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LINKAGE MAP CONSTRUCTION AND ANALYSIS OF FRUIT SIZE IN SWEET (*Prunus avium* L.) AND SOUR (*Prunus cerasus* L.) CHERRY

presented by

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has been accepted towards fulfillment
of the requirements for the

Doctoral degree in Plant Breeding and Genetics - Department of Horticulture

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May 3, 2006

Date

MSU is an Affirmative Action/Equal Opportunity Institution

LINKAGE MAP CONSTRUCTION AND ANALYSIS OF FRUIT SIZE IN SWEET (*Prunus avium* L.) AND SOUR (*Prunus cerasus* L.) CHERRY

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Plant Breeding and Genetics - Horticulture

2006

ABSTRACT

LINKAGE MAP CONSTRUCTION AND ANALYSIS OF FRUIT SIZE IN SWEET (*Prunus avium* L.) AND SOUR (*Prunus cerasus* L.) CHERRY

By

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Maximizing fruit size is critical for profitable sweet (*Prunus avium* L.) and sour (*Prunus cerasus* L.) cherry production, yet little is known about the genetic control of this trait. Fruit size varies widely between cherry cultivars, and significant variation exists among genetically identical fruit due to environmental and cultural differences. A more thorough understanding of the genetic control of fruit size may be used to design future management and genetic improvement strategies to increase cherry fruit size.

This research examined the mesocarp cellular differences between five cultivars representing a broad range of fruit size in sweet cherry. Both cell number and cell size were significantly different ($P < 0.05$) between cultivars. However, the relationship of cell number with fruit weight and diameter was significantly and positively correlated while cell size was not correlated with either measure of fruit size. Cell number was stable during the three years of this study and in two different locations. Differences in cell number due to environmental variation were examined in fruit from three of the same cultivars that were significantly different ($P < 0.001$) in fruit size. In this case, fruit size differences were attributed to a difference in cell size rather than cell number, confirming the identification of cell number as the primary genetic component resulting in fruit size differences between cultivars.

To study the genetic control of fruit size in cherry, linkage maps were constructed for reciprocal crosses between the sweet cherry cultivars 'NY 54' and 'EF'. The linkage

maps consist of 8 linkage groups (LG) for the 'EF' parent (479.1 cM) and 10 LG for the 'NY 54' parent (308.9 cM). The average distance between marker loci and largest gaps are 7 cM and 29 cM for 'EF' and 8 cM and 34 cM for 'NY 54', respectively. Fourteen of the sweet cherry linkage groups could be aligned with the reference *Prunus* map based on shared SSR markers.

QTL analysis of fruit size traits was performed using the 'NY 54' × 'EF population. For mesocarp length, one QTL (*mlength1*) was identified on 'EF' linkage group 6 (LG 6) and one on 'NY 54' LG (y) (*mlength2*), explaining 18.3% and 37.4% of the total phenotypic variance, respectively. Three QTL were identified for mesocarp cell length, on 'EF' LG 6 (*clength1*) and 'NY 54' LG 6 (*clength2*) and LG (y) (*clength3*). The QTL explained 17.4, 16.8, and 16.8% of the phenotypic variation, respectively.

A targeted mapping approach, using SSR loci previously mapped to LG 6 in other *Prunus* species was used to develop a linkage map for the 'UF' × 'Surefire' sour cherry population. A QTL three cM from the *S* locus explaining 26.4% of the phenotypic variation was identified in the 'UF' × 'Surefire' population. Additionally, a fruit shape QTL was also located on LG 6, co-segregating with the CPSCT012 marker and explaining up to 22.6% of the phenotypic variation for fruit shape.

To my wife Mercy Olmstead, and my parents Peter and Patricia Olmstead.

ACKNOWLEDGMENTS

I wish to thank my major professor, Dr. Amy Iezzoni, and the members of my dissertation committee, Dr. Greg Lang, Dr. Dechun Wang, and Dr. Kyung-Hwan Han, for their guidance and support during this research project.

