ABSTRACT

MOLINA BRAVO, RAMÓN. Genetic and Quantitative Analysis of Red Raspberry (*Rubus idaeus*) for Heat Tolerance and Longer Chilling Requirement. (Under the direction of Bryon R. Sosinski and Gina E. Fernandez.)

Despite the high level of interest for growing raspberries (*Rubus idaeus*) in the southeastern US, production is limited by the lack of adapted, high quality cultivars. Breeding efforts are underway for increasing cultivar availability, however developing improved cultivars in *Rubus* is a slow and time-consuming process. In order to expedite the slow, but effective, breeding process, more molecular breeding tools need to be developed. Cultivars adapted to the southeastern US need to tolerate warm summers, and winters with wide temperature fluctuations. To address this issue, a genetic mapping population that segregates for tolerance to both climatic conditions has been developed from a cross between (*R. parvifolius* × ‘Tulameen’) × ‘Qualicum’. This population was used for the construction of a genetic linkage map and for quantitative trait loci (QTL) analysis for heat tolerance, and for tolerance to fluctuating winter temperatures. Seven linkage groups were identified and were anchored to the already existing map. The majority of the linkage groups identified were of similar genetic size, and anchor markers were located at similar genetic distances relative to other markers in linkage groups. For significant QTL analysis, accurate phenotypic screening in the population is crucial. Because heat tolerance is a difficult trait to measure, a protocol was developed using chlorophyll fluorescence to assess heat tolerance. The protocol was used to measure heat...
tolerance in the mapping population, and after QTL analysis, 3 regions explained ~35% of the variation. Appropriate chilling requirement is necessary in woody perennials for tolerance to temperature fluctuations in the winter. Therefore, this trait was analyzed for the location of important QTL on the constructed map. Chilling requirements in the mapping population were estimated by measuring bud break in greenhouse conditions. These estimates were subjected to quantitative trait analysis, and three QTL were found in two separate season evaluations. In most cases, co-localization of these QTL occurred in the same region on the map. These regions explained the majority of the variation of the trait (100-64.5%). Other important horticultural traits segregated in the \((R.\ parvifolius \times \text{‘Tulameen’}) \times \text{‘Qualicum’} \) cross, and were evaluated for QTL analysis as well. The horticultural traits of importance were growth habit, prickle density, fruit color, fruit shape, and fruit size. In most cases, two field evaluations were performed. Several regions were identified as significant and the majority of the QTL were co-localized to the same region of the linkage map. In summary, this research has established a protocol that measures heat tolerance without relying on visual assessment, and has mapped important QTL for further molecular studies. This research has drawn a baseline foundation for the development of molecular technologies in improving heat tolerance and tolerance to winter temperature fluctuations in \textit{Rubus}. Future research should focus on these regions to develop closely linked molecular markers for marker assisted breeding.
Genetic and Quantitative Analysis of Red Raspberry (*Rubus idaeus*) for Heat Tolerance and Longer Chilling Requirement

by

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DEDICATION

I recognize that my journey would not have been possible without the aid of two people that supported all of my endeavors throughout my life, my father and my mother, Ing. Raúl Molina Galaz, and Ing. María Elena Bravo Almanza. I am and will be in eternal gratitude for their love, encouragement, and advisory. I truly believe that my accomplishments have been made possible because of my beloved parents.

Quiero reconocer a dos personas que han apoyado mis esfuerzos y emprendimientos en mi vida profesional, que sin ellos mi aventura escolar no habría sido posible. Esas dos personas son mis queridos padres, Ing Raúl Molina Galaz e Ing. María Elena Bravo Almanza. Estoy en deber perpetuo por su amor, su formación en mi persona y su demostración de entusiasmo hacia todos los aspectos de mi vida. Gracias a ellos, mis logros se han convertido en realidad y por ésto estoy eternamente agradecido.
BIOGRAPHY

Ramón Molina Bravo was born in Ciudad Juarez, Chihuahua, Mexico on October 27th, 1979. Ramón grew up and completed his basic education in Ciudad Juarez. Upon completing his high school education, Ramón decided to venture outside of Mexico for his higher education, and moved to the nearby city of Las Cruces, New Mexico, where he attended New Mexico State University (NMSU). Ramón received his Bachelor of Science in Horticulture, and during the pursuit of his bachelor’s, Ramón was inspired to continue his studies in plants with a more specialized focus in genetics and breeding. He attributes this inspiration to one very special Professor at NMSU, Dr. Champa Sengupta-Gopalan. Dr. Sengupta-Gopalan’s work in the genetics of the symbiotic nitrogen-fixing bacteria was extremely fascinating to Ramón. He saw the potential application of this research, and how this could lead to better quality of life. Ramón continued his studies at NMSU, and accepted an assistantship in the Horticulture Master’s program under the advisory of Dr. Chris Cramer. Ramón worked on molecular marker techniques and screening a fungal disease called pink root in onion. Dr. Cramer, a former NCSU graduate himself, highly recommended for Ramón to continue studies at NCSU. After Ramón visited with the Department of Horticulture at NCSU and received acceptance, he saw incredible potential in the molecular studies of heat tolerance in raspberry, which has culminated to the fascinating work discussed herein.

After completing his doctorate, Ramon is venturing to Costa Rica to work for the National University of Costa Rica as a visiting professor continuing research in *Rubus* and teaching classes in applied molecular biology.
ACKNOWLEDGEMENTS

This has been an amazing four years of life dedicated to the pursuit of the advancement of understudied crops, such as the raspberry. I believe that there are many specialty crops that contain a substantially high potential for the betterment of society. I am truly grateful to have imprinted my contribution to the world of Rosaceae genetics and genomics. I dedicate this study to all the community that supports my work and similar work in the understanding, betterment, and improvement of fruit and nut crops.

To my ever-patient advisors, Dr. Gina Fernandez and Dr. Bryon Sosinski, I cannot express my gratitude in words. Both of you have been astonishing advisors, supporters, and friends in my endeavors. I am very grateful to Bryon for exposing me to the Rosaceae Community at the Rosaceae Genomics Conference 4 in Pucón, Chile, and allowing me to meet and greet the people that give life to the world of Rosaceae genetics and genomics. Thank you Bryon for supporting my unforgettable experience in Palmerston North, New Zealand. These two experiences have given my work and perception a very special flavor of enthusiasm and personal growth. I give special thanks to Dr. Emily Buck and Dr. Susan Gardiner of Plant and Food Research in New Zealand. Thank you for bringing me into your laboratory and allowing me to become a part of the project. My experience at Palmerston North, New Zealand was absolutely unforgettable, and I believe I have grown to be a better researcher because of our interaction. Thank you Gina, for all your guidance, and the opportunity to present at the Southern Region ASHS. It is crucial for us as young scientists to expose ourselves to the rest of the community, and I am grateful you have rendered that
exposure. I will miss making my desserts at your gatherings; the crowd at these events was always very appreciative of my gestures.

I dedicate this work to my colleagues to whom I consider close friends and companions. I will be forever grateful to Jessica Spencer. Thank you for joining team Rubus, and helping me forge my heat screening protocol into something much greater than I had imagine. I hold you as one of the most important people in life. I want to send a very special thank you to my former colleague, Megan Ulmer, whom I keep as one of my closest and dearest friends. The framework of my laboratory work was possible because you were able to trellis my thoughts with your witty advice. My sincerest appreciation goes to my friend and former colleague, Dr. Per McCord. Thank you, Per, for all your advice, help, and intelligent dialogue inside and outside of the lab. You are responsible for all my training in generating molecular markers; you are a very special researcher. I admire your abilities; you are the MacGyver of the laboratory world. I want to thank my former colleague and friend Dr. Monica Santa-Maria, to whom I owe great collaboration in the lab. I want to recognize my current colleague, Michelle Kim for her support and taking care of the laboratory duties for when I was in the lost space of thesis writing. I would like to thank Lis Meyer for opening my now never-ending curiosity in plant taxonomy. I am grateful to you, but still blame you for my lollygagging at the parks and landscapes everywhere I go.

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In September of 2008, the \textit{Rubus} breeding program received a special visitor from the National University of Costa Rica, Rafael Orozco. I want to thank you Rafael for venturing your way to North Carolina. It is because of you that I have been offered the visiting professor position at the National University of Costa Rica. I appreciate all your great networking efforts, as well as all your hard work. Without it, this opportunity would not have been possible. I am looking forward to our new endeavors as a research team.

Lastly, I want to acknowledge an important person in my personal life, Rudy Dominguez. Thank you, Rudy for the memories of us in North Carolina, and your brave decision to pursue a future together, even though this future did not materialize. You were a part of my journey as much as I was.
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CHAPTER I

Literature Review

INTRODUCTION

Raspberry (*Rubus idaeus*) is an economically and nutritionally important crop in America, with a very wide variety of markets. Although fresh raspberries are available year round in stores across the country, the southeastern part of the United States does not have a major producer despite its proximity to major markets (Fernandez personal comm.). Increasing demand, proximity to markets and the development of cultivars adapted to the region would result in increased potential for making North Carolina a major producer of raspberries in the eastern US market. In addition, raspberry fruits have high levels of antioxidants, and a wide range of different antioxidant types (Mullen et al. 2002). In the southeastern US, the most limiting factor of production is the lack of adapted cultivars (Moore 1997). Typical seasonal conditions in southeastern states, like North Carolina, are warm humid summers, and winters with wide temperature fluctuations. In order to establish major production in this area, cultivars will require tolerance to both of these conditions, which are very atypical of available raspberry cultivars (Ballington and Fernandez 2008). Furthermore, domestication of red raspberry (*R. idaeus*) has resulted in a reduction of genetic diversity, with most modern cultivars being genetically similar and adapted to the climates like that of the pacific north west of the US because *R. idaeus* is naturally adapted to these climatic conditions (Graham et al 2004; Graham and McNicol 1995; Jennings 1988). Moreover, heat tolerance is becoming an increasingly important trait with the advent of global climate change.
The raspberry breeding program at North Carolina State University (NCSU) focuses on developing germplasm adapted to the warm humid summers and cultivars with long chilling requirements in order to tolerate fluctuating winter temperatures. The most successful source for heat tolerance in the breeding program has been from *R. parvifolius*, a species indigenous to the warm temperate regions of eastern Asia (Jennings 1988; Williams 1950). In addition to heat tolerance, *R. parvifolius* incorporates longer chilling requirements when crossed with cultivated varieties (Ballington and Fernandez 2008; Williams 1950). One of the most promising combinations developed in the NCSU breeding program is (*R. parvifolius* × ‘Tulameen’) × ‘Qualicum’. The progeny from this modified backcross segregates for chilling requirement and heat tolerance, as well as a myriad of other traits, and has been the primary focus for molecular research in the breeding program. Preliminary data indicate that, in addition to segregation of heat tolerance and chilling requirement, the progeny of the (*R. parvifolius* × ‘Tulameen’) × ‘Qualicum’ cross segregates for several types of antioxidants (see appendix B), perhaps because *R. parvifolius* fruits are orange, whereas the other progenitors have red fruits.

**Breeding in Raspberry**

Breeding advancements in *Rubus* can be a long and slow process which, depending on the species, are hindered by different ploidy levels, self incompatibility, apomixis, and poor germination (Graham and Woodhead 2009). However, *Rubus* has high potential for further and novel genetic improvements. *Rubus* is one of the most diverse genera in the plant kingdom with more than 500 species distributed throughout the world (Jennings 1988). The
domestication of *Rubus* has occurred in 5 of the 12 subgenera. The two most noteworthy subgenera for this study are *Idaeobatus* and *Rubus* (formally *Eubatus*), which contain the domestic raspberries and blackberries, respectively (Jennings 1988). The subgenus *Idaeobatus* has over 200 species that occur in all parts of the globe, but most of the diversity is in temperate and subtropical regions of eastern Asia (Jennings 1988). Thus, all this diversity provides *Rubus* with incredible potential for improving important commercial traits.

Traditional breeding efforts are time consuming, therefore the use of molecular tools that will allow breeders to screen germplasm at the seedling stage and expedite cultivar development. Using conventional breeding methods in raspberry, the breeding cycle begins with the crossing of complementary parents to combine traits that are more suitable for the growing environment than either parent alone (1 year). The resulting seedlings are planted in the field, grown for 2 to 3 seasons and then evaluated. At the end of this cycle, typically less than 5% of those seedlings are “selected” based on combinations of desirable traits. The “selections” are propagated and placed in 2 to 3 different locations for further evaluation. In subsequent years, the selections are rated on plant characteristics, berry weight and quality, climatic adaptation, and disease and pest resistance. This stage can take 3 to 5 or more years. From this initial group of “selections”, the most promising are placed in replicated trials and placed in 2 or more sites for 3 to 5 years. An “advanced selection” which consistently provides high quality and quantity fruit combined with excellent plant health and vigor may be released by the program and assigned a cultivar name. Thus, the entire process using conventional breeding methods normally takes 10 to 15 years. The development of a suitable genetic linkage map encompassing both informative and simply inherited markers as well as
QTL will increase the precision and speed of breeding, especially for traits that are polygenic (quantitative) and are strongly affected by the environmental. This will ultimately lead to a set of molecular markers that are useful for marker assisted breeding (MAB). By employing MAB, the time and cost of the breeding process may be reduced by selecting only those genotypes at the seedling level in the greenhouse. This saves space and time by reducing the number of plants to evaluate and maintain in the field.

**Molecular Tools in Breeding**

Linkage maps have been utilized for identifying chromosomal regions that contain genes controlling simple traits (single gene) and quantitative traits using quantitative trait loci (QTL) analysis (QTL mapping) (Collard et al. 2005; Mohan et al. 1997). By using the presence or absence of molecular markers in place of or in addition to phenotypic selection, crop improvements are more effective, efficient, reliable, and cost-effective than with conventional breeding alone (Collard et al. 2005). In QTL mapping, once tight linkages between markers and QTL are established, these can be used as tools for trait diagnosis. Similarly for mapping simply inherited traits, establishing tight linkage between the marker and the trait can aid in trait diagnosis. MAB renders breeders several advantages: (1) Selection is performed more efficiently by eliminating variable phenotypic evaluations that vary due to environmental effects. (2) The technique saves time by substituting multiple-year replicated field trials for molecular tests in the early stages of evaluation and selection at the seedling stage. (3) The technique reduces linkage drag by minimizing transfer of undesirable alleles in backcrossing practices. Saving time is especially useful because of the biennial
nature of raspberry. Saving time is already a crucial factor in herbaceous crop improvement, and due to their long juvenile phases, saving time is even more so important in woody perennials (Dirlewanger et al. 2004). Theoretically, selecting for a single QTL can be beneficial in plant breeding when such a QTL accounts for the largest proportion of phenotypic variance (Ribaut and Betran 1999; Tanksley 1993). This is supported through reports that polygenic traits can be selected using qualitative traits as selectable markers (Tanksley 1993). One study in maize supports this by showing associations between QTL regions and mapped qualitative loci (Beavis et al. 1991). Therefore, a single QTL with a relatively large proportion of variance can be treated as a qualitative trait and can have a positive effect when applying marker selection. Although, theoretically, all tightly linked QTL can be used in MAB, due to the cost of utilizing such technologies, no more than three closely linked QTL are typically used (Collard et al. 2005; Ribaut and Betran 1999).

**Identification of QTL in Raspberry**

Raspberry is a member of the Rosaceae, an extraordinarily large family, containing many economically important genera (Dirlewanger et al. 2004). Molecular research in this family has historically been scarce, particularly for the genus *Rubus*, especially in comparison to other plant species like *Arabidopsis* or rice. The three most agriculturally important subfamilies in Rosaceae are Rosoideae, Amygdaloideae, and Maloideae. Some of the representative members of these subfamilies are strawberry, and rose in Rosoideae, peach and cherry in Amygdaloideae, and apple and pear in Maloideae. *Rubus idaeus* is an important species for the Rosoideae subfamily of the Rosaceae because of its small genome (275 Mb).
and its diploid inheritance (2n=2x=14) as well as its early evolutionary establishment in Rosaceae (Graham et al. 2007; Potter et al. 2002). Graham et al. (2004; 2006) published the first linkage map of cultivated raspberry, and have made great contributions to the molecular research in *Rubus*. The map included six QTL affecting important horticultural traits. A total of 19 QTL regions were reported, most of which were for several fungal pathogen resistance. One of these QTL affects cane pubescence (gene H), and has also been shown to be closely associated with resistance to cane botrytis and spur blight on the same map region in linkage group 2 (Graham et al. 2006). Graham et al. (2006; 2004) also mapped density of prickles (commonly referred to as spines in other literature), rust spot resistance, and cane spot resistance. Most of these traits were associated with 2 QTL regions on the map.

**Research Objectives**

The molecular breeding work in this study is focused upon mapping two important complex traits (heat tolerance, and tolerance to fluctuating winter temperatures), plus other important horticultural traits (fruit color, fruit shape, fruit size, growth habit, and prickle density). As a result, the molecular mapping work will contribute enormously towards a greater understanding of the genetics of important horticultural traits in raspberry. Establishing marker-phenotype relationships is crucial for allowing marker-based selection in breeding. Constructing a genetic linkage map provides the basis for these relationships and thus allows manipulation of both single gene and quantitative traits in breeding programs.

