of an allele at the corresponding locus.

Table 3.2: LSmeans calculated using PROC MIXED in SAS for Crown Freezing Tolerance scores (CFT) for each cultivar in the association mapping population and mean for the crown freezing tolerance scores.

Cultivar	CFT	Cultivar	CFT
Beltsville Selection 279**	0.38	Fulgrain	3.05
RL800**	0.76	Florida 500	3.05
Dawn**	0.83	Brooks	3.14
Tabor**	1.01	Arlington	3.15
Ogle**	1.02	Coronado	3.17
Trispernia**	1.11	Delair	3.23
Dallas**	1.18	Fulghum Clav. 3228	3.23
Verde**	1.19	Culberson	3.28
Bond**	1.20	Desoto	3.29
Markton**	1.24	Norton	3.42
Gold Rain**	1.25	Carolee	3.48
Osage**	1.33	Madison	3.71
Richland**	1.33	Quincy Grey	4.00
Victoria**	1.41	Forkedeer	4.20
Landhafer**	1.49	Coker 242	4.32
Kanota	1.57	Windsor*	4.56
Green Russian	1.62	Victorgrain*	4.60
Sante Fe	1.72	Winter Fulghum*	4.81
Clinton	1.84	Suregrain*	4.83
Tift	1.90	Cliff*	4.84
Florida 167	1.96	Rodgers*	4.84
Fulgrain Strain 3	1.97	Winter Turf Clav. 1234*	5.08
Sioux	2.04	Pioneer*	5.18
Morota	2.25	Excel*	5.20
Red Algerian	2.25	Checota*	5.25
Houston	2.53	Lee*	5.43
Floriland	2.62	Ozark*	5.85
Aurora	2.78	Norline*	6.13

Table 3.2 (continued)

Ceirch du Bach	2.85	Winter Turf CIav. 1570*	6.61
Corbit	2.94	Simpson*	6.93
Fulghum CIav. 1204	2.98	Coker 716*	6.98
Red Rustproof	3.05		
<b>Average CFT</b>			<b>3.09</b>

\* Cultivars that test significantly ( $\alpha=0.05$ ) the same as the winter hardy check (Norline)

\*\* Cultivars that test significantly ( $\alpha=0.05$ ) the lower than the winter tender check

(Fulghum CIav. 1204)

Table 3.3: Mixed Linear Model data given by TASSEL 2.1 for marker trait association for crown freezing tolerance. Marker locus, the Model and Error have degrees of Freedom of 1, 10, and 52 respectively.

Locus	Allele Size <sup>†</sup>	F_Marker	p_Marker	MS_Error	Rsq_model	Rsq_marker
AM102	220	13.7268	5.13E-04	1.7	0.5	0.131
HVM20	143	10.9102	0.0017	1.47	0.57	0.091
JAO4636	277	7.2682	0.0094	0.97	0.72	0.04
AM114	260	6.6551	0.0128	0.86	0.75	0.032
AME177	189	5.7687	0.0199	1.52	0.55	0.049
AM1	219	5.492	0.023	1.04	0.7	0.032
JAO4234	260	4.4554	0.0396	1.38	0.6	0.035
AME178	182	4.2886	0.0433	1.34	0.61	0.032
xncs15-3	233	4.2748	0.0437	1.1	0.68	0.026

<sup>†</sup> Denotes the allele length in base pairs (bp)

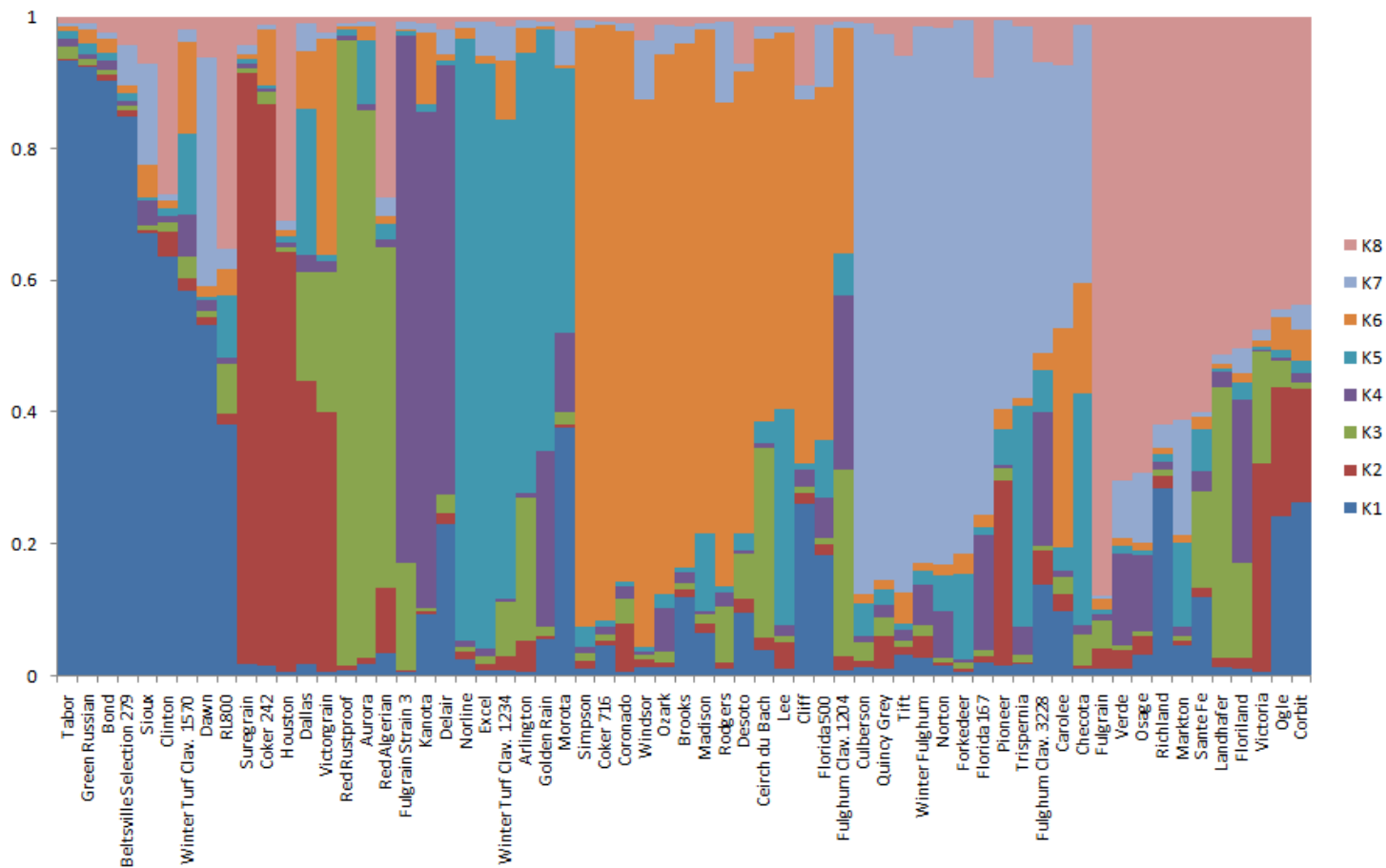


Figure 3.1: STRUCTURE 2.3 output for K=8 subpopulation level. Each Subpopulation level is indicated by a different color and cultivars belonging to each subpopulation level are located below.