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CHAPTER ONE

Introduction and Review of Literature

INTRODUCTION

Sweet (*Prunus avium* L.) and sour (*P. cerasus* L.) cherries are produced in most agricultural regions of the world where temperate crops can be grown. The genus *Prunus* contains many economically important tree fruit and nut crops, including peach [*P. persica* (L.) Batsch], almond [*P. dulcis* (Miller) D.A. Webb], European plum (*P. domestica* L.), Japanese plum (*P. salicina* Lindl.), and apricot (*P. armeniaca* L.). Approximately 1,850,000 mt of cherries were produced worldwide in 2005 (FAOSTAT data, 2005). The United States produced 250,000 mt, 13.5% of the world total. Four states (Washington, Oregon, California, and Michigan) produce over 95% of U.S. sweet cherries, while Michigan alone accounts for over 65% of the U.S. sour cherry production (NASS-USDA, 2005). Like other members of *Prunus*, sweet and sour cherries are classified anatomically as a drupe, originating from a single carpel (Esau, 1977). The pericarp, enlarged ovary tissue, is composed of three tissue types; the endocarp, mesocarp, and exocarp. The sclerified endocarp (stone or pit) contains the single seed. The mesocarp is fleshy, consisting of multiple layers of highly vacuolated parenchyma cells. The specialized cuticular cells comprising the exocarp (skin) are typically only a few cell layers thick.

Importance of Fruit Size in Production

The early progenitors of modern sweet cherry cultivars probably had small fruit similar in size to wild sweet cherry forest trees. These wild sweet cherry types, collectively referred to as Mazzard, have very small fruit averaging 1-2 g in weight. As a result of selection and domestication, the fruit of modern cultivars can be over 10 g. This

increase is particularly striking, considering that many of the cultivars grown today are only a few generations removed from the landraces from which they were originally selected (Iezzoni et al., 1990). Improvements in cultural practices have contributed to the increase in fruit size. For example, the average size of 'Bing' fruit achieved in commercial production has increased in recent years, although the cultivar has been fixed genetically by vegetative propagation since its introduction in the 1870s. However, the 10x or greater increase in fruit size, compared to wild members of the species also has a significant genetic component. Early sweet cherry breeders recognized relatively uniform distribution of fruit size among progeny from different crosses suggesting a quantitatively inherited trait (Fogle, 1961; Hansche et al., 1966; Lamb, 1953; Matthews, 1973).

Sour cherry is tetraploid ($2n = 4x = 32$), whereas most cultivated *Prunus* are predominantly diploid ($2n = 2x = 16$). Sour cherry is believed to have arisen multiple times through natural hybridization between ground cherry (*P. fruticosa* Pall.; $2n = 4x = 32$) and unreduced gametes from sweet cherry ($2n = 2x = 16$) (Beaver and Iezzoni, 1993; Brettin et al., 2000; Olden and Nybom, 1968). *Prunus fruticosa* has small fruit; however, some larger fruited selections have been bred in Russia (Iezzoni et al., 1990). It is not known if the origin of today's sour cherry cultivars occurred through hybridization with large or small-fruited sweet cherries. However, it is likely that certain landrace varieties are the result of recent backcrossing with sweet cherry. Currently, the sour cherry industry in the United States is based almost entirely on one genotype, 'Montmorency', a 400-year-old selection from France that averages 4-6 g in fruit weight (Iezzoni, 1988, 2005).

Large fruit size is an essential component of profitable fresh market sweet cherry production. Currently, fruit size is the primary criterion by which fresh cherries are graded for sale, with fruit averaging over 29 mm in diameter worth nearly twice as much ($/kg) as fruit less than 24 mm in diameter (Whiting et al., 2005, 2006). Therefore, sweet cherry breeding efforts have long focused on the development of cultivars with larger fruit. The fruit quality of many sour cherry cultivars is superior to 'Montmorency', and there has been increased producer interest in, and consumer acceptance of sour cherries marketed for fresh consumption (Lang et al., 2003). As this market develops, a premium will likely be placed on large sour cherries. Therefore, future breeding efforts may be directed toward selection of larger sour cherry varieties for fresh market production. However, little effort has been directed toward understanding the influence of cell number and size on fruit development and final size in cherry.

Factors Influencing Fruit Size

Various methods of quantifying fruit size have been employed in past research. Fruit weight, length, diameter, and volume are all relatively simple measures of size. However, the relationship between these measurements and cellular components of fruit size is not clear. This is of particular importance in relation to quantitative trait loci (QTL) identification, where the reliability of phenotypic data is of utmost importance to genetic analyses.

Fruits of *Prunus*, including cherry, generally exhibit a characteristic double sigmoid growth curve, consisting of three developmental stages (Lilleland and Newsome, 1934). Stage I is characterized by rapid and exponential fruit size increase, stage II by a

lag period of size increase coinciding with endocarp hardening, and stage III by a second period of exponential size increase ending with harvest. These stages have been assigned arbitrarily based on external fruit size increase measurements. However, the delineation between the developmental phases is not distinct and does not necessarily coincide with physiological development (Chalmers and van den Ende, 1973; Coombe, 1976; DeJong and Goudriaan, 1989; Gage and Stutte, 1991).