The construction of the genetic linkage map in raspberry has a direct application in breeding and cultivar development, but its application can potentially be extended to other
Rosaceae crops. This project is geared towards fulfilling two major long-term goals: (1) to develop a protocol to reliably test *Rubus* material for heat tolerance; (2) to develop inexpensive, closely linked molecular markers that can be used to rapidly screen germplasm for effective improvement of germplasm for heat tolerance, high chilling requirement, and other important horticultural traits (fruit color, fruit shape, fruit size, growth habit, and prickle density). In the former case, heat tolerance has only been measured via visual assessment in the field. Therefore there is a need to develop a protocol for heat tolerance and thus a part of this research was focused on using chlorophyll fluorescence to assess heat tolerance. In the latter case, many traits can be screened in the early stages of plant development allowing breeders to curtail large test populations before plants are evaluated in the field.

The research described herein entails examining the (*R. parvifolius* x ‘Tulameen’) x ‘Qualicum’ population to map QTL regions of horticultural importance, principally heat tolerance, and longer chilling requirements. Additionally, QTL regions or single marker analysis for fruit color, fruit shape, prickle density, flower color, and growth habit (erect to prostrate) will be developed. This work, in the short term will require refinement of the QTL regions for future characterization of putative sequences to design closely linked molecular markers. In the long term, these will be converted to sequenced based markers, for cheap and rapid screening of germplasm for effective selection of these important traits.

*Heat Tolerance:* In North Carolina, heat tolerance is an essential trait for the cultivation of *Rubus* (Williams 1950). With the advent of global climate change (i.e. global warming), heat tolerance is becoming an increasingly important trait not only in *Rubus* but
also throughout different taxa. The effect of high temperatures on growth and development is complex due to the influence of various factors, such as the diurnal temperature range and water stress (Stoddard 2006; Paulsen 1994). All plant processes are sensitive to heat and can cause irreversible damages. Heat stress is associated with significant reduction in photosynthetic activities (Stafne et al. 2001; Nagarajan et al. 1998; Fernandez and Pritts 1994; Harding et al. 1990), membrane stability of both the cell and the organelles, especially the chloroplast and thylakoid membranes (Hurkman and Tanaka 1987), and interference with evapotranspiration (Stafne et al. 2001; Christiansen 1978). Reduction in plant growth directly attributed to high temperatures is well established in horticultural crops such as tomato (Adams et al. 2001), grape (Chaumont et al. 1997), and strawberry (Kadir and Sidhu 2006).

In red raspberry, the rate of net CO₂ assimilation drops significantly when temperatures rise from 20 to 30°C, while stomatal conductance remains the same (i.e. no change in evapotranspiration) (Stafne et al. 2001; Fernandez and Pritts 1994). High temperatures adversely affect photosynthesis processes due to sensitivity to thermal inhibition (Henning and Brown 1986). Whether heat stress occurs early or late in the season, high temperature inhibits thylakoid activities principally near the photosystem II (PSII) reaction center (Berry and Björkman 1980). Screening methodologies and molecular dissections of heat tolerance have been studied extensively in cereal crops, particularly in wheat and maize (Dhanda and Mujal 2006; Maestri et al. 2002; Yang et al. 2002; Fokar et al. 1998; Ottaviano et al. 1991; Blum and Ebercon 1981). Limited studies have been done on strawberry (Kadir and Sidhu 2006; Ledesma et al 2004), tomato (Willits and Peet 2001), and a mix of tropical and sub-
tropical fruits (Weng and Lai 2005; Yamada et al. 1996). To date, no published screening methods have been developed for any fruit crops, including members of *Rubus*.

*Tolerance to Temperature Fluctuations in Winter*: Due to the different uses of terminology relating to dormancy, in this context the term dormancy will refer to endodormancy (or rest), which is the dormancy of buds due to internal physiological blocks (Westwood 1993). These physiological blocks prevent growth even under favorable environmental conditions and terminate after exposure of chilling temperatures above freezing (between 0°-7°C) (Westwood 1993). Many important horticultural deciduous species require an accumulation of chilling hours in order to break bud dormancy, such as *Vitus* (grape), *Ribes* (currant), *Carya* (pecan), *Malus* (apple), *Pyrus* (pear), *Prunus* (peach, almond, plum, apricot and cherry), and *Rubus* (raspberry and blackberry) (Westwood 1993). Middle-latitude temperate species of deciduous fruits and nuts are adapted to winters that fluctuate between cold and warm temperatures by requiring long chilling periods to break bud dormancy (Westwood 1993). By requiring long chilling hours to break dormancy, winter injury can be avoided because lateral bud growth (in the case of *Rubus*) will not occur in midwinter even under warm conditions that favor growth until the plant accumulates the appropriate chilling hours. On the other hand, a short chilling requirement will allow growth under warm conditions in midwinter but cause death of lateral buds when the temperature decreases after mid-winter thaws. Therefore in middle latitude species, this can lead to severe crop loss because lateral buds give rise to fruit bearing structures in *Rubus*. However, in lower latitude temperate species, low chilling is a desirable trait because plants are able to break dormancy and grow after a short exposure to cold as typically occurs in these regions.
Thus, the QTL mapping of this trait will be beneficial to for the study and breeding of this trait in many important horticultural crops within Rosaceae and across different plant genera.

**APPROACH:**

The mapping population employed in this study was generated from a cross between \( (R. \textit{parvifolius} \times \text{‘Tulameen’}) \times \text{‘Qualicum’} \) (Fig. 1) and its full-sib progeny will be referred as the mapping population in this text. ‘Qualicum’ (Daubeney and Kempler 1995) was generated from a cross between the cultivars ‘Glen Moy’ and ‘Chilliwack’ (Daubeney 1987), both of which are adapted to climates of the northern US and Canada. ‘Tulameen’ (Daubeney and Anderson 1991) was developed by the Agriculture Canada breeding program in British Colombia and was generated from a cross between ‘Nootka’ and ‘Glen Prosen’. The latter is a cultivar from the Scottish Crop Research Institute (SCRI), and contains black raspberry \( (R. \textit{occidentalis}) \) in its derivation. As mentioned above, the \( (R. \textit{parvifolius} \times \text{‘Tulameen’}) \times \text{‘Qualicum’} \) cross is regarded as a promising combination in which the progeny segregate for heat tolerance and tolerance to fluctuating winter temperatures (Ballington and Fernandez 2008). This same progeny also segregates for prickle density, fruit color, flower color, fruit shape, and growth habit (prostrate, upright, and semi-upright). There are 450 individuals in the mapping population and have been planted in replicated trials in the field at the Sandhills Research Station in Jackson Springs, North Carolina (N. 35° 11.1471 W. 0.079° 40.629 elevation 567’ (173m)). Two hundred random individuals from this cross are currently propagated in-vitro via meristem culture for replicated trials, and were used for generating the genetic linkage map and quantitative trait analysis.
Construction of Linkage Map: Leaf DNA was extracted using a modified 2% CTAB method developed by Graham et al. (2003). The genetic linkage map was constructed based on AFLP markers and SSR markers. The AFLP markers were developed using the technique developed by Vos et al. (1995). SSR markers have already been developed for Rubus, and existing primers pairs have been pre-screened for the NQ population and have determined that these markers are transferable onto the existing Rubus map (Graham et al 2004). For SSR markers, we used a modified 3 primer tailing system by fluorescently labeling the universal M13 primer (Shuelke 2000). Single point analysis was performed using MapQTL 5 ® (Van Ooijen 2004) to determine any association between the molecular markers and the trait. Interval mapping (Lander and Botstein 1989) and composite interval mapping analysis were performed using standard algorithms implemented in MapQTL ® 5 (Van Ooijen 2004).

Screening of Traits

Thorough phenotypic screening replicated over time and/or over environments is utmost important for accurate QTL mapping (Collard et al. 2005). Therefore phenotypic screening protocols for chilling requirement and heat tolerance were repeated over time. Prickle density, fruit color, flower color, fruit shape, and growth habit, were screened visually in the field or in the lab.

Heat Tolerance Screening: Many studies have screened populations for heat tolerance by measuring chlorophyll fluorescence (Kadir and Sidhu 2006; Weng and Lai 2005; Willits and Peet 2001; Yamada et al 1997), electrolyte leakage using conductivity meters, (Dhanda and Munjal 2006; Rehman et al. 2004; Srinivasan et al. 1996; Ottaviano et al. 1991), cell
viability measured by reduction of tetrazolium triphenyl chloride (TTC) (Dhanda and Munjal 2006; Porter et al 1995), and by measuring phenotypic or morphological data such as grain-filling duration (Yang et al. 2002).

Heat tolerance was screened in the mapping population using chlorophyll fluorescence. Measurements generated from this method were used to distinguish heat tolerant individuals. Chlorophyll fluorescence is routinely used as a physiological parameter that correlates well with heat tolerance (Kadir and Sidhu 2006; Weng and Lai 2005; Knight and Ackerly 2002; Srinavasan 1996; Yamada et al. 1996; Yang et al. 1996; Belkhodja et al 1994). Chlorophyll fluorescence analysis is simple, non destructive, and allows analysis of many samples within a short time. These advantages make the technique ideal for large scale screening of populations (Srinivasan et al. 1996). Chlorophyll fluorometers indirectly assess the damage of PSII by measuring absorbance of reemitted fluorescence (Genty et al. 1990). Studies that measured plant membrane stability detected significant differences among diverse plant species of legumes (Srinivasan et al 1996), and birch (Ranney and Peet 1994) or among different genotypes of wheat (Dhanda and Munjal 2006; Ibrahim and Quick 2001), and grape (Kadir et al. 2007). These studies determined significant correlations that corroborate with field measurements or with measurements from other methodologies such as reduction of TTC or chlorophyll fluorescence. For example, studies that measured chlorophyll fluorescence to quantify heat stress detected differences among species of temperate, tropical and subtropical regions (Weng and Lai 2005; Yamada et al. 1996); differences among leguminous crops (Srinivasan 1996); and differences among genotypes
within a cultivated species (Kadir and Sidhu 2006; Ranney and Peet 1994). All studies found strong correlations with other heat stress measurements or with observational data.

During the summer of 2008, a protocol for screening leaves was developed using an OS1-FL chlorophyll fluorometer (Opti-Sciences, Hudson, NH, USA) specifically for *Rubus*. The parameter that is commonly used in heat tolerance analysis is Fv/Fm, which is the ratio of variable fluorescence to maximal fluorescence (Weng 2006). In order to obtain variable fluorescence (Fv), leaves are adapted to dark conditions to measure the difference of initial (Fo) and maximum (Fm) fluorescence according to the manufacturer’s instructions. Fully expanded primocane leaves of similar age showed “stress” most consistently using the fluorometer under high temperature conditions. Fluorescence was measured using the OS1-FL fluorometer on detached leaves of similar age and size. Other studies have shown that detached leaves behave similarly to leaves attached to the plant (Willits and Peet 2001; Srinivasan et al 1996; Smillie et al 1987). Willits and Peet (2001) showed that Fv/Fm decreases quadratically with increasing temperature in detached leaves and in greenhouse studies, supporting lab approaches. Weng (2006) favored a more controlled environment because Fv/Fm can be underestimated in warm field conditions. For this study, detached leaves were placed immediately in clear plastic bags in 100% humidity, and heat-treated (45°C) for 30 min to assess chlorophyll fluorescence under laboratory conditions. In 2007, a subset of the population was screened using this method, and we have detected significant differences among these individuals. Thus, we have determined that the population is segregating for the heat tolerance trait based on this method. In 2008, 196 field-grown
individuals from the mapping population were screened using the method for quantitative trait (QTL) evaluation.

A potential drawback to the approach is that there are no established controls using chlorophyll fluorescence for raspberry to denote tolerance or susceptibility to heat. However the research presented herein presented an excellent opportunity to establish these controls. The NCSU *Rubus* breeding program released a heat tolerant cultivar, ‘Mandarin’ in 1955 (Ballington and Fernandez 2008). Although ‘Mandarin’ lacks desirable fruit characteristics, it consistently performs well in the warm climate of North Carolina (Ballington and Fernandez) and has a high carbon assimilation rate (A) at temperatures as high as 35°C (Stafne et al. 2000). Stafne et al. (2000) have also shown that selections from the same cross as that of the heat tolerant parent (*R. parvifolius* × ‘Tulameen’) can sustain an intermediate A at 35°C. Thus, the experiments in Chapter II further broaden the knowledge and applicability of these two genotypes and their abilities to perform well in hot environments.

*Chilling Requirement:* Chilling requirements was measured using a modified method described by Warmund and Krumme (2005) in blackberry. Chilling requirement models indicate that temperatures between 0 and 7°C are the most effective but vary in criteria for determining chilling inception (Warmund and Krumme 2005; Drake and Clark 2000; Richardson et al. 1974). The model used as a standard is the Utah chilling model described by Richardson et al. (1974) based on peach. However this model does not accurately predict chilling inception in *Rubus* (i.e. the starting point of chilling requirement) (Warmund and Krumme 2005). This inaccuracy is attributed to the lack of an appropriate parameter to determine the point of inception in *Rubus*. Warmund and Krumme (2005) have developed a
modified Utah chilling model (model 5), where chilling inception is at the first incidence of -2.2 °C which is a parameter based on a blackberry model developed by Yazzetti and Clark (2001). Warmund and Krumme (2005) show that this model had the smallest variation in time of inception and accumulated hours in blackberry. In the research presented in Chapter IV, a very similar model, model 2, was used to calculate the chilling hours in the mapping population. Model 2 has the same chilling inception parameter as model 5, but chilling hours are weighted differently depending on the range of temperature, and in certain ranges, accumulated chilling hours can be subtracted in model 5. Model 2 is not weighted, and adds one chilling unit for every hour of exposure of temperatures within 0 to 7°C. The North Carolina State Climate Office continuously monitors chilling requirement based on several of these models (including the model used here), and was used to determine point of chilling inception in the field (State Climate Office of North Carolina, NC State University. CRONOS [internet database] available at http://www.nc-climate.ncsu.edu/cronos/). This model served only as a guide to determine the initial accumulated hours in the field.

Seven- to ten-node sections were collected after establishment of chilling inception with some chilling hours, and are placed in coolers. Sections are removed from coolers every 8 to 16 days (200 to 400 chilling hours), placed under greenhouse conditions with bottom heat and kept hydrated (misting). Chilling requirements were calculated based on percentage of observed bud-break. In our first year’s screening (winter of 2006-2007), we determined that the NQ population segregates for this trait with a wide range of variability, from as little as 200 to over 1200 chilling hours. However, most *Rubus* species have been reported to have chilling requirements that range from 800 up to 1800 hours (Westwood 1993). Because plant
material was transplanted in late spring of 2006, material was not readily available and only 2 wood sections per data point were taken. In the winters of 2007-2008 and 2008-2009, three wood sections for every accumulated hours interval for each genotype were screened, rendering a better estimate of the chilling hour requirement.

As a potential disadvantage to this method, calculating chilling requirement in a controlled environment may not reflect genotypic responses in the field due to fluctuating environmental conditions. However, observations were made in the field for visual correlations between observed bud break measured using the method by Warmund and Krumme (2005) and bud break in the field. Nevertheless, this is not a major concern because the model only provides the starting point for chilling hours, and differences among individuals is the major factor of consideration for the purposes of QTL analysis. Moreover, chilling inception was well established in most cuttings that were screened in our experiments. However, cuttings from the blackberry cultivar ‘Kiowa’ (Moore and Clark 1996) were used as a low chilling control (Carter et al. 2006), and compared to raspberry, chilling inception occurred later in the winter.

**Pubescence and Prickle Density:** Cane pubescence is determined by gene $H$, which gives rise to fine hairs on the surface of canes (genotype $HH$ or $Hh$), and in homozygous recessive individuals ($hh$), hairs are absent (glabrous). Homozygous dominant ($HH$) individuals are rarely found due to linkage with a lethal recessive gene (Jennings 1967). Pubescence of canes is designated as present ($Hh$) or absent ($hh$) by visual examination.

Breeding for prickle free canes is a main concern for developing cultivars that are easier to handle (i.e. easier to prune and to harvest). There are several major genes that confer
prickles (Jennings and Ingram 1983; Jennings 1988). One of these is the s gene, and is dominant for prickles. ‘Glen Moy’, a parent of ‘Qualicum’, and ‘Glen Prosen’, a grandparent of NC497, are homozygous recessive for gene s and is a prickle free cultivar (Fig. 1). *R. parvifolius* is known to carry gene(s) for the prickle free trait and are of particular interest because its segregation ratio in F1 hybrids suggested that these are dominant genes (Jennings and Ingram 1983). However, these genes have not been genetically characterized. In our studies, density of prickles was scored visually on a scale of 0 to 5, where 0 is no prickles and 5 is densely covered in prickles. Approximately twenty-five percent of the mapping population is prickle free, however both parents are prickly. Although the prickly trait has been previously mapped (Graham et al. 2004), completely prickle free individuals were not present in the population for that particular study.

**Fruit Color:** Raspberries (*R. idaeus*) have a variety of colors that range from yellow to dark red. Orange-red berry color shows predominance of pelargonidin glycosides, while red berries have predominance of cyanidin-3-rutinoside and cyaniding-3-glucosyl-rutinoside (two anthocyanins found only in red raspberry) (Jennings 1988). In our studies, the mapping population has a wide array of different colors. These color differences suggest that segregation of different anthocyanins is occurring in the mapping population, and therefore mapping of genes closely related to synthesis of the different types of anthocyanins is possible. The fruit color was categorized using the Royal Botanical Colour Charts. The range is yellow (11A), pink (51B), orange (23A), bright red (45A) or dark red (59A). This color variation shows possible difference in types of anthocyanins. A short study on the segregation of fruit color and health related compounds were performed on a subset of the
population (See appendix B). We were able to establish that the progeny segregate for
different types of phyto-chemicals, and that there is a relationship between chemical type and
color.

**Impact of Research**

*Rosaceae Community Database (GDR).* A centralized and curated community
database is critical to facilitate comparative research into Rosaceae genomics and genetics
data. The GDR serves as a communications hub for the community and incorporates much of
the family's publicly available structural and functional genomics knowledge. The GDR is a
robust and widely used database, but further improvements to enhance its utility to
researchers is underway. Work is on-going to expand its gene, allele, trait, map, marker, and
segregation data sets and prepare for the addition of genome sequence and gene expression
data using the GMOD toolkit (http://www.gmod.org). This database is essential to translate
the investment in genomics and genetics into rosaceous crop improvements. The map that
has been generated in this investigation will be uploaded onto the GDR and make an
important contribution to the genetic and genomic resources for raspberry, since to date,
these have been scant. The data from our investigations will be incorporated into the GDR.

Development of varieties for NC would help establish a major production area on the
east coast and fulfill these closer to markets. The downstream effects would have several
advantages including: less time in transit (less fuel used and longer post harvest life), local
production, add a high value crop to the North Carolina rural economy, and increase
consumption of healthy produce. Available molecular technology that is cheap and useful
makes valuable tools for breeders for many fruit crops that require tolerance to adverse conditions.

This study is unique as it is the first to screen heat tolerance in *Rubus* as well as the first to carry out a molecular assessment for this trait in *Rubus*. This is paving the way and bringing recognition to an understudied but important Rosaceae crop with potential to extend its applications onto other members of the family. This also emphasizes the importance of comparative mapping and genomics within Rosaceae (See Appendix A). As a model system, raspberry would have considerable application because of its diploid inheritance and small genome size (275 Mb). Moreover, compared to most woody perennials, raspberry has a short juvenile phase, thus generation time is shorter. This renders raspberry as a model system with the particular advantage for faster testing and evaluations over other well-studied diploid systems within Rosaceae, such as peach, apricot cherry, and European plum. Many other Rosaceae crops such as plum, blackberry, and strawberry, are complex in their genetic nature. Therefore this study has a high impact to look for heat tolerance on more complex plant systems since baseline knowledge on a simpler system has been established. This is the first study to establish a method for measuring heat tolerance in *Rubus* using chlorophyll fluorescence. Additionally, this is the first study to perform a quantitative trait analysis of heat tolerance in *Rubus*. 
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Figure 1. Dendrogram of the parental history of the mapping population.
CHAPTER II


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Abstract. Despite the high interest in growing raspberries in the southeastern US, production is limited due the lack of cultivars adapted to warm humid conditions in the summer. Breeding for heat tolerance is crucial for production in southeastern states like North Carolina. However there are no established protocols for measuring heat tolerance in Rubus. Heat tolerance is a difficult trait to measure by visual assessment and requires several years
of evaluation. A detached leaf protocol was developed to assess heat tolerance in *Rubus* by measuring leaf chlorophyll fluorescence using a dark adapted test. In order to establish the heat screen protocol for field measurements, several preliminary experiments were performed on genotypes with known heat tolerance to establish baseline physiological responses. Preliminary experiments helped determine optimum leaf collection time, critical heat shock temperature (Tc), minimum duration of dark adaptation, and heat shock duration. We established that collection time should be before leaf temperature reached 28 °C, Tc was determined at 43.7 °C, dark adaptation time was 15 min, and heat shock duration was 30 min. These parameters were used for screening a large population (196 genotypes) planted in warm humid conditions. The segregation of the population was essentially normal, and thus quantitative analysis is a plausible approach for studying heat tolerance in this *Rubus* population. Additionally, ‘Mandarin’ and ‘Qualicum’ were used as heat tolerant and heat susceptible controls, respectively. In field experiments, a significant difference in leaf chlorophyll fluorescence was found between the two controls (0.38 vs 0.63, for ‘Qualicum’ and ‘Mandarin’, respectively). When compared to the segregating population, the tolerant parent, NC497, and ‘Mandarin’ showed the highest Fv/Fm values, while ‘Qualicum’ expressed the lowest. Therefore, a protocol to measure heat tolerance in *Rubus* has been established. This protocol has great potential application to other Rosaceae, and it is not dependent on visual assessment. However, the application of this protocol is probably not well suited for breeders who require screening very large populations (over 400 individuals).
Introduction

In the southeastern US, one of the most limiting factors of raspberry production is the lack of cultivars adapted to warm, humid summers (Moore, 1997). In order to establish major production in this area, cultivars will require tolerance to these conditions, which are very atypical of available raspberry cultivars (Ballington, and Fernandez, 2008). In fact, domestication of red raspberry (*R. idaeus*) has resulted in a reduction of genetic diversity, with most modern cultivars being genetically similar and adapted to the climates like that of the Pacific Northwest (Graham et al., 2004; Graham and McNicol, 1995; Jennings, 1988). In North Carolina, heat tolerance is an essential trait for the cultivation of *Rubus* (Williams, 1950). Cultivars that cannot tolerate the warm conditions of North Carolina tend to be short-lived, and liable to die before the plant is able to flower (Ballington and Fernandez, 2008; Jennings, 1988).

Although there are reports of variation for heat tolerance in fruit crops (Ballington and Fernandez, 2008; Quamme, and Stushnoff, 1983; Williams, 1950), breeding and selection for this trait has not been emphasized. Moreover, with the advent of global climate change, heat tolerance is becoming an increasingly important trait for many other crops. Screening methodologies and molecular dissections of the genetic control of heat tolerance have been studied well in cereal crops, particularly in wheat and maize (Blum and Ebercon, 1981; Dhanda and Mujal, 2006; Fokar et al., 1998; Maestri et al., 2002; Ottaviano et al., 1991; Yang et al., 2002), and some studies on heat tolerance using chlorophyll fluorescence in horticultural crops which include strawberry (Kadir and Sidhu, 2006; Ledesma et al., 2004), tomato (Willits and Peet, 2001), grape (Kadir et al., 2007), and a mix of tropical and sub-
tropical fruits (Weng and Lai, 2005; Yamada et al., 1996). However, very little information is currently available on *Rubus* and other Rosaceae genera. The raspberry breeding program at North Carolina State University (NCSU) focuses on developing germplasm adapted to the warm humid summers. The most successful source for heat tolerance in the program has been from *R. parvifolius*, a species that is considered to be heat tolerant and is indigenous to the warm temperate regions of eastern Asia (Jennings, 1988; Moore 1997; Williams, 1950), and was used to generate segregating populations for the development of this technique.

All plant processes are sensitive to heat, which can cause irreversible damage due to extended heat exposure. Extended heat exposure in raspberry shortens the lifespan of the plant and cause loss of vigor (Jennings, 1988). Heat stress in small fruit crops is associated with significant reduction in photosynthetic activities (Fernandez, and Pritts, 1994; Kadir and Sidhu, 2006; Stafne et al., 2001), and interference of evapotranspiration (Christiansen 1978; Stafne et al., 2001). Reduction in plant growth directly attributed to high temperatures is well established in horticultural crops such as tomato (Adams et al., 2001), grape (Chaumont et al., 1997), and strawberry (Kadir and Sidhu, 2006). In red raspberry, the rate of net CO₂ assimilation drops significantly when temperatures rise to as little as 21 °C, while stomatal conductance remains the same (i.e. no change in evapotranspiration) (Fernandez and Pritts, 1994; Stafne et al., 2001). Whether heat stress occurs early or late in the season, high temperature inhibits thylakoid activities principally near the photosystem II (PSII) reaction centers (Berry and Björkman, 1980). All of these factors ultimately lead to a reduction in yield, and a shorter life span of the plant, emphasizing the importance of heat tolerance in crops.
Chlorophyll fluorescence is routinely used as a physiological parameter that correlates well with heat tolerance (Belkhodja et al., 1994; Kadir et al., 2007; Kadir and Sidhu, 2006; Knight and Ackerly, 2002; Srinavasan, 1996; Weng and Lai, 2005; Yamada et al., 1996; Yang et al., 2002). Chlorophyll fluorescence analysis is simple, non-destructive, and allows analysis of many samples within a short time (Srinivasan et al., 1996). These advantages make the technique ideal for large scale screening of populations, such as large populations for genetic mapping or for selecting elite material in a breeding program. Light is captured by light harvesting chlorophyll proteins, which transfer their energy to photosystem I and II (PS I and II). Approximately 3 to 9% of this energy is re-emitted as fluorescence, primarily by PS II. After the leaf has been subjected to a short dark adaptation period, the pools of intermediates for the electron transport pathway return to a common level; at this point the minimal chlorophyll fluorescence (Fo) is determined. Upon illumination of a dark-adapted leaf, there is a rapid rise of fluorescence emission from PSII, referred to the maximal fluorescence (Fm), followed by a series of short oscillations (Kautsky and Hirsch, 1931). Many studies show that plants subjected to practically any stress will change the level of chlorophyll fluorescence (Genty et al., 1990; Larcher and Neuner, 1989; Oberhuber and Edwards, 1993; Schreiber and Neubauer, 1987; Smilie and Nott, 1982). Therefore, chlorophyll fluorescence is an indirect measurement of damage to PSII.

Here we describe a non-destructive, relatively rapid screening method for field-grown raspberries using chlorophyll fluorescence as a way to measure heat tolerance. This method was developed for the purpose of application to a segregating population intended for quantitative trait analysis. The protocol was modified from that of Yamada et al., (1996),...
where detached leaves were subjected to a brief heat shock. No protocols for screening heat
tolerance have been established in raspberry. Therefore, several baseline physiological
experiments were performed to determine the most logistically and scientifically sound
manner to measure heat stress in this study.

Materials and Methods

All field material for this study was planted at the Sandhills Research Station (SRS) in
Jackson Springs, North Carolina (N. 35° 11.1471 W. 0.079° 40.629 elevation 567’ (173 m)).
This region is located between the coastal plains and the piedmont area, where the maximum
temperature was 31.4 °C in July and 32.1 °C in Aug averaged over the past 4 years (State
Climate Office of North Carolina 2009). The field soil type is Candor Sand type with a 0 to
4% slope with a pH of 5.7. Recommended nitrogen applications were 90 to 112 kg·hec⁻¹ and
lime amendments of 1255 kg·hec⁻¹. Watering was applied as needed throughout the growing
season to equal approximately 1 inch·week⁻¹ from May to Sept. by overhead water reels.

Plant material. The population used for this study was generated from a modified backcross
between (R. parvifolius × ‘Tulameen’) (NC497) and ‘Qualicum’ (Daubeny and Kempler,
1995). Four hundred seedlings were germinated in the greenhouse in January 2006, and
planted in June 2006 at SRS (including parents and controls). One hundred ninety six of
these seedlings were randomly selected for chlorophyll fluorescence stress analysis with a
dark adaptation test. Several cultivars were used as controls in our field screening experiment
and were tested in addition to our seedling population. ‘Mandarin’, NC497, and ‘Kiowa’
(Moore and Clark, 1996), a blackberry cultivar adapted to warm climates, were used as
positive controls in field experiments. ‘Qualicum’ does not tolerate heat (Ballington and
Fernandez, 2008), and was used as the heat susceptible control in field experiments as well. A core group of potted heat tolerant genotypes, ‘Mandarin’ (Stafne et al., 2000; Williams, 1950), *R. parvifolius* and NC497, and heat susceptible cultivars ‘Latham’ and ‘Glen Rosa’ were used to determine baseline physiological responses. This group was chosen because of their performance based on the breeder’s observations.

Fluorescence was measured using a modulated chlorophyll fluorometer (OS1-FL, Opti-Sciences, Hudson, NH, USA). Photosynthetic efficiency was measured by the parameter Fv/Fm, where Fv=Fm-Fo. Minimal fluorescence state (Fo) is defined as the point when all antenna sites are open, i.e. have undergone dark-adaptation. Maximal fluorescence (Fm) occurs when all antenna sites are closed under a light saturation flash. Under stress conditions, chlorophyll fluorescence levels change, mostly due to an increase in Fo (Krause and Weis, 1991). Damage to the light harvesting complexes is expressed as a decrease in Fv/Fm. Dark adaptation was performed using special dark-adaptation clips. These clips can attach to leaves and allowed measurements at a uniform distance, while a special sliding shutter excluded light. All measurements were performed on first fully expanded primocane leaves for all experiments.

**Heat Shock Protocol**

The protocol was modified from that of Yamada et al., (1996), where detached leaves were subjected to a brief heat shock. As mentioned above, no protocols for screening heat tolerance have been established in raspberry. Therefore, several baseline physiological experiments were performed on potted plants. Although potted plant conditions may not reflect the same fluorescence response in the field, these baseline experiments enabled the
determination of the most logistically and scientifically sound manner to measure heat stress in the 196 field-grown individuals in this study. The baseline experiments were performed to determine diurnal variation in tolerance, critical temperature, minimal dark adaptation, and heat shock duration for our core group of *Rubus* genotypes.

*Diurnal Variations in Tolerance.* The Fv/Fm was measured on several attached leaves of genotypes to monitor changes due to diurnal effects. Four ‘Mandarin’, two ‘Latham’, one ‘Glen Rosa’, two NC497, and two *R. parvifolius* plants, with at least two readings per plant, were measured from 6:30 am to 4:40 pm every 45 min in June 2008. Leaf temperatures were recorded for every reading using an Omegascope® infrared handheld thermometer (Omega®, Stamford Connecticut, USA) following manufacturer’s instructions.

*Critical Temperature.* The critical temperature (Tc) is defined as the temperature at which Fo starts to increase sharply, thus the Fv/Fm decreases sharply (Weng and Lai, 2005). Critical temperature for several *Rubus* genotypes was determined by submerging two detached leaves of two ‘Mandarin’, two ‘Latham’, one ‘Glen Rosa’, two NC497, and two *R. parvifolius* potted plants at increasing temperatures (intervals of 5 °C at a rate of approximately 1 °C per minute) in a water bath with a rotary shaker. Two dark-adaptation clips were placed on each of two leaves per each cultivar for dark adaptation and Fv/Fm chlorophyll fluorescence parameter was measured every 40 min for a total of 6 hours.

*Dark Adaptation.* Minimal required dark-adaptation was determined on two detached leaves of two ‘Mandarin’, two ‘Latham’, one ‘Glen Rosa’, two NC497, and two *R. parvifolius* plants, with two measurements per leaf (two dark-adaptation clips per leaf). The Fv/Fm was
measured on leaves once after 45 min, every 20 min for 180 min, every 10 min for 30 min, every 5 min for 25 min, and every minute for four minutes.

Heat Shock Duration. Two dark-adaptation clips were placed on two detached leaves from field grown NC497, ‘Mandarin’, and ‘Qualicum’ plants and placed in clear plastic bags with a moist paper towel and taken into the lab for heat shock. The Fv/Fm measurements were recorded at room temperature. Bagged leaves were then submerged in a water bath with a rotary shaker at 45 °C. The Fv/Fm values were then recorded every 20 min for 100 min to determine reasonable heat shock durations for screening a larger population. Two detached leaves from four ‘Mandarin’, two ‘Latham’, two NC497, and two R. parvifolius in potted, gravel bed conditions were subjected to the same experimentation to determine differences in heat shock duration in potted- versus field-grown plants.

Detached Leaves. To test the effects of measuring Fv/Fm in attached versus detached leaves, several genotypes (‘Mandarin’, ‘Latham’, and NC497) with attached and detached leaves were studied. The Fv/Fm was measured in attached and detached leaves from potted material in lab conditions every 40 min for six hours, where two detached leaves from each genotype were placed in a clear plastic bag at 100% humidity. The same experiment was repeated using field grown plants of ‘Mandarin’, NC497, and ‘Qualicum’ except that only detached leaves were tested. All measurements were taken at 25 °C, and initial dark-adaptation was for 20 min for both experiments.

Heat Screening Field-Grown Plants. The protocol employed, as described above, was developed using results from baseline experiments using the core group of potted plants. Field screening was performed on the population of 196 individuals from the NC497 ×
‘Qualicum’ cross by randomly selecting approximately one third of the population each day for chlorophyll fluorescence analysis. The study population was tested every week from 22 July 2008 until 13 Aug. 2008. The first fully expanded leaf from two randomly selected primocanes were detached from each plant, wrapped in a moist paper towel and placed in a clear plastic bag. Bags were taken into lab conditions where leaves were adapted using 2 dark-adaptation clips per leaf for 15 to 20 min and subsequently the Fv/Fm was measured using the chlorophyll fluorometer. These same leaves were then submerged in a water bath with a rotary shaker at 45 °C. Leaves were taken out after 30 min, allowed to reach room temperature, and the Fv/Fm was recorded.