Although the defined developmental stages do not always correspond with physiological development, anatomical and morphological changes in the fruit are thought to follow the general pattern of three stages of development (Coombe, 1976; Gage and Stutte, 1991; Jackson and Coombe, 1966; Ragland, 1934; Tukey and Young, 1939; Yamaguchi et al., 2002b). Stage I, from anthesis to the beginning of endocarp sclerification, is a period of rapid cell division and initial cell enlargement. By stage II, cell division has generally ceased, cell enlargement slows considerably, and endocarp sclerification occurs, as well as a general thickening of parenchyma cell walls throughout the mesocarp. Stage III is characterized by renewed cell enlargement, either radially or tangentially, depending on cell location in relation to the endocarp or exocarp. Cell layers closest to the endocarp enlarge in a radial direction, while exocarp cell layers enlarge tangentially as fruit surface area increases with increasing size.

Larger sized fruit have been associated with increased cell numbers, increased cell size, and increased intercellular spaces (Coombe, 1976). However, previous research suggests that the role of intercellular space in *Prunus* final fruit size increase is negligible (Jackson and Coombe, 1966; Tukey and Young, 1939). The role of both mesocarp cell number and cell size in relation to total fruit size has been examined. The bulk of this

research has been in apple (*Malus domestica*), where biennial bearing of many cultivars has necessitated annual fruit thinning by hand or by chemical application. From this body of work, evidence is conflicting as to whether cell number or cell size relates to large fruit size. Increased fruit size within the same genotype after hand-thinning has been attributed to differences in cell number (Bain and Robertson, 1951; Bergh, 1985,1990; Goffinet et al., 1995; Martin et al., 1964; Westwood et al., 1967), cell size (Al-Hinai and Roper, 2004; Atkinson et al., 2001), or a combination of both (Denne, 1960). However, between genotypes, differences in cell number and/or size have rarely been documented. Only recently, the importance of increased cell division in domesticated apple, as compared to related wild species, has been documented (Harada, et al. 2005).

In *Prunus* species, fruit mesocarp size has been associated with both cell number and cell size. Although both cell number and size have been correlated with total fruit size in experiments with a single cultivar (Bradley, 1959; Coombe, 1976; Jackson and Coombe, 1966; Tukey and Young, 1939), relative differences in fruit cell number and size between different cultivars have rarely been measured. When comparisons have been made between cultivars of varying sizes, cell number was associated with overall fruit size (Scorza et al., 1991; Yamaguchi et al., 2002a, 2002b, 2004). Alternatively, significant fruit size differences within the same cultivar and without a corresponding increase in cell number have been reported for other drupe species. In olive (*Olea europaea* L.), development of fruit under irrigated and non-irrigated conditions, or after water deficit had been applied, resulted in overall fresh fruit size differences but no

significant difference in cell number in the fruit (Costagli et al., 2003; Rapoport et al., 2004).

Interestingly, these reports for *Prunus* species are similar to that of the model fruit plant, tomato (*Lycopersicon esculentum* L.). In tomato, the gene underlying a major QTL (*fw2.2*) contributing to fruit size difference between wild and cultivated species has been cloned and shown to influence cell division and therefore final fruit size (Frary et al., 2000; Nesbitt and Tanksley, 2002).

Prunus Linkage Map Construction

Significant improvement in average fruit size has been made in new cultivars in all *Prunus* species. However, even with gain from selection being relatively high for this quantitative trait, breeding long-lived perennial *Prunus* tree species is costly and time-consuming. Long juvenility periods and gametophytic self-incompatibility (de Nettancourt, 1971) (peach being a notable exception), as well as large space requirements, significantly reduce progeny numbers that can be evaluated in a given time period (Fogle, 1975). Marker-assisted selection (MAS) may hold the greatest promise for improving selection efficiency in sweet cherry. However, efforts to develop suitable linkage maps, and more importantly, identifying QTL for important agronomic traits in sweet cherry, have lagged far behind other fruit crops.