Statistical Analyses. All correlation coefficients were estimated using PROC CORR in SAS (SAS Institute, Cary, NC). Differences among different cultivars for the parameter Fv/Fm and temperature were tested using PROC MIXED. The critical temperature was determined using PROC NLIN in SAS.

Results

Diurnal Variations in Tolerance. ‘Mandarin’ and R. parvifolius retained the highest Fv/Fm throughout the day, while ‘Latham’ retained the lowest Fv/Fm. The parent NC497 was intermediate between ‘Latham’ and R. parvifolius (Fig. 1). A strong negative correlation was found between leaf temperature and Fv/Fm, i.e. as leaf temperature increased the parameter Fv/Fm decreased. The correlation coefficient over all cultivars was -0.72, while the highest correlation was found in ‘Latham’ and ‘Mandarin’ with values of -0.95 and -0.92, respectively. Differences in the parameter among cultivars were only marginally significant (p=0.03), while difference in Fv/Fm over time was highly significant (p<0.0001). Leaf
temperature was not significantly different among cultivars, but was significant over time (p<0.0001). Although not statistically significant, the leaf temperature in ‘Latham’ was numerically the lowest during peak hours. In ‘Mandarin’ leaf temperatures were highest and escalated up to 41.1 °C, while those of ‘Latham’ only reached a high of 38.9 °C. ‘Glen Rosa’ had sporadic Fv/Fm values, probably due to low number of reps. Therefore ‘Glen Rosa’ was excluded in this analysis. Based on the mixed model, we observed a significant interaction among cultivars after temperatures went beyond 28.3 °C. Therefore, to avoid any bias due to daily temperature stress, collection of leaves in the field was carried out in the morning before ambient temperatures reached 28 °C.

**Critical Temperature.** Based on the LNIL model in SAS, the critical temperature was the point where both regression lines intersected at 43.7 °C (± 0.27, p<0.0001) over all cultivars (Fig. 2). Therefore, to adjust for any fluctuations in temperature during trafficking of samples, heat shock temperature was set at 45 °C.

**Dark Adaptation Duration.** The parameter Fv/Fm was significantly different among cultivars (p=0.0181), and dark adaptation times (p=0.0068). Although there is a general drop in Fv/Fm among all cultivars at the 5-min interval adaptation, there was only a significant difference in Fv/Fm at 1-min intervals for dark adaptation (p=0.0039) (Fig. 3). Despite the statistical analysis, dark adaptation for field studies was kept to a minimum of 15 min to avoid false measurements of the minimal fluorescence (Fo).

**Heat Shock Duration.** In the core group of potted genotypes, ‘Mandarin’, *R. parvifolius*, and NC497 had respectively the highest Fv/Fm throughout the entire experiment, while ‘Latham’ had the lowest Fv/Fm (Fig. 4). In the field planted genotypes, the Fv/Fm values decreased
faster in field planted material than in the potted plant material (Fig. 4 and 5). The heat susceptible cultivar, ‘Qualicum’ had an average Fv/Fm value of 0.432 at 40 min of heat shock (Fig. 4), while that of ‘Latham’ was 0.640 (Fig. 5). Similarly for the tolerant cultivar, ‘Mandarin’, a faster rate of decline in Fv/Fm was observed in field grown plants versus potted plants (approximately 5 times faster in field conditions verses potted in gravel bed). Therefore, there was a notable difference in Fv/Fm values between potted material and field grown material. Heat shock duration was decided at 30 min in order to logistically manage the large number of field samples, while still able to detect a reasonable difference in Fv/Fm.

**Detached Leaves.** Several reports have shown that in many species detached leaves behave no differently from attached leaves, including several rosaceous species (Glynn et al., 2002; Weng and Lai, 2005; Yamada et al., 1996). This was also the case for Rubus in our experiments. No significant change in Fv/Fm was observed after 6 hours, and there was no significant difference between attached and detached leaves at room temperature (data not shown). No difference in Fv/Fm values between field and potted material was observed, and no significant change in Fv/Fm was observed after 100 min in leaves collected from field material (data not shown).

**Population Screening.** The average Fv/Fm values with heat shock treatment were significantly different between the tolerant control and the susceptible control; ‘Qualicum’ had an average value of 0.363, while ‘Mandarin’ and NC497 averaged at 0.632 and 0.631, respectively. ‘Kiowa’ had a moderate Fv/Fm value of 0.576, with heat shock treatment. Both NC497 and ‘Mandarin’ had the highest average Fv/Fm values while ‘Qualicum’ had the lowest among all genotypes tested. Although there was a slight numerical difference at room
temperature, the average Fv/Fm values were not significantly different between the susceptible and tolerant controls. Among the controls, the highest average Fv/Fm values with 25°C treatment were found in ‘Mandarin’ and ‘Kiowa’ at 0.799 and 0.796, respectively; while the lowest average Fv/Fm values were found in NC497 and ‘Qualicum’ at 0.785 and 0.772, respectively. The correlation between the Fv/Fm values at the 25°C and 45 °C treatments was significant but weak (r=0.14, p<0.0001). This implies that values measured at room temperature do not reflect tolerance to heat and that the heat shock treatment is still necessary for “unmasking” damage due to extensive heat exposure. The distribution of individual Fv/Fm values of the population for both treatments was essentially normal (Fig. 5), however, the spread of the distribution is noticeably wider in the 45 °C treatment. Figure 6 shows the average Fv/Fm values for all genotypes with the 45 °C treatment, where the blue line is a normal distribution, and the red line is a fitted distribution of the population. The behavior of this distribution is essentially normal and was suggestive for meaningful quantitative analysis. The mean Fv/Fm value in the mapping population was 0.600. A little over 6000 readings were performed in the field screening study and because of the numerous repetitions, we were able to calculate powerful estimates of important variance components. Table 1 summarizes the variances at both treatment temperatures for the control plants (‘Mandarin’, NC497 ‘Kiowa, and ‘Qualicum’) and the individuals (196 full-sibs from the NC497 × ‘Qualicum’ cross). This allowed us to evaluate the consistency of the method and make considerations for future experiments. The highest variation occurred in Fv/Fm values from leaf to leaf (σ²_L) in all cases, except for the plant to plant variance from the controls at the 45 °C treatment (σ²_p = 1.0901). At 25 °C treatments all variance components
were small, and in most cases showed a ten-fold reduction in comparison to the 45 °C treatment. This is expected since the Fv/Fm values at the 25 °C treatments were very similar (fig. 5). The within leaf variation ($\sigma^2_C$) was relatively small for both controls and individuals at 25 °C. The within leaf variance at 45 °C was similar between the controls and the individual plants, except for the 25 °C treatment, variation was higher in the controls.

Discussion

*Diurnal Variations in Tolerance.* A strong negative correlation was observed between temperature and Fv/Fm values, which indicates that the ambient temperature should be considered when collecting leaves for field studies. Leaf collection at mid-day conditions could result in an interaction response among cultivars and misleading tolerance measurements. A significant interaction was observed among cultivars after temperatures rose above 28.3°C. Therefore, to avoid any bias due to daily temperature stress, collection of leaves in the field was carried out in the morning before ambient temperatures reached 28°C. Other studies on legumes have developed similar approaches to avoid these diurnal responses (Srinivasan et al., 1996). Although not statistically significant, the leaf temperatures in ‘Latham’ were numerically the lowest during peak hours, while in ‘Mandarin’ leaf temperatures were highest. This could be due to an inability for ‘Latham’ to tolerate higher temperatures, thus triggering more transpiration to maintain viable temperatures under heat stress conditions. Other studies support this phenomenon; Stafne et al., 2000 tested the cultivar ‘Mandarin’, and several selections from crosses identical to that of the heat tolerant parent NC497 (*R. parvifolius* $\times$ ‘Tulameen’) and found no difference in stomatal conductance among the genotypes when subjected to high temperature.
Considering the effects of water stress, in this study all genotypes were irrigated at the beginning the experiment, thus the Fv/Fm values could be reflecting water stress during peak hours. However, in contrast to many other environmental stresses, Fv/Fm is not sensitive to early or moderate water stress in most plants (Bukhov and Carpentier, 2004; Živčák et al., 2008), therefore water stress in this experiment is likely negligible.

In this particular experiment, the parent NC497 showed similar Fv/Fm values from those of ‘Latham’, a heat susceptible genotype. Preliminary experiments using potted material also showed Fv/Fm values lower than expected in tolerant genotypes (data not shown). However, in field conditions (see population screening below) NC497 showed Fv/Fm values comparable to that of heat the tolerant control. This difference perhaps is due to aberrant potting conditions, i.e. packing of soil, space limitation for roots, differences in plant maturity, etc. Thus, field conditions for screening heat tolerance using this method are a more accurate representation of the phenotype. Furthermore, field conditions allow proper acclimatization of plants, and screening at the field stage is more reliable because heat tolerance in potted conditions may not transfer at the field level.

The heat tolerant parent is one-half *R. parvifolius*, while ‘Mandarin’ is one-quarter of this species and showed higher Fv/Fm values. In some instances ‘Mandarin’ showed higher Fv/Fm values than the full species. Although no definitive records exist, the *R. parvifolius* species in the genetic background of ‘Mandarin’ likely originated from different source than that of NC497. The different levels of heat tolerance suggest that there are diverse levels of tolerance to heat within the species. Thus, with only one-quarter in its background, the particular *R. parvifolius* in ‘Mandarin’ is attributing exceptional tolerance to heat. Moreover,
‘Mandarin’ has also been shown to have a high carbon assimilation rate (A) at 35 °C, while those of selections of (R. parvifolius × ‘Tulameen) show intermediate A (Stafne et al., 2000).

Critical Temperature. The critical temperature is plant species specific and may vary with the season (Weng and Lai 2005), for this reason, it was necessary to determine the Tc specific to Rubus during the growing season. We determined that Tc is 43.7 °C. Although the critical temperature is not reflective of realistic conditions, Weng and Lai, (2005) tested numerous genera with different climate adaptations, and found no correlation between heat tolerance and critical temperature value. Thus this temperature signifies only a point at which heat damage is conspicuously detectable by chlorophyll fluorescence. Weng and Lai, (2005) have also determined very similar critical temperatures in other rosaceous genera, specifically, Prunus (42 °C), Rosa (46 to 47 °C), and Pyrus (44 to 45 °C). They also found that the seasonal changes in Tc for grass species varied significantly, i.e. the Tc in Jan. through Feb. was much lower than in July. However this was not the case for woody species, where Tc tended to remain the same in both seasons. In cotton, the same heat stress treatment as in our field experiments (30 min at 45 °C, but using leaf discs) shows a significant reduction in Fv/Fm, while showing no change in Fv/Fm at 40 °C or at 42.5 °C (Crafts-Brandner and Law, 2000). The same study measured carbon exchange rates (CER) and Rubisco activity pre- and post-treatment at 45 °C, and found that all three parameters (CER, Rubisco activity, and Fv/Fm) had been significantly reduced with no recovery. This indicates that the Tc must be a point at which photosynthetic activities are irreversibly damaged, and are detectible only when the cells have been exposed to the specific temperature.
Dark Adaptation Duration. Although there is a general drop in Fv/Fm among all cultivars at the 5-minute interval adaptation, there was only a significant difference in Fv/Fm at 1-minute intervals for dark adaptation (Fig. 3). Although statistical analysis revealed that 5 min was adequate, dark adaptation for field studies was kept to a minimum of 15 min to avoid any bias measurements of non-adapted leaves due to unopened antenna sites. In other words, there is an overestimation of the minimal fluorescence (Fo). This was very pronounced at the 1-minute interval because very likely antenna sites were saturated. Although in our study a 15-minute dark adaptation was chosen, future studies may be able to perform treatments at shorter dark-adaptation durations to enable more time efficient testing.

Heat Shock Duration. Heat shock duration was chosen at 30 min in order to logistically manage the large number of field samples, while still able to detect a reasonable difference in Fv/Fm. The variability within each sample also increased at longer heat shock durations (after 30 min). Therefore, to avoid any possible sample interaction, heat shock durations should be no longer than 30 min. Other studies have also noted increased variability in Fv/Fm values particularly at the Tc (Crafts-Brandner and Law, 2000). When comparing the heat shock duration of potted material (Fig.4) and field material (Fig. 5), the rate at which Fv/Fm decreases is faster in field material. Although all plant material was exposed to warm outdoor conditions, this difference in rate suggests that field conditions are causing more long-term damage to the leaf, and is being reflected at the Tc.

Population Screening. A little over 6000 readings were performed in the field screening study and we were able to calculate significant estimates of important variance components. This allowed us to evaluate the consistency of the method and make considerations for future
experiments. The within leaf variance at 45 °C was similar between the controls and the individual plants, except for the 25 °C treatment, where variation was higher in the controls. The cause of this high variation is uncertain. Although all leaves were tested at the same developmental stage (first fully expanded primocane leaf), in most cases the highest variance was observed in the leaf to leaf variance within plants ($\sigma^2_L$), this suggests that for future evaluations experiments must include more measurements from different leaves within plants rather than more measurements within leaves.

The distribution of values of the mapping population is essentially normal (Fig. 6). This is very encouraging for quantitative trait analysis and finding significant QTL associated with heat tolerance (Collard et al., 2005). There were no transgressive segregants in the population, i.e. no individuals outperformed either parent as heat tolerant or heat susceptible. This suggests that ‘Qualicum’ is not contributing any genes to the heat tolerance trait in the population. A similar screening study was performed in maize, except cellular membrane stability was used an indicator of thermotolerance, a type of heat tolerance expressed at the seedling stage (Ottoviano et al., 1991). A similar distribution curve was generated where variability tended towards normality, a typical pattern of quantitative traits. However, unlike our Rubus mapping population, Ottoviano et al., (1991) found individuals that exceeded both parents in tolerance and susceptibility. The mean Fv/Fm value in the mapping population was 0.600, and approximately 52% of the population was above this limit. We propose that plants that have an average Fv/Fm below 0.600 after a heat shock treatment are considered heat susceptible, and conversely, those with values above 0.600 are heat tolerant.

**Conclusion**
Heat stress is a major limitation to the production of many crops including raspberry (Ballington and Fernandez, 2008; Fernandez and Pritts, 1994). However, this is the first study to establish a method for measuring heat tolerance specifically in *Rubus* that is non-dependent on visual assessment. To our knowledge, very few cultivars are listed as heat tolerant in the USDA-ARS *Rubus* germplasm repository, and those that have been identified have only been evaluated through visual assessment. The large difference in Fv/Fm values between tolerant and susceptible controls in field measurements supports this method as a strong indicator of heat tolerance. Moreover, this study supports observations of poor and excellent heat tolerance in the field from ‘Qualicum’ and ‘Mandarin’, respectively. Thus we have also demarked ‘Mandarin’, and NC497 as heat tolerant standards, and ‘Qualicum’ as a heat susceptible standard. This will be particularly useful for breeders in field evaluations and in future heat tolerance studies as established controls. However the method is perhaps not well suited for screening a large amount of material in an established breeding program. Because it is necessary to apply a 30 minute treatment at 45 °C to discern heat tolerant types, screening more than 200 individuals with this method would be tedious and time consuming. Therefore, finding molecular markers linked to heat tolerance will be a more efficient approach for aiding in breeding.

In molecular breeding and quantitative trait analysis, reliable and accurate phenotyping is crucial for detection of quantitative trait loci or QTL (Collard et al., 2005). Reliability of QTL is dependent upon reliable phenotypic data. Additionally, visual assessment of heat tolerance can be difficult and breeders can only ascertain the extremes. Therefore, this protocol allows for significant application in detecting QTL. Because very few studies have
focused on measuring heat tolerance in Rosaceae crops, potential application to other genera within this family exists.
Literature Cited


Table 1. Summary of variance components estimates of the Fv/Fm values for heat shock treatments at 25°C and 45°C. Summary compares variances of control plants (‘Qualicum’, ‘Kiowa’, NC497, and ‘Mandarin’), and individuals from the mapping population (196 full-sibs from the NC497 × ‘Qualicum’ cross). Table includes plant variance ($\sigma^2_P$), week variance within plant ($\sigma^2_W$), leaf variance within plant within week ($\sigma^2_L$), and clip variance within leaf, plant and week ($\sigma^2_C$).

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*, **, *** Significant at $P \leq 0.05$, 0.01, or 0.001, respectively
Figure 1. Diurnal variation in tolerance of potted *Rubus* plants. Chlorophyll fluorescence parameter Fv/Fm was measured on dark-adapted leaves every 45 min for 10 hours and 20 min in outdoor potted conditions June 2008.
Figure 2. Critical temperature (Tc) over all genotypes determined by the intersection of the regression lines between the fast moving portion (open circles) and the slow moving portion (black circles) of the curve.
Figure 3. Dark adaptation duration curve. Fv/Fm measurements were taken in intervals of 20, 10, 5 and 1 min. Fv/Fm values decrease due to inaccurate estimation of Fo.
Figure 4. Effects of the Fv/Fm over time of detached, dark-adapted leaves from several *Rubus* genotypes measured at 20-minute intervals at 45 °C. Genotypes were potted and in gravel bed conditions.
Figure 5. Effects of the Fv/Fm over time of detached, dark-adapted leaves from several *Rubus* genotypes measured at 20-minute intervals at 45 °C. Genotypes were planted at the Sandhills Research Station, in Jackson Springs, NC in June 2006, and tested in Aug. 2008.
Figure 6. Distribution of all Fv/Fm values for all measurements in field screening experiments for the 25 °C and the 45 °C treatments.
Figure 7. Distribution of Fv/Fm values averaged over four weeks for all genotypes at 45 °C treatment. The blue line represents a normal distribution, while the red line is a fitted curve to the distribution of this population.
CHAPTER III

Construction of a genetic linkage map of raspberry (*Rubus idaeus* L.): Quantitative analysis of heat tolerance, prickle density and growth habit

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ABSTRACT

Despite the high level of interest for growing raspberries (*Rubus idaeus*) in the southeastern US, production is limited by the lack of cultivars adapted to warm, humid summers. Breeding efforts are underway for increasing cultivar availability, however breeding improvement in *Rubus* is a slow and time-consuming process. In order to expedite the slow, but effective breeding process, more molecular breeding tools should be developed. To address this issue, a genetic mapping population has been developed from a cross between (*R. parvifolius* × ‘Tulameen’) (NC497) × ‘Qualicum’. This population was used for the construction of a genetic linkage map and for quantitative trait loci (QTL) analysis of heat tolerance. Seven linkage groups were identified and were anchored to the already existing map. The majority of the linkage groups identified were of similar genetic size, and anchor markers were located at similar genetic distances relative to other markers for all linkage groups. For significant QTL analysis, accurate phenotypic screening in the population is crucial. Chlorophyll fluorescence to assess heat tolerance in the mapping
population, and after QTL analysis, three regions were associated with trait, the QTL on group explained the highest percent variation (15.9%). Other important horticultural traits segregated in the NC497 × ‘Qualicum’ cross, and were evaluated for QTL analysis. These traits were growth habit, and prickles density. Two field evaluations were performed for growth habit and several regions were identified as significant. One QTL co-localized to the same region of the linkage map for growth habit in both growing seasons. Important QTL have been mapped for heat tolerance and should be considered for further molecular studies. This research has drawn a baseline foundation for the development of molecular technologies in improving heat tolerance in *Rubus*. Future research should focus on these regions to develop closely linked molecular markers for marker assisted breeding.

INTRODUCTION

Raspberry (*Rubus idaeus* L.) production has mostly been limited to cool temperate regions, however there is increasing interest to grow raspberries in other regions of the US. This concentration in production can be partly attributed to the lack of cultivars adapted to warm temperate regions like that of the southeastern US. Moreover, the domestication of raspberry has resulted in a reduction of genetic diversity, with most cultivars adapted to climates like that of the Pacific Northwest, a climate in which the *R. idaeus* is naturally adapted (Graham et al. 2004; Graham and McNicol 1995; Jennings 1988). Perhaps most notably, the biggest limitation is achieving tolerance to heat in the field. In raspberry, the consequence of long term exposure to high heat shortens the lifespan of the plant, and can even cause death before fruiting (Jennings 1988). *Rubus idaeus* is so sensitive that the rate of
net CO₂ assimilation drops significantly when temperatures rise above 21°C, while evapotranspiration remains the same (Stafne et al. 2001; Fernandez and Pritts 1994). High temperature also inhibits thylakoid activities near the photosystem II reaction centers limiting photosynthesis and therefore plant growth (Berry and Björkman 1980). These factors contribute to a reduction in yield and a shorter life span of the plant causing a limitation in production areas. Additionally, with the advent of global warming, heat tolerance is becoming an increasingly important trait in many other crops.

At the North Carolina State University Rubus Breeding Program, efforts are underway to develop raspberry cultivars adapted to the warm humid conditions of the southeastern US (Ballington and Fernandez 2008). Traditional breeding efforts in Rubus can be quite time consuming with initial field evaluations alone taking up to 3 years, and cultivar releases typically taking 10 to 15 years. This emphasizes the need for the use molecular tools in marker assisted breeding (MAB) to expedite the breeding process. Establishing a strong gene to phenotype relationship is important in marker development, and consequently this will facilitate the breeding process because selection is based on genotype, not phenotype. Developing a genetic linkage map can greatly improve the speed and precision of breeding by mapping of traits and consequently developing markers tightly associated with the trait (Collard et al. 2005).

Accurate quantitative trait loci (QTL) are highly dependent on thorough and accurate phenotypic screening (Collard et al. 2005). Therefore, in order to screen for heat tolerance, a highly dependable and accurate system for measuring heat tolerance is necessary. Chlorophyll fluorescence is routinely used as a physiological parameter that correlates well
with heat tolerance (Kadir et al. 2007; Kadir and Sidhu 2006; Weng and Lai 2005; Knight and Ackerly 2002; Yang et al. 2002; Srinavasan 1996; Yamada et al. 1996). Chlorophyll fluorescence analysis is simple, non destructive, and allows analysis of many samples within a short time (Srinivasan et al. 1996). These advantages make the technique ideal for large scale screening of populations, such as large populations for genetic mapping or for selecting elite material in a breeding program. In a previous study, we reported the development of a protocol for the screening of heat tolerance specifically for *Rubus* using chlorophyll fluorescence (Chapter II), which was used in this study for measuring the trait in the mapping population and consequently for quantitative genetic analysis. The mapping population for this study was generated from a cross between (*R. parvifolius* × ‘Tulameen’) (NC497) and ‘Qualicum’, and segregates for heat tolerance. Additionally, the NC497 × ‘Qualicum’ cross-segregated for other important horticultural traits and we describe the quantitative analysis of density of prickles (also referred to spines in other literature), and cane growth habit.

**Prickle Density and Pubescence**: Breeding cultivars with prickle free canes that are easier to manage (i.e. easier to prune and to harvest), is a main concern for breeders and growers. Both parents in this study exhibit prickly canes, but the population from this cross segregate for the trait from densely prickly to prickle-free. There are several major genes that confer prickles in red raspberry (*R. idaeus*) (Jennings and Ingram 1983; Jennings 1988). One of these is the *s* gene, and is dominant for prickles. *R. parvifolius* is known to carry gene(s) for the prickle free trait and are of particular interest because its occurrence in F1 hybrids suggested that these are dominant genes (Jennings and Ingram 1983). However, these genes have not been genetically characterized. Cane pubescence is determined by gene *H*, which
gives rise to fine hairs on the surface of canes (genotype \( HH \) or \( Hh \)), and in homozygous recessive individuals (\( hh \)), hairs are absent (glabrous). Homozygous dominant (\( HH \)) individuals are rarely found due to linkage with a lethal recessive gene (Jennings 1967). Gene \( H \) is also known to have a pleiotropic effect on prickles; the presence of the gene slightly increases density of prickles while also decreasing the size of prickles (Jennings 1962; Keep et al. 1977).

**Growth Habit:** Breeding for erect canes is much more desirable for breeders and growers. Erect canes can allow easier trellising and more efficient harvesting. In addition to heat tolerance, and prickle density, the mapping population segregated for different growth habit, where most of the progeny had a decumbent growth. This trait is likely coming from the parent NC497 as it exhibits thin, spindly canes, whereas ‘Qualicum’ exhibits more upright canes.

Thus far, heat tolerance in *Rubus* has not been characterized molecularly; this is the first study on quantitative analysis for heat tolerance in raspberry. Here we report the construction of a genetic linkage map in raspberry, and the detection of quantitative trait loci for heat tolerance, prickle density, and growth habit.

**MATERIALS AND METHODS**

**Plant Material and Heat Screening:** The population used for this study was generated from a modified backcross between NC497 and ‘Qualicum’. The heat tolerance trait was integrated into the tolerant parent, NC497, via *R. parvifolius*, a species native to the warm humid regions of southeastern Asia (Ballington and Fernandez 2008). ‘Mandarin’ (Williams
1950) is a heat tolerant raspberry cultivar with a similar genetic makeup, species-wise, to the mapping population (Ballington and Fernandez 2008), and ‘Kiowa’ (Moore and Clark 1996) is a blackberry cultivar that consistently performs well in warm climates. These two genotypes were used as positive controls while ‘Qualicum’ (Daubeny and Kempler 1995) was used as a heat susceptible control as it performs poorly in warm climates (Ballington and Fernandez 2008). Four hundred seedlings of the mapping population were germinated in the greenhouse in January 2006, and planted in June 2006 at the Sandhills Research Station (SRS) in Jackson Springs North Carolina, including parents and controls. This region is located between the coastal plains and the piedmont area, where the maximum temperature was 31.4°C in July and 32.1°C in August averaged over the past 4 years (State Climate Office of North Carolina, NC State University. CRONOS [internet database]). The field soil type is Candor Sand type with a 0-4% slope with a pH of 5.7. Recommended amendment applications were 80-100lbs N/ac and 0.5 tons lime per acre. Watering was applied as needed throughout the growing season to equal approximately 1 inch per week from May to September by overhead water reels. One hundred ninety eight individuals from the mapping population were randomly selected for the construction of the genetic linkage map and 196 of these were used for chlorophyll fluorescence stress analysis with a dark adaptation test (measured as the ratio between the variable fluorescence and maximum fluorescence or Fv/Fm) as described in Chapter II.

*Pubescence and Prickle Density:* Pubescence of canes were designated as present (*Hh* or *HH*) or absent (*hh*) by visual examination, and all individuals in the mapping population were pubescent. Density of prickles was scored visually on a scale of 0 to 5, where 0 is no
prickles and 5 is densely covered in prickles. Although the prickly trait has been previously mapped (Graham et al. 2004), completely prickle free individuals were not present in the population for that particular study.

**Growth Habit:** The entire population of 196 seedlings was scored during two growing seasons, the summers of 2007 and 2008. In 2007, the growth habit of the canes were mostly decumbent and very few canes reached a height greater than 15 cm, and thus a simple scoring system from 0 to 3 was used, where 0 signified that most canes were decumbent and 3 that most canes were erect. In 2008, the general growth habit of the population changed, although most canes were still decumbent. In 2008 the overall height of the canes was 40 cm. Thus the scoring system was adjusted to 0 to 4 to reflect the change in growth habit. This change from decumbent to upright growth habit in the second year is common in erect blackberry genotypes (Fernandez and Ballington 1999).

**Molecular Marker Development:** Purification of total genomic DNA was extracted using a 2% CTAB method from young leaves for the 198 mapping individuals (Graham et al. 2003). Amplified fragment length polymorphisms (AFLP) were generated based on the protocol developed by Vos et al. (1995). Five hundred ng of template DNA from the mapping population and parents was digested with 12 U of EcoRI and 8 U of MseI. Pre-selective amplification reactions were performed as indicated by Vos et al. (1995). To maximize the number of polymorphic markers, a preliminary screen of 171 different selective amplification primer combinations was performed on 6 of the progeny. The selective primer combinations were prioritized according to the number of polymorphic markers and clarity of the fragments present in the gel. The 5’ end of the EcoRI selective
amplification primer was labeled with either infrared dye IRDye™ 800 or IRDye™ 700 (LI-COR, Lincoln, Nebraska). AFLP products were denatured and resolved in an 8% acrylamide gel that ran for approximately 3 hours in a 4300 DNA Analysis System (LI-COR, Lincoln, Nebraska). Simple sequence repeat (SSR) markers developed by Graham et al. (2004) were used for the development of the linkage map and to anchor linkage groups to their already existing (Graham et al. 2006). Polymerase chain reactions (PCR) for SSR were carried out using the three-primer tailing system described by Schuelke (2000), where the IRD label was bound to the 5’ end of the M13 universal primer. The PCR for SSR fragments were performed using 20 to 50 ng of DNA from the 196 plants in the mapping population and the two parents. Reactions were carried out in a 10 µl reaction with 0.2 pmol forward primer, 1.0 pmol reverse primer, 1.0 pmol M13-universal IRD labeled primer, 0.2 mM of each nucleotide, 1X standard buffer with MgCl₂, 0.5 U Taq DNA polymerase (Roche) in a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems) for 15 cycles with a denaturing step at 94.0°C for 30s, an annealing at 59°C for 30s, and an extension at 72.0°C for 1 min, then 25 cycles with denaturing at 94.0°C for 30s, annealing at 50°C for 30s, and extension at 72.0°C for 1 min, and a final extension at 72°C for 7 min.

**Linkage Map Construction and QTL Analysis:** Segregation analysis was carried out on the 198 individual plants of the mapping population. The linkage map was constructed by analyzing data from the polymorphic SSR and AFLP fragments in the mapping population using JoinMap 3.0 and setting the population type as CP for cross pollinated crops (Van Ooijen and Voorrips 2001). The expected segregation ratios of the molecular markers (either 1:1 for aaxab and abxaa, or 3:1 for abxab) were tested for goodness-of-fit with a Chi-square
SSR markers developed by Graham et al. (2004) were used to reference and anchor linkage groups to the map reported by Graham et al. (2006). Recombination units were converted into genetic distances using the Kosambi function. The QTL analysis was conducted using MapQTL® 5 (Van Ooijen 2004) and a logarithm of odds (LOD) score of 3.0. QTL for heat tolerance were first detected with interval mapping and, whenever possible, regions were further analyzed by using the MQM function analysis (composite interval mapping) in MapQTL and using significant markers found in interval mapping as cofactors. Significant markers for Prickle density and growth habit were first analyzed by single point analysis by using the Kruskal-Wallis function in MapQTL. Significant marker regions were then further analyzed by the interval mapping procedure.

RESULTS

Marker Data and Map Construction: Thirty-nine SSR markers were tested on the mapping population. Ten of these were monomorphic, seven had no or sporadic amplification, one was heterozygous, and twenty were polymorphic. However, two of the polymorphic markers did not amplify the expected SSR region using the M13-tailing approach, and consequently were not mapped. One hundred seventy one AFLP selective primer combinations were prescreened using 6 of the progeny, 39 of these combinations met the selection criteria. Twelve of these 39 selective primer combinations were chosen for the construction of the genetic linkage map (Table 1). A total of 341 AFLP markers were scored. Deviations from Mendelian expectations were significant for 157 of these markers, which were removed from the analysis. Thus 184 AFLP markers were used for the construction of
the map. The *Rubus* map reported here has a total length of 607 cM. Linkage groups were of similar size to the groups reported in Graham et al. (2006). Linkage group 6 had poor coverage of markers and therefore this group is shown fragmented. This fragmentation was likely due to the lack of marker saturation and although the linkage of the markers remained at a LOD score of 7.0, and JoinMap 3 could not determine the linkage phase of the markers. The majority of the markers segregated in one of the parents, in this case NC497. Two SSR markers from Graham et al. (2004), Rubus157 and Rubus228a, have now been mapped to groups 2 and 3, respectively.

**Heat Tolerance**: The distribution of Fv/Fm values for the population was essentially normal, with Fv/Fm values (averaged for each genotype over time) ranging between 0.63 and 0.38 (Chapter II, Fig. 6). No transgressive segregants were observed in the distribution, and the normal distribution suggests a typical pattern of that of quantitative traits. Three QTL regions were found associated with heat tolerance in linkage groups 1, 5 and 7 (Table 2). The QTL on group 1 however, explained most of the variation (15.9%), and had a LOD score of 4.17. The other two QTL accounted for approximately 10% of the variation.

**Prickle Density**: Approximately twenty percent of the mapping population is prickle-free, but the majority of the progeny (~50%) were mildly prickly (score of 4 or 3), while a small percentage was sparsely prickly or very densely prickly (score of 1, 2 or 5) (Fig. 3). Two QTL were detected for prickle density one of which explains a large portion of the variation (Table 2). The largest percentage of the explained variation was attributed to the QTL found on group 7, explaining approximately 75% of the variation. The QTL on group 3 was found in a similar location as a QTL for prickles (or spines) reported by Graham et al.
(2006), although the explained variation and LOD scores were lower in comparison to the other QTL for prickle density. In Graham et al. (2006) this same QTL for prickles (or spines) on group 3 co-localizes with three QTL for disease resistances.

*Growth Habit:* The growth habit of the progeny in the growing season of 2007 was mostly decumbent with few of these expressing erect canes (Fig. 2). Although the habit in the growing season of 2008 was mostly decumbent, the overall height of the canes had increased, and changes in the scoring system were made to reflect this increase (Fig. 2). Nevertheless, only a few plants exhibited a completely erect phenotype as in the first growing season. Two QTL for each growing season were associated with linkage group 2 and the regions were closely positioned to each other (Fig. 2). Both of the QTL on group 2 explained approximately half of the variation, 39% the first growing season, and 58.6% the second growing season. Phenotypic data from both growing seasons generated a second QTL, however these were associated with two different linkage groups. In the first growing season (Growth-07), the second (minor) QTL was associated with group 5 with a LOD score of 3.02 and explaining 7.2% of the variation. In the second season (Growth-08), a minor QTL was found on group 3, explaining 14% of the trait with a LOD score of 4.50.

**DISCUSSION**

Molecular research in raspberry has been steadily growing, providing more potential tools that can be implemented in molecular breeding and expanding research in this understudied crop. Currently, there are three genetic linkage maps available in raspberry
(Sargent et al. 2007; Pattison et al. 2007; Graham et al. 2004). The most elaborate map has been developed by Graham et al. (2004; 2006; 2009), and offers extensive coverage with various QTL regions for valuable traits. Here we report a fourth linkage map of raspberry, and is the first study to look at heat tolerance in *Rubus* using chlorophyll fluorescence for QTL analysis. The application of this study for developing heat tolerant cultivars can have important impact for production, since growing regions would expand to the southeastern US and places with similar climatic conditions. Additionally, other important traits had significant QTL regions associated to the linkage groups, and will help further research, as localization of QTL can represent the first step for molecular characterization of genes underlying the trait (Ganal et al. 1989). Validation of the linkage map was achieved by assessing co-linearity of SSR and SSR-EST markers onto the existing map, along with the co-localization of a QTL for prickles (or spines) that was mapped at a similar genetic distance, close to marker Rubus103a (Graham et al. 2006).

*Map Construction:* Similar to the map developed by Graham et al. (2004; 2006), most of the markers segregated in one of the parents, in this case NC497. This is likely due to the integration of *R. parvifolius* that is contributing to more heterozygous regions in the progeny and thus more polymorphisms came from NC497. However, to a lesser extent, ‘Qualicum’ has *R. occidentalis* in its parentage (Daubeny and Kempler 1995) The *Rubus* map reported here has a total length of 607 cM, while the total length of that of Graham et al. (2006) was 669 cM. Consistent with the available map (Graham et al. 2006), most of the linkage groups are of similar size, while the SSR markers had the same order and approximately the same position within the groups. However, there were some notable differences in the linkage
groups. For example, linkage group 1 reported in Graham et al. (2006), the genetic distance was 125 cM, while group 1 in this study is only 64 cM. Perhaps this is due to a lack of markers that segregate in this region, and the fact that most of the markers in this region were closely linked, approximately 1.5 cM apart on average. A similar explanation can be applied to linkage group 4; group 4 in Graham et al. (2006) was 85 cM, while in this study group 4 is 55 cM. Linkage group 6 had poor coverage of markers and therefore this group is shown fragmented. Based on the SSR anchor markers Rubus123a, Rubusleaf102, and Rubusleaf97, it is likely that group 6A corresponds to the upper portion of group 6, while 6B corresponds to the lower portion of group 6 (Graham et al. 2006) (Fig. 1). Additionally, our linkage group 6 was fragmented into 3 segments, where group 6B2 is likely part of group 6B. This fragmentation was likely due to the lack of marker saturation and although the linkage of the markers remained at a LOD score of 7.0, JoinMap 3 could not determine the linkage phase of the markers. Although linkage group 6 in this study had limited recombinant markers, the genetic distance agrees with the previously published map (Graham et al. 2006). Moreover, in the map published by Graham et al. (2006), there is a large gap in the middle portion of group 6, therefore it has been difficult to identify recombinant markers in this group. Linkage group 7 was slightly denser and larger than group 7 reported by Graham et al. (2006), but was of similar size (69.3 cM vs. 61cM).

Heat Tolerance: In the southeastern US, heat tolerance in the field is an essential trait for the production of raspberry (Ballington and Fernandez 2008; Moore 1997; Williams 1950). The heat tolerance trait was screened following the chlorophyll fluorescence protocol previously reported (Chapter II), and resulted in a distribution that was essentially normal,
while showing a distinct difference in tolerance in the two parents (Chapter II, Fig 6). This result was encouraging for QTL analysis, as simple or non normal distributions tend to result in the phenotypic information outweighing the genetic information (Van Ooijen 2004). There was no transgressive segregation in this distribution, and the normal behavior of the distribution is typical of that of quantitative traits. Both parents represented the extreme values of the distribution of the screen with Fv/Fm values of 0.38 and 0.63 for ‘Qualicum’ and NC497, respectively. This suggests that ‘Qualicum’ is not contributing any complimentary genes to the heat tolerance trait ( Tanksley 1993). Additionally, both NC497 and ‘Mandarin’ contain R. parvifolius in their background. ‘Mandarin’ was used as a positive control in the protocol development, and showed significant and consistent heat tolerance that was comparable to the resistant parent (Chapter II). Thus this helps further validate the significance of the results of the heat screening in the mapping population. Three QTL were found associated with the trait, and the highest percent explained variation was 15.9% for the QTL on group 1. Since chlorophyll fluorescence is an indirect measure of the efficiency of the light harvesting complexes (Krause and Weise 1991), we would expect that the identified QTL regions encode genes involved in the stabilization of photosynthetic proteins. As mentioned in Chapter II, in order to “unmask” the effects of extended heat exposure in raspberry leaves, a brief heat shock at 45°C was necessary. Crafts-Brandner et al. (2000) has shown that in cotton, the exact heat shock treatment as in Chapter II (45°C for 30 minutes) causes irreversible damage to Rubisco, and stops carbon assimilation. Therefore, NC497 could be contributing allele products that stabilize photosynthetic proteins such as Rubisco, or enzymes that help form more stable lipid membranes.
**Prickle Density:** The QTL for density on group 3 in our linkage map was located at a similar genetic distance as the one reported by Graham et al. (2006), close to SSR marker Rubus103 (Fig. 1). The presence of prickles have been observed to be tightly associated with several fungal disease resistances in *Rubus* (Jennings 1988). Graham et al. (2006) found three QTL that were associated with fungal resistance to rust, didymella, and botrytis overlapping the same region as the QTL for prickle density on group 3. Thusly, inferences on these fungal resistances can be made between the map reported herein, and the map reported by Graham et al. (2006). However a second QTL for prickle density reported by Graham et al (2006) was not detected in our linkage map, perhaps because this region is associated with gene H, and the glabrous phenotype does not segregate in our mapping population. The prickle-free phenotype is attributed to gene s, where a homozygous recessive genotype renders prickle-free canes. Approximately 20% of the progeny is completely prickle-free. A little over 50% are mildly prickly, while few are densely prickly or sparsely prickly (~15% each) (Fig 3). This phenotypic distribution suggests that each of the parents was heterozygous for gene s, and the majority of the progeny are heterozygous expressing incomplete dominance or the involvement of other genes. The segregation of gene s in the population is possible because both ‘Glen Prosen’ and ‘Glen Moy’ are prickle-free cultivars, i.e. each grandparent of the parents is known to carry two recessive alleles for gene s (Chapter I, Fig. 1). ‘Tulameen’ received the recessive allele from ‘Glen Prosen’ (Daubeny and Anderson 1991), and ‘Qualicum’ inherited the allele from ‘Glen Moy’ (Daubeny and Kempler 1995). However, there are several major genes that confer the prickle free trait in addition to gene s. These are gene Sf (Rosati et al. 1986), $S_{TE}$ (Hall et al. 1986) and $S_{fL}$ (McPheeters and Skirvin 1983;
Rosati et al. 1988). Moreover, there are other genes present in *R. parvifolius* that are known to suppress the prickly phenotype and convert glands into soft prickles (or spines) (Jennings 1988; Jennings and Ingram 1983). These different genes provide several potential genetic explanations for the second QTL found on group 7, which had a large significant effect on prickle density (Table 2).

*Growth Habit:* Growth habit was evaluated across two growing seasons, and two QTL regions were detected in both seasons (Fig 1). The QTL model explained a large percentage of the variation in both 2007 and 2008. Although the growth habit changed in 2008, one QTL in each of the seasons was found associated with group 2, and slightly overlapped the same region. Thus, this reinforces the association between this region and the trait. In the first growing season, a QTL on group 5, the QTL only explained a small portion of the variation (7.2%), and although the Kruskal-Wallis test showed a significant association to markers (*p*≤0.005), in the interval mapping procedure the LOD score was small (3.02). These two minor QTL however could be reflecting the change in growth habit between the two years or the change in the scoring system. Nonetheless, the change in scoring system may not be the reason for this change because the distribution of the values in the scoring system, essentially were kept unchanged (Fig. 2).

Heat stress is a major limitation to the production of many crops including raspberry (Ballington and Fernandez 2008; Moore 1997). This is the first study to identify QTL associated with heat tolerance in *Rubus*. Breeding for heat tolerance is essential for production in warm temperate regions such as North Carolina, and most of the southeastern US (Moore 1997). To our knowledge, very few cultivars are listed as heat tolerant in the
USDA-ARS *Rubus* germplasm repository, and these listings have mostly been supported through visual assessment in the field. There is high interest in raspberry production in the southeastern US, and one of the major limitations is a lack of heat tolerant cultivars (Ballington and Fernandez 2008; Moore 1997). Because conventional breeding is an effective but slow process, marker assisted breeding (MAS) will help expedite the breeding process. The first step in route to molecular characterization and selectable marker development for traits of interest is the establishment of QTL regions (Ganal et al. 1989). In this study, several QTL have been identified associated with an essential trait for growing raspberries in North Carolina, i.e. heat tolerance, plus two important horticultural traits, growth habit and prickle density. Our constructed map is well corroborated by the similarity in size of the proposed linkage groups, and the similarity in the position of anchoring SSR markers to the well-established map of red raspberry (Graham et al. 2004; 2006; 2009). Additionally, a QTL associated with prickle density (or spine density) is associated with the same linkage group and is in close proximity to an SSR anchoring marker in both linkage maps. Although the QTL for heat tolerance explained a maximum of 15.9% of the variation of the trait, a large percentage of the variation in prickle density, and growth habit at both evaluated years were explained by the QTL models.

Future prospects include further saturation of the map to complete the construction of linkage group 6, and any other unsaturated regions. Because heat tolerance is a complex trait that can affect several major biological processes (Stoddard 2006; Paulsen 1994), a screening of the population using a different methodology, such as measuring membrane permeability...
(Srinivasan et al. 1996), should be implemented. This will enable the location of other important QTL involved in heat tolerance and or further corroborate the current QTL.
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Figure 1. Genetic linkage map of raspberry. Significant LOD intervals (>3.00) for heat tolerance (Heat), prickle density (Prick), growth habit in the first season (Growth-07), and growth habit in the second season (Growth-08) are shown by solid bars. Suspected associations are shown as hairlines after solid bars.
LG2

0.0  E45M37_122.4
10.7  Rubus107a
22.0  Rubus194h
29.0  E45M33_124.3
30.1  E42M32_63.8
37.9  E42M32_155.4
42.1  E45M35_394.0
43.4  E35M32_338.1
52.5  E32M32_154.4
54.5  E45M35_249.8
57.4  E45M33_99.8
62.5  E45M37_85.4
63.9  E45M35_259.1
65.7  E38M40_337.5
70.3  E45M35_254.2
72.2  E42M32_104.8
76.9  Rubus157
81.9  E43M32_184.7
90.5  E35M32_121.1
Figure 2. Population distribution of growth habit of raspberry canes measured in July 2007 and July 2008 of the mapping population, NC497 × ‘Qualicum’. Growth habit score of 0 is decumbent, score of 1 is semi-erect, score of 2 or 3 is mostly erect or erect.
Figure 3. Distribution of prickle density in canes of the mapping population NC497 × ‘Qualicum’. Density score of 0 is prickle free, a density score of 5 is densely prickly.
Table 1. Summary of different selective amplification primers for generating amplified length polymorphisms in the construction of genetic linkage map. Nine EcoRI (AC, CA, CC, CT, GG, GT, TA, TC, and TG) and seven MseI (AA, AC, AG, CA, CC, and GC) selective primers with a 2 base extension were used but only 12 combinations out of all the different possible combinations from these primers were utilized in the construction of the map.

<table>
<thead>
<tr>
<th>Primer Combination</th>
<th>Primer Code</th>
</tr>
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<tbody>
<tr>
<td>Eco+AC MseI+AC</td>
<td>E32M32</td>
</tr>
<tr>
<td>Eco+CA MseI+AC</td>
<td>E35M32</td>
</tr>
<tr>
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<td>E45M35</td>
</tr>
<tr>
<td>Eco+TG MseI+CG</td>
<td>E45M37</td>
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</table>
Table 2. Summary of all QTL detected for traits heat tolerance (Heat), prickle density (Prick), and growth habit for the two study years (Growth-07, Growth-08) in raspberry (*R. idaeus*). A LOD score of 3 or higher was considered significant. Last column shows the percent of the explained variation by the QTL.

<table>
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<tr>
<td>Growth-08</td>
<td>3</td>
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<td>14.0</td>
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<tr>
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<tr>
<td>Prick</td>
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<td>75.9</td>
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</tbody>
</table>
CHAPTER IV

Quantitative analysis of tolerance to temperature fluctuations in winter and berry characteristics in raspberry (*Rubus idaeus* L.)

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ABSTRACT

One of the major limitations to growing raspberries in parts of the southeastern United States is their inability to tolerate fluctuating winter temperatures. Other perennial plants have adapted to these conditions by having high chilling requirements. However, the to achieve this trait in a breeding program it takes many years of field observation and selection. To address this issue, QTL analysis was performed on a previously developed map of *R. idaeus* based on a genetic mapping population from a cross between (*R. parvifolius* × ‘Tulameen’) × ‘Qualicum’. The mapping population was used for quantitative trait loci (QTL) analysis of chilling requirement. Chilling requirement was evaluated for three non-growing seasons and three QTL were found in two separate season evaluations. In most cases, co-localization of these QTL occurred in the same region on the map. Several of these regions explained a large percentage of the variation of the trait for each season (26.8 to 69.0%). Other variably important horticultural traits segregated in the (*R. parvifolius* × ‘Tulameen’) × ‘Qualicum’ cross, and were evaluated for QTL analysis as well; these were
fruit color, fruit shape, and fruit size. In most cases, two field evaluations were performed. Several regions were identified as significant and the majority of the QTL were co-localized to the same region of the linkage map. This research has drawn a baseline foundation for the development of molecular technologies in improving tolerance to fluctuating winter temperatures in *Rubus*. Future research should focus on these regions to develop closely linked molecular markers for marker assisted breeding.

INTRODUCTION

In the southeastern US, the most limiting factor for raspberry production is the lack of adapted cultivars. Typical seasonal conditions in southeastern states, including North Carolina, are warm humid summers, and winters with intense temperature fluctuations. In order to establish major production in this area, cultivars will require tolerance to both of these conditions, which are very atypical of available raspberry cultivars (Ballington and Fernandez 2008). In fact, domestication of red raspberry has resulted in a reduction of genetic diversity, with most modern cultivars being genetically similar and adapted to the climates like that of the Pacific Northwest because *R. idaeus* is naturally adapted to these climates (Graham et al 2004; Graham and McNicol 1995; Jennings 1988). Following fulfillment of chilling, fluctuations in temperature in the winter cause premature bud break when temperatures increase and expose fruiting laterals to winter damage after late frosts (Westwood 1993; Jennings 1988). This can cause complete loss of the crop when plants are not tolerant. Woody perennial plants can tolerate these fluctuations by requiring long chilling hours to break winter dormancy (Westwood 1993). Thus, for regions such as North Carolina,
longer chilling requirements (also described as a longer period of rest completion) are desirable for tolerance to winter fluctuations. However, low chilling requirement in *Rubus* is also important in other economic growing regions such as Florida, southern Mexico and Chile. Because of the mild winters in these regions, chilling requirements are not met and plants exhibit a high incidence of bud suppression (Jennings 1988). Therefore, breeding for region-specific chilling requirements is important for substantial production of *Rubus*, however this is also true for many other woody perennial plants (Westwood 1993).

In the North Carolina State University *Rubus* Breeding Program, efforts are underway for developing cultivars with longer chilling requirements, but because of the biennial nature of *Rubus*, breeding for important traits is a time consuming process. Initial field trials alone take up to 3 years and cultivar development typically takes 10 to 15 years. This emphasizes the need for the use molecular tools to expedite the breeding process. Establishing a strong genotype to phenotype relationship is important in developing useful, molecular markers that are tightly linked to the trait. Consequently this facilitates the breeding process because selection is based on the genotype, not the phenotype, which can exhibit genotype by environment variability (Collard et al. 2005). Molecular research in *Rubus* has been scant, and tools such as a genetic linkage map would greatly expand the tool set for future marker assisted breeding.

Very few studies have looked at dormancy, and chilling requirement at the molecular level, and thus molecular and genetic mechanisms are poorly understood and have mostly focused on seed dormancy of annual plants (Rohde and Bhalerao 2007). Perhaps the best characterized system that closely pertains to this study is for flowering time in *Prunus*. 
Because flowering time is one of the most important agronomic traits in *Prunus*, there have been several genetic and quantitative studies focused on the trait, as well as the characterization of an *evergrowing* mutant in peach (Sánchez-Pérez et al. 2008; Silva et al. 2005; Bielenberg et al. 2004; Wang et al. 2002). Blooming time is inherited quantitatively in the majority of fruit species (Anderson and Seely 1993). Thus, a quantitative analysis method to studying chilling requirement in *Rubus* is a plausible approach.

One of the most notable qualities of raspberry fruit is the high level of antioxidants, which is largely attributed to anthocyanins and other plant-derived compounds such as ellagitannins, and flavonols (Mullen et al. 2002; Cao et al. 1997). Raspberries have a variety of colors that are attributed to different anthocyanins and can range from yellow to dark red. Orange-red berry color shows a predominance of pelargonidin glycosides, while red berries have predominance of cyanidin-3-rutinoside and cyanidin-3-glucosyl-rutinoside (two anthocyanins found only in red raspberry) (Jennings 1988). The plant material in this study is of a complex nature and the cross involves three different species, all three bear different color fruits. The cross involves *R. ideaus*, *R. parvifolius*, and *R. occidentalis* and have red, orange and black colored fruits respectively. The mapping population has a wide array of different colors, showing possible differences in types of anthocyanins. These color differences suggests that segregation of anthocyanin genes is occurring, and therefore one can map regions that are closely related to synthesis of the different types of anthocyanins.

In a previous study (Chapter III), a genetic linkage map of raspberry was developed and used for the detection of important QTL associated with heat tolerance and other variably important traits. In this study, the same genetic map was utilized to perform quantitative trait
analysis of chilling requirement. The same progeny from this population, in addition to segregating for chilling requirement, segregate for several berry characteristics: berry color, size, and shape, and quantitative analysis was performed on these traits as well. This is the first study to analyze the chilling requirement in raspberry for the identification of quantitative trait loci (QTL).

MATERIALS AND METHODS

**Plant Material:** The mapping population used for this study was generated from a modified backcross between (*R. parvifolius* × ‘Tulameen’) (NC497) and ‘Qualicum’. In addition to heat tolerance, the mapping population segregates for different chilling requirements (Ballington and Fernandez 2008). ‘Kiowa’ (Moore and Clark 1996) is a blackberry cultivar that was used as a low-chilling control to evaluate the chilling model (described below) (Carter et al. 2006). Four hundred seedlings of the mapping population were planted in June 2006 at the Sandhills Research Station (SRS) in Jackson Springs North Carolina, including the parents and ‘Kiowa’. One hundred ninety eight individuals from the mapping population were randomly selected for mapping studies.

**Genetic Linkage Map and QTL Analysis:** Generation of the molecular markers and the development of the genetic map used in this study was described in Chapter III, and was used in this study for quantitative analysis of all traits described. The mapping analysis was conducted using MapQTL® 5 (Van Ooijen 2004). Quantitative trait loci for chilling requirement were first detected with interval mapping and, whenever possible, regions were further analyzed with composite interval mapping by using the MQM function in MapQTL.
Cofactors were selected by using the automatic cofactor selection function in MapQTL. Significant markers for berry color, berry size, and berry shape were first analyzed by single-point analysis by using the Kruskal-Wallis analysis in MapQTL. Significant marker regions were then further analyzed in MapQTL by the interval mapping procedure.

**Chilling Hour Requirement Screening and Determination:** Chilling requirement (or rest completion) in the mapping population was measured using the method described by Warmund and Krumme (2005) with minor modifications. In the method described here, chilling hours are allowed to accumulate under refrigeration instead of the field. Chilling requirements were measured on the 196 genotypes of the mapping population during 3 non-growing seasons, the winters of 2006-07, 2007-08, and 2008-09. Time of chilling inception and accumulated chilling hours were calculated using standard model 2, where chilling inception occurs at the first incidence of -2.2°C and one chilling hour accumulates every hour at temperatures between 0 and 7°C (Warmund and Krumme 2005). This model was used to determine the optimal collection time, and the initial accumulated chilling hours in the field. The accumulated chilling hours and the point of chilling inception at Jackson Springs, NC were monitored through the CRONOS Internet database (State Climate Office of North Carolina, NC State University. CRONOS [Internet database] available at [http://www.nc-climate.ncsu.edu/cronos/](http://www.nc-climate.ncsu.edu/cronos/)). Wood sections were collected when at least 100 chilling hours have been accumulated in the field to ensure all genotypes have entered dormancy. Eight- to ten-node wood sections were collected from the middle portion of the floricanes (or reproductive canes) of the 198 individuals in the mapping population. Wood sections were then kept under refrigeration at 4°C and kept in hydrated conditions. Three randomly selected
wood sections from each genotype were removed from refrigeration and placed in trays with Oasis Wedges (Smithers Oasis Company, Kent, Ohio). Trays were then placed in the greenhouse on heating mats at 25°C and kept under misted conditions (10 sec every 10 minutes). Position and direction of the trays were changed every week to normalize any unknown variability in misters and heating mats. Percent bud break was measured every week for four weeks. A bud was defined as broken when the first leaf expanded and became visible as it unfolded from the bud. All buds were counted, except for the bud inside the Oasis Wedge, lest the bud had died due to lack of air. This process was repeated each time buds accumulated 200 to 400 chilling hours (approximately every 8 to 16 days). Chilling requirements were estimated for each genotype, plus the parents and the control by calculating confidence intervals about each data point using SAS (SAS Institute, Cary, NC) and looking for significant changes in bud break percentage as described in Carter et al. (2006). For example, when bud break percent increases from 10% at 200 hours to 25% at 400 hours, and continues to increase in the subsequent accumulated hours, chilling requirement is between 200 to 400 hours. In the first season, very little material was available for collection, as plants had been transplanted in the field in June of 2006, and had a low number of canes. For this reason, only two wood sections per genotype per data point were used for estimating chilling requirements.

*Fruit Color:* The progeny that resulted from the cross NC497 × ‘Qualicum’ exhibited a broad variety of colors that ranged from yellow to dark red. The fruit color was categorized using the Royal Horticultural Society Colour Chart (Royal Horticultural Society 1966). The color scores given by these charts were yellow (11A), pink (37A), orange (23A), bright red
Berry color is not a fixed trait, i.e. depending on the developmental stage, fruit color can darken or change. For example, yellow-orange berries can transition to bright red when fully ripened, even though orange and red berry colors indicate different anthocyanins (Jennings 1988). Therefore, fruit color was scored at both ripening stages (denoted as transition 1 when berry pigments are starting to express, and transition 2 when berries are fully ripened). For example, berries that were yellow at transition 1, turned light or dark orange in transition 2. Quantitative analysis was performed using the categorical number as the quantitative value for color. Two growing seasons were evaluated for fruit color (summers 2007 and 2008).

_Fruit Shape and Size:_ Fruit shape in the progeny was either round, semi-conical or conical with some intermediates. The size of the fruits had similar variability, where relative size was small, medium or large. The size and shape of the fruit was scored on a scale of 1 to 3, where 1 was round or small, and 3 was conical or large. The berry shape and size of the parent NC497 was small and round, while that of the other parent, ‘Qualicum’, was large and conical. Because the berries in the progeny tended to be small and round, or conical and large, a correlation matrix of all the traits was generated using the PROC CORR function in SAS. Fruit size and shape were scored during two growing seasons (summer 2007, and 2008).

**RESULTS**

A total of 22 QTL were identified in this study and are summarized on Table 1. Most QTL explained a large percentage of the variation of the various traits, except for those QTL
for berry size in the second season. Each of the QTL for the second season roughly explained 18% of the variation. Briefly, the nomenclature used for each QTL consisted of a descriptive name (for example Chilling), and a number after the descriptor that represents the season the phenotypic data was evaluated. In one rare case, a lower case letter was used after the season number to represent to separate QTL located on the same linkage group in the same season.

*Chilling Requirements:* Chilling requirements were estimated during three non-growing seasons. The general trend was normal for all seasons except for the first season, where most genotypes had a low chilling. Some data points were missed during all seasons because of insufficient bud break or no bud break and therefore chilling requirement could not be determined. The correlation between each of the seasons was positive and significant, although correlations were applicable to approximately 50% of the population (Table 2). The chilling requirement of the parents was 800 and 400 for ‘Qualicum’ and NC497, respectively. The range of chilling requirement in the progeny however, was much longer (Fig. 2), ranging from 200 hours to 1600 hours. This shows that there was transgressive segregation in the mapping population for the chilling trait. ‘Kiowa’ was used as a low chilling control, but the estimated chilling requirement differed for each season. In the first season, ‘Kiowa’ was collected later in the season (approximately at 300 chilling hours in the field estimated by model 2). The second and third seasons, ‘Kiowa’ was collected at approximately after 100 accumulated chilling hours in the field (estimated by model 2 as well). In the first season’s tests, ‘Kiowa’ met its chilling hours approximately after 300 hours, however, the second and third season, percent bud break was lower than expected at
all data points and chilling requirement was estimated at 400 and 700 hours (for seasons two and three, respectively).

The majority of the variability was explained by the QTL in the model for all three seasons (Table 2). The QTL in the second growing season (Chilling2) had the lowest percent explanation, each accounting for 16.0, 21.7 and 26.8% of the variation (Table 1). Quantitative trait loci were located on the same three linkage groups for the second and third season (Chilling2, and Chilling3). One QTL for all the seasons was localized to group 3, however Chilling1 was associated with different molecular markers within that group. One QTL from the second and third seasons (Chilling2 and Chilling3) co-localized to the lower portion of linkage group 3 and further supports the potential involvement of this region to chilling hour requirement (Fig. 1). Two QTL were found in the first season. The one on linkage group 3 had a larger LOD score and explained most of the variation (7.75 and 61.7%, respectively) The second QTL associated with group 5 had a minor effect at a LOD score of 4.35 and explained 17.5% of the variation.

**Fruit Color:** In summary, the berries were yellow, orange, pink, bright red, or dark red, and changed color depending on the ripening stage. Despite the differences in berry color at the two transitional stages (transition 1 is initial expression, transition 2 is ripened) and the different season evaluations, the same QTL were detected for both stages and both years. Therefore, we report a quantitative analysis on the consolidated data set of the two years on the first stage of color development (Table 1). The consolidated fruit color scores for both transitional stages are summarized in Figure 3. The two stages essentially differed only by numerical position, as the two distributions appear to have shifted (Fig. 3). This
strong similarity was also reflected in the correlation matrix, where $r = 0.87$ ($p<0.0001$) (Table 2). Each QTL explained approximately 31 and 45% of the variation. The QTL on group 6A explained a large proportion of the variation, and had an unusually large LOD score (16.68). Because of the distinctive nature of the data, there is a possibility that the phenotypic data is outweighing the genetic data (Van Ooijen 2004). The QTL on group 5 accounted for 31.2% of the variability of color, and showed linkage to berry size and shape (Fig. 1).

**Fruit Shape and Size:** There was a significant positive correlation between berry shape, and size but only within each growing season (Table 2). In other words, berry shape and size did not correlate between seasons. For the quantitative analysis, the QTL regions generally co-localized to the same linkage groups for both shape and size in both seasons (Fig. 1). The most significant QTL were those located on group 5, where both season evaluations of shape and size co-localized to the same region (Fig. 1). Two QTL for size (Size1) were found on group 1 (QTL Size1a, and Size1b). Quantitative trait locus Size1a co-localized with a QTL for size for the second season (Size2), while Size1b co-localized with Shape1. Although significant markers were found in the single point analysis (Kruskal-Wallis function in MapQTL) for size in the second season (Size2) on group 3, there was not enough information available in neighboring markers to specify a significant QTL region in interval mapping, and therefore this result is not shown on Figure 1 or Table 1. The missing information for the second season for shape (Shape2) resulted in phenotypic data overweighing the genetic data for some of the QTL on group 3. This section is shown after
the line of the solid bar in Figure 1. The high number of iterations, and the high local LOD scores support the occurrence of this phenomenon in this region (Van Ooijen 2004).

DISCUSSION

*Chilling Requirements:* Chilling requirement ranged from 200 to 1600 hours, and exceeded the hours of both parents, thus transgressive segregation occurred in the population. Barrientos and Rodriguez (1980) have also observed transgressive segregation in open pollinated progenies of the red raspberry cultivar ‘Malling Exploit’. This implies that several genes are likely involved in the chilling requirement trait and can affect the trait differently. In the first season’s tests, ‘Kiowa’ met its chilling hours approximately after 300 hours, as reported in Carter et al. (2006). However, in the following evaluations, the chilling hours differed from the expected (400 and 700 hours vs. 200 to 300 hours). The reason for this variation is unknown, but the phenomenon could be due leaf attachment at the time of collection (Clark pers comm.)

Accurate QTL are highly dependent on thorough phenotypic screening (Collard et al. 2005). Therefore, this study measured chilling hour requirement over three seasons. One QTL from the second and third seasons (Chilling2 and Chilling3) co-localized to the lower portion of linkage group 3 and strengthens the possible involvement of this region to chilling hour requirement (Fig. 1). Although co-localization did not occur in the same map position as in Chilling2, and Chilling3, a QTL for the first season’s screening of chilling was also found on linkage group 3. This further supports the involvement of this group in plant chilling requirement. A QTL for each season also co-localized to the same region on group 7 (Fig 1),
and supports the involvement of this region in chilling requirement. Overall, taken over the three seasons, the model supports the involvement of groups 1, 3, and 7, particularly the lower portions of groups 3 and 7 in the map. Moreover, several QTL explained a large percentage of the variability of the trait (up to 69.0%, Table 1).

Graham et al. (2009) have studied different stages of reproductive development of red raspberry from blooming to fruiting in the field and under row covers. Many QTL were found to be associated with the different developmental reproductive stages of raspberry, and a large portion of these QTL were associated with group 3 of the map (Graham et al. 2009). In the construction of the genetic linkage that was used in this study (Chapter III), SSR markers from the map by Graham et al. (2006) were used to anchor all 7 linkage groups. Therefore, comparisons can be made between the two maps. The findings of Graham et al. (2009) of QTL for time of bud break and development on group 3, and chilling requirement on the same group in our studies could be further supporting the associations of the chilling requirement trait, since time of bud break would be closely linked with rest completion.

Several studies have examined the genetics and genomics of blooming time as a quantitative character in Prunus (Silva et al. 2005; Verde et al. 2002; Ballester et al. 2001; Dirlewanger et al. 1999; Joobeur et al. 1998). Although, no direct comparisons among groups can be paralleled, similar numbers of QTL affecting blooming time in peach and almond have been identified. Joobeur et al. (1998) found four QTL affecting blooming time in four separate linkage groups (groups 1, 4, 6 and 7) of the ‘Texas’ × ‘Earlygold’ (TxE) Prunus map. This same trait has been analyzed quantitatively in other Prunus maps and direct references to groups were drawn using conserved anchor markers, therefore it was possible to
compare the different *Prunus* maps regardless of their different genetic backgrounds.

Dirlewanger et al. (1999) found two QTL regions associated with blooming time, one of which mapped to a comparable position to the QTL found on group 7 of the TxE reference map (Joobeur et al. 1998). Verde et al. (2002) found one QTL linked to blooming time and mapped very closely to the QTL on group 4 of the TxE map. Moreover, Tardy Nonpareil is a late-blooming bud sport of almond and one major gene, the *Lb* gene, has been shown to be associated with the trait (Grassely 1978). Ballester et al. (2001) first mapped the *Lb* gene located on group 4, on a position of the map comparable to that of the QTL identified by Joobeur et al. (1998) and Verder et al. (2002) on the same linkage group. It is worth noting that the genetic background of these maps is of diverse origin (involving at least three different species), yet several QTL and a single major gene were located at similar positions on groups 4 and 7. Because bloom time in *Prunus* and chilling requirement in *Rubus* is measured by the same method, we could expect similar results and find parallels among loci or genes controlling rest completion in *Rubus*. Silva et al. (2005) has mapped several candidate genes onto the *Prunus* map by using degenerate primers based on genes described in *Arabidopsis* to control flowering and vernalization. Two of these candidate genes were found to be linked to markers in QTL regions for blooming time, one of which was *PrpFAR1*, a *Phytocrome A* gene involved in the flowering time through the photoperiod pathway. Another candidate gene, *prdMADS1*, was mapped very close in position to the peach *Evergrowing* gene on group 1. The *Evergrowing* phenotype is a mutation that causes terminal buds to continue growing throughout the season until killed by low temperatures, while basal buds become floral and require low chilling hours (Wang et al. 2002). Because
these genes are highly conserved even among different plant families, mapping these same genes onto the *Rubus* map, assuming there is co-localization, further supports the QTL regions, and helps develop selectable markers.

Fruit Color: The mapping population is of a complex background involving three species, *R. idaeus*, *R. parvifolius*, and to a lesser extent, *R. occidentalis*, all three bear different colored fruit. ‘Qualicum’, the male parent, has *R. occidentalis* in its parentage (Daubeny and Kempler 1995), while NC497 is one half *R. parvifolius*. Berries from both *R. occidentalis* and *R. idaeus* mostly contain cyanidin pigments, while *R. parvifolius* berries mostly contain pelargonidins as the major pigments (Jennings 1988). A broad range of colors segregated in the population, and significant QTL were found. Two QTL accounted for the variation of fruit color. Both QTL roughly accounted for 40% of the variation, however, the QTL on group 6A had an unusually large LOD score (16.68) and explained a larger portion of the variation (44.9%). Additionally, a large number of iterations were necessary for analysis in these regions. Thus there is a possibility that due to the distinct nature of the scoring system, the phenotypic data is outweighing the genetic data. Usually, this is eliminated by single point analysis or by increasing the number of neighboring loci in interval mapping (Van Ooijen 2004). However this was not the case, and increasing the number of loci was limited since group 6 is sparsely saturated. Usually, regions with abnormally high LOD values identified in the interval mapping procedure can be eliminated in the single point analysis (Kruskal-Wallis analysis in MapQTL). In spite of this, the validity of this region’s involvement in berry color is still supported by the single-point analysis test, and perhaps would be a better-fitted model. The QTL on group 5 accounted for 31.2% of the
variability of color, and showed linkage to other berry traits, such as size and shape. Thus it suggests that many berry traits are controlled by loci in this region. A series of genes control the expression of anthocyanin concentrations in the fruit, but the most important one is gene $T$. Two recessive alleles of gene $T$ ($tt$) give a very low concentration of anthocyanins, and give rise to yellow colored fruit and stems with non-pigmented spines. Although completely yellow fruits were not present, approximately 24% of the population exhibited yellow fruits with a pinkish to reddish blush. This suggests that the two parents could be heterozygous for gene $T$, thus one of the QTL might be associated with this gene.

*Fruit Shape and Size:* Although berry shape and size did not correlate between seasons, the majority of the QTL for berry size and berry shape co-localized to the same region for both seasons (Fig. 1). In group 3 for example, co-localization of QTL for both size and shape in both seasons, except for size during the second season (Size2). However, in single-point analysis, single markers were found to be associated to the same region in group 3 for Size2. This strengthens the positive correlation coefficient between berry shape and size (Table 2), as well as the putative involvement of this region in both traits.

Breeding for chilling requirement is important in fruit crops, including *Rubus*. Appropriate chilling requirement is dependent upon geographical location, and requires breeders to select for each trait to produce cultivars adapted to particular winter conditions. In southeastern states, such as North Carolina, plants will require longer periods of rest completion in the winter to avoid the climate’s severe fluctuations in temperature. To facilitate the breeding of this trait, molecular tools should be considered to expedite the breeding process. This study has established a baseline for the development of these tools.
Three QTL associated with the chilling requirement have been identified, and in most cases consistently in different seasons. One future strategy that can further strengthen and accelerate the development of these tools is by comparative mapping studies with the well-established *Prunus* maps. Within Rosaceae, comparative mapping studies have been developed between *Prunus* and *Malus*, and among the *Prunus* species, and strong collinearity has been found (Dirlewanger et al. 2004). Thus comparative mapping between *Rubus* and *Prunus* is encouraging for further development of the QTL regions and of the *Rubus* maps.
REFERENCES


Table 1. Summary of all QTL detected for traits heat chilling requirement (Chilling), berry size (Size), and berry color (Color) in raspberry (*R. idaeus*). The number after the name descriptor of the QTL signifies the season of evaluation, the letter after the season number represents a QTL on the same group. Chilling requirement was evaluated over three non-growing seasons. Qualitative traits (Color, Shape, Size) first analyzed by single point analysis, then further analyzed by interval mapping. Last column shows the percent of the explained variation by the QTL.

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<th>QTL</th>
<th>Group</th>
<th>LOD</th>
<th>Percent</th>
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Table 2. Pearson’s correlation coefficients for all traits, chilling requirement (Chilling), fruit color (Color Transition), relative fruit shape (Shape), and relative fruit size (size). The number after the descriptor represents the season evaluation number for the trait. Coefficients are shown in the first row of the trait descriptors, the second row shows the p-value under the null, and the third row is the number of observations.

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Figure 1. Genetic linkage map of raspberry. Significant LOD intervals (>3.00) for all traits, chilling requirement (Chilling), berry color (Color), berry shape (Shape) and size of berries (Size) are shown by solid bars. Suspected associations are shown as hairlines after solid bars. The number after the trait descriptor denotes the season the trait was evaluated, while the lowercase letter after the season number signifies that there were more than 1 QTL from the same season on the same group.
Figure 2. Distribution of chilling hour requirement for the mapping population NC497 × ‘Qualicum’. Chilling hour requirement was tested in the population in the winter of 2006-07 (Chilling1), 2007-08 (Chilling2), and 2008-09 (Chilling3).
Figure 3. Distribution of berry color at two transitional stages for the mapping population. Transition 1 is the initial expression of color, and transition 2 is the color expressed when fully ripened. The numbers in the x-axis correspond to the scores determined by the RHS Colour Chart (Royal Horticultural Society 1966).
Figure 4. Distribution of relative berry shape and size for the mapping population. Both berry shape and size was scored from a scale of 1 to 3 with 0.5 increments. A score of 1 signifies that berries were round for shape, and small in size, while 3 is conical in shape and large in size.
APPENDICES
APPENDIX A

Transfer of SSR and SSR-EST markers from Rosaceae genera onto *Rubus*.

SUMMARY

Since the late 1980s, genome comparisons (i.e. comparative mapping) across different plant genera have shown that the organization of genes within genomes has remained conserved over long evolutionary periods (Devos and Gale 2000). Complementary (DNA) molecular markers act as anchor points (transferable markers) that link different linkage maps and cross-map the genomes to allow comparisons among distinctively different linkage maps. Studies that focus on relationships among genomes have shifted from whole map comparisons to specific chromosomal areas (and even the DNA sequence itself) and have included a plethora of plant species (Jung et al. 2008, submitted; Phan et al 2007; Kalo et al. 2004; Ventelon et al. 2001), however most comprehensive data comes from the grasses (Devos and Gale 2000). Comparative mapping has greatly expanded the knowledge of structural genome changes, and duplications. These comparisons will eventually lead to an increase in available information in (conserved) molecular functionality in plants. Within Rosaceae, comparative mapping studies have been developed among several of the *Prunus* species, and between *Prunus* and *Malus* (Jung et al. 2009; Dirlewanger et al. 2004). Among the *Prunus* species, comparisons among several maps show that these maps are essentially collinear, thus the *Prunus* genus can be regarded as a single genetic entity (Dirlewanger et al. 2004). *Prunus* and *Malus* comparative mapping studies also detected large collinear
segments (Jung et al. 2008). Comparative mapping of other plant families, such as in Solanaceae (Doganlar et al. 2002), Poaceae (Devos and Gale 2000), and Brassicaceae (Lukens et al. 2003), consistently indicate that there is little reconstruction of genomes within families. Results in Rosaceae are demonstrating the same trend (Dirlewanger et al 2004). Synteny analysis across species provides insight on the evolutionary relationships between different lineages and also the opportunity to study the relationship between genome structure and function of genomic regions. The collinear regions conserved between organisms also facilitate marker saturation and framework map development in other crosses (mapping populations) and species. Comparative mapping also allows the location of different major genes, QTL in a unique map, and candidate gene approaches. For example, micro-collinearity data between Arabidopsis and rice has been utilized in the improvement of phylogenetic resolution of the expansin gene family (Sampedro et al. 2005). In other words, understanding structural similarities and differences among genomes of different species will allow the knowledge obtained in one species to expedite research and discoveries in other species. This is particularly advantageous for genomic studies involving rosaceous crops considering that,

**Mapping Research in Rosaceae**

Currently, the Rosaceae family does not have a sequenced reference genome to serve as a starting point for genome wide and targeted genomics. Impetus on three genera within Rosaceae have been the primary focus for sequencing and mapping: Peach, strawberry, and apple. An overview of the mapping resources throughout Rosaceae is summarized in Table 1.
It is surprising considering that Rosaceae genomes are generally small in comparison to other plant family model species (rice, maize, tomato, soybean, *Medicago*, poplar) (Bennett and Leitch 2005) and that many members within this family are of great agricultural importance. Smaller genomes are usually more advantageous because these are easier to analyze and require less allocation of resources. In fact, completely sequencing the (diploid) strawberry, raspberry, and apple genomes combined (a total of ~1,200 Mb) would require a resource allocation of only 10% greater than that committed to soybean alone (a genome size of 1,100 Mb). Alternatively, comparative mapping can provide a convenient way to extend information onto other species. For this reason, comparative genomic mapping within Rosaceae will greatly increase the information of functionality via molecular marker comparisons from closely and distantly related species. With the peach and apple genome sequences slated to be completed in 2009, and the large database of molecular markers for both *Prunus* and *Malus*, further expanding the knowledge in *Rubus* by map comparisons, is advantageous. Anchor markers allow comparisons between different species across genera and even across families. One of the most notable examples within Rosaceae has been realized between *Prunus* and *Malus*. A total of 30 loci (24 RFLPs detected by the same probes and 6 isozyme genes) of a *Prunus* map have homologous counterparts in an apple map (Maliepaard et al. 1998). In three of the *Prunus* linkage groups (G1, G3, and G4) there were three or more anchors with apple linkage groups (L5, L8, L9, L10, L13, and L17). This doubling of anchor markers is likely due to genome duplications (Dirlewanger et al. 2004; Maliepaard et al. 1998). These and similar reports support the idea that apple has likely paleopolyploid properties. Marker order was generally identical, and one linkage group of
Prunus usually corresponded to two of the apple linkage groups considered as homoeologous. The most outstanding resources are available for peach, apple and strawberry. Below is a brief summary of the research advancements of each genus.

**Peach:** A large number of genes with fundamental horticultural importance have been described in peach. Numerous genetic marker maps are available in peach, including a densely saturated map based on an interspecific cross between peach and almond (Verde et al 2005; Dirlewanger 2004). This map has an excess of 1000 molecular markers and phenotypic characters and serves as the standard for linkage and physical mapping in peach. The peach physical map spans over 90% of the peach genome (Zhebentyayeva et al 2008), and is publicly available in the Genome Database for Rosaceae (GDR).

**Strawberry:** Due to the varying number of polyploidy in the genus Fragaria, genetic linkage maps are based on the diploid *F. vesca* and serves as the model for the cultivated octoploid strawberry (*Fragaria × ananassa*). The strawberry EST database is comprised of approximately 20,000 GenBank accessions, with a more accessions in progress.

**Apple:** Many molecular mapping studies have been performed covering linkage groups with over 900 molecular markers (Liebhard et al. 2003; Xu and Korban 2000). There is an exorbitant amount of available (GenBank) EST markers that have been developed in apple. Over 155,000 apple ESTs developed by Gasic et al. (2006), and an additional 150,000 ESTs generated by HortResearch (New Zealand). As a consequence of this resource, researchers will be able to further extend the genetic and genomic characterization of the many important horticultural traits within this family. It is worth noting that collectively these goals impact the cultivation of raspberry in the southeastern US, as well as broadens the
knowledge and understanding of the Rosaceae genome evolution in the field of Plant Biology.

**Research Objectives**

Comparative mapping research in our program is underway in collaboration with researchers at HortResearch in Palmerston North, New Zealand. Many SSR and EST-SSR markers from several other Rosaceae members including apple and pear (Maloideae), strawberry (Rosoideae), and *Prunus* (Amygdaloideae) have been screened for marker transferability in pursuit of finding universally conserved markers (Table 2, and Fig 1). The project will further research conserved markers across genera in Rosaceae to understand evolutionary changes and tag common agriculturally important traits. The short-term goal of the comparative genomics aspect of this project is to identify markers that are linked to traits of interest in related rosaceous species, and transfer these onto *Rubus* for further molecular analysis. The long-term goal of this project is to aid in the development of a global genetic map for Rosaceae to refine candidate gene approaches within the family. This will also further extend the genetic and genomic characterization of the many important horticultural traits within this family. It is worth noting that collectively these goals impact the cultivation of raspberry in the southeastern US, as well as broadens the knowledge and understanding of the Rosaceae genome evolution in the field of Plant Biology.
REFERENCES


Table 1. Current map resources available in Rosaceae.

<table>
<thead>
<tr>
<th>Species DNA</th>
<th>Maps</th>
<th>Large Insert libraries</th>
<th>EST resources</th>
<th>Sequence Anchor pts</th>
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<td>High density genetic map&lt;0.7cM interval Genetically anchored physical map &gt;80% genome coverage</td>
<td>3 BAC libraries &gt; 20X total coverage (Clemson)</td>
<td>100,000 sequences</td>
<td>~4000 mapped marker sequences</td>
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<td>1 BAC library ~ 5X coverage (Clemson)</td>
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<td>1 BAC library 16X coverage (Clemson)</td>
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<td>Sweet Cherry (diploid)</td>
<td>Low density map</td>
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<tr>
<td>Apple dihaploid ‘Golden Delicious’ proposed for genomic sequencing</td>
<td>High density maps, 2 reference maps Framework physical Map</td>
<td>BAC library 12X coverage, (UNI)</td>
<td>&gt;300,000 sequences</td>
<td>&gt;500 mapped sequence anchor points</td>
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<td>Strawberry 8x <em>F. vesca</em> inbred proposed for genomic sequencing</td>
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<td>~ 500</td>
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<td>Several medium density genetic maps</td>
<td>1 BAC library, 4X coverage. T. Debener, Hannover, Germany</td>
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Table 2. Summary of results of the number and percent of markers that transferred and segregated within the raspberry population from the cross between NC497 × 'Qualicum'.

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* Sequence of putative function available

** Excludes Rubus to emphasize transferability from other genera

*** Analysis of this information is still in progress
Figure 1. The Rosaceae lineages (Potter et al. 2002)
APPENDIX B

Detection of important phyto-chemicals associated with human health in a segregating population of *Rubus idaeus*

SUMMARY

One of the most notable qualities of raspberry fruit is the high level of antioxidants, which is largely attributed to anthocyanins and other plant-derived compounds such as ellagitannins, and flavonols (Mullen et al. 2002; Cao et al. 1997). In addition to the high levels of anthocyanins, raspberries contain high levels of vitamin C and total phenolics that form a complex of anti-carcinogenic compounds (Anttonen and Karjalainen 2005; Mullen et al. 2002). Although cyanidin-3-sphoroside and cyanidin-3-glucoside are the two main anthocyanins in raspberry fruit, Mullen et al. (2002) have identified as many as 11 different anthocyanins, all of which are considered important antioxidants. Raspberry fruit can contain high levels of ellagitannins, and upon hydrolysis, releases ellagic acid (Rommel and Wrolstad 1993). Ellagic acid is a phenolic compound that harnesses antiviral activity (Corthout et al. 1991), and protects against cancers of the colon (Coates et al. 2007), lung and esophagus (Stoner and Morse 1997). In comparison to walnut and pecan, raspberries can contain up to three times more ellagic acid (Maas et al. 1991). Raspberries also contain flavonols including quercetin and kaempferol, both of which possess antioxidant activity (Anttonen and Karjalainen 2005). Quercetin in particular, is a powerful antioxidant and has arterial blood pressure reducing properties (Duarte et al. 2001), and inhibits the growth of
human breast carcinoma cells (Choi et al 2001). In spite of the overwhelming health benefits, and the high free radical scavenging capacity, the bioavailability and consumption of dietary anthocyanins in humans is low (Chun et al. 2005; Mazza et al. 2002; Wu et al. 2002). This presented an opportunity to further study these compounds for breeding purposes with the goal of the nutriceutical improvement of raspberry fruit quality. The compounds for the preliminary research measured the presence and relative amounts of different anthocyanins: total cyanidins, total pelargonidin, peonidin, and proanthocyanidin. The future research will also include other important compounds, which are total flavonoids, and three amino acids (phenylalanine, tyrosine, and tryptophan).

A subset of the mapping population (Chapter II and III) was evaluated for traits that are implicated in human health and nutrition (phenylalanine, tyrosine, tryptophan, total cyanidins, total pelargonidin, peonidin, proanthocyanidin, and total flavonoids). For the initial study, berries were macerated with an equal volume (g/ml) of 1% formic acid in 50:50 methanol:water. After centrifugation, 500ul of the supernatant dried in a speed vacuum, and then reconstituted in 250ul of 1% formic acid in 50:50 methanol:water. Analysis was performed by LC-PDA-MS/MS on a Thermo (San Jose, CA) LTQ ion trap mass spectrometer fitted with electrospray interface. Very briefly, a 5ul injection of the sample is made onto a 250x2mm Phenomenex Polar RP column, equilibrated in 95:5 A:B, where A = 1% formic acid in water, and B = 1% formic acid in acetonitrile. A 50 minute gradient to 70% B eluted the components of interest. The flow rate is 250ul/min throughout. Data was extracted with Xcalibur 2.0 software. Relative abundance of each compound is calculated by the integration of peak areas, which will allow comparisons among samples for further QTL
analysis. A subset of the NQ population has been screened for the myriad of the different compounds using this procedure (table 4). Preliminary multivariate statistics were prepared within XLSTAT to generate a dendrogram. Distinct differences in amounts and types of compounds were found. Figure 4 shows the dendrogram of the different berry samples with a picture taken at harvest. The two parents of the mapping population, (R. parvifolius x ‘Tulameen’) (NC497) and ‘Qualicum’, are into distinct clades, showing that there is considerable metabolic differences in the berries of the parents. The diagram also strongly suggests that there is grouping of individuals with lighter pigmentation (samples 3, 10, 11, 14, 15, and 16), which supports that there is segregation in the production of these compounds in the NQ population and that there is an association between color and antioxidant type
REFERENCES


comparative map of white lupin (*Lupinus albus* L.): identification of QTLs for anthracnose resistance and flowering time, and a locus for alkaloid content. DNA Res 14: 59-70

Sampedro J, Lee Y, Carey RE, dePamphilis C, Cosgrove DJ (2005) Use of genomic history to improve the phylogeny and understanding of births and deaths in a gene family. Plant J 44: 409-419


Table 1. Relative abundance of different amino acids, anthocyanins, and flavonoids in a subset of berries in the NQ population.

Abundance is calculated as the integrated area under the curve for each peak. * Includes 5 different cyanidins, ** Includes 2 different pelargonidins, *** Includes 3 different flavonoids, + *R. parvifolius* x ‘Tulameen’ (NC497).

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Figure 1. Dendrogram using multivariate analysis of different berry compounds in a segregating sub-sample from the \((R. \textit{parvifolius} \times \textit{Tulameen}) \times \textit{Qualicum}\) population.