Peach is the best genetically characterized member of *Prunus*. This is partly because peach is the most economically important member of the genus, but also because it is self-fertile and its juvenility period is shorter than most other *Prunus* species (3 years versus 5-7 for sweet cherry). Self-fertility permits more amenable linkage mapping

populations such as backcross and F_2 to be used, rather than the F_1 pseudo-testcross structure commonly used in cherry (Wang et al., 1998). However, self-fertility also has limited the heterozygosity among cultivated peaches, and many *Prunus* linkage mapping populations were developed from interspecific crosses between almond and peach (Aranzana et al., 2003; Bliss et al., 2002; Dirlewanger et al., 2004a; Foolad et al., 1995; Howad et al., 2005; Jauregui et al., 2001; Joobeur et al., 1998). Additional interspecific crosses have been made between peach and related *Prunus* species such as *P. davidiana* (Carr.) Franch., *P. ferganensis* (Kost. & Rjab.) Y.Y. Yao, and *P. cerasifera* Ehrh. (Dettori et al., 2001; Dirlewanger et al., 1996, 2004b; Foulongne et al, 2003).

One drawback to the use of linkage maps based on interspecific crosses has been the high level of marker distortion, with up to 46% of the markers on these maps deviating from the expected segregation ratios and indicating preferential inheritance of certain genomic regions (Bliss et al., 2002; Foolad et al., 1995; Joobeur et al., 1998). In many cases, the marker distortion has been attributed to gametophytic selection due to homologous pairing problems between species during meiosis. However, the presence of an active gametophytic self-incompatibility locus in many *Prunus* members also has been implicated in the high percentage of markers exhibiting distorted segregation ratios (Bliss et al., 2002; Foulongne et al., 2003; Joobeur et al., 1998; Lambert et al., 2004; Vilanova et al., 2003).

Intraspecific linkage mapping populations also have been developed for other *Prunus* members. Various peach and peach rootstock populations that segregate for agronomic traits of interest such as tree architecture, peach-nectarine characters, and nematode resistance, have been used to develop genetic linkage maps (Abbott et al.,

1998; Chaparro et al., 1994; Dirlewanger et al., 1998; Gillen and Bliss 2005; Lu et al., 1998; Shimada et al., 2000; Yamamoto et al., 2005). In almond, mapping populations were developed to identify bloom and self-incompatibility traits (Ballester et al., 1998, 2001; Joobeur et al., 2000). Similarly, in apricot, there are populations which segregate for self-incompatibility and plum pox virus resistance (Hurtado et al., 2002; Lambert et al., 2004; Vilanova et al., 2003).

In sour cherry, parental linkage maps have been developed for the 'Rheinische Schattenmorelle' ('RS') × 'Erdi Botermo' ('EB') population (Wang et al., 1998). In sweet cherry, Stockinger et al. (1996) developed a RAPD marker-based linkage map of a microspore-derived callus culture population. However, because of the marker system, this map is not comparable with other *Prunus* linkage maps, and phenotypic analysis of important fruit and tree traits are not possible for QTL studies. Similarly, Boskovic et al. (1997, 1998) reported isozyme-based interspecific maps of sweet cherry × *P. incisa* Thunb. ex Murr. and sweet cherry × *P. nipponica* Matsum., but the marker density is not conducive to QTL studies. Only Dirlewanger et al. (2004a) has developed a linkage map from a 'Regina' × 'Lapins' sweet cherry cross that can be compared with current linkage maps from other *Prunus* species using shared markers. However, 'Regina' (S_1S_3) and 'Lapins' (S_1S_4') are only partially compatible, and the loss of one pollen gametophytic class is likely to distort marker segregation ratios around the self-incompatibility locus.

Prunus Reference Map

One of the interspecific *Prunus* populations, a cross between 'Texas' ('T') almond × 'Earlygold' ('E') peach, is considered the reference *Prunus* linkage map

because of the saturation of markers (0.92 cM average distance) and high number of polymorphic simple sequence repeat (SSR) markers located on the map (Dirlewanger et al., 2004a; Howad et al., 2005). The 'T' × 'E' population was developed originally by Joobeur et al. (1998) and consists of 88 F_2 progeny generated by selfing one individual from the original cross. The first generation of this map (Joobeur et al., 1998) consisted of 246 RFLP and isozyme markers, covering a total distance of 491 cM over the expected haploid chromosome number (x = 8) of linkage groups (LG) for diploid *Prunus*. As new libraries of SSR markers were developed, they were subsequently added to the 'T' × 'E' map (Aranzana et al., 2003; Dirlewanger et al., 2004a). Additional RFLP probes from *Arabidopsis thaliana* were also added (Dirlewanger et al., 2004a). Currently, the map consists of 562 markers covering 519 cM, with an average marker density of 0.92 cM, and the largest gap of 7 cM (Dirlewanger et al., 2004a). The map distance is similar to the predicted genome size of peach, 5.3×10^8 base pairs (Dickson et al., 1992). Most recently, individuals from the 'T' × 'E' population were used in a selective bin mapping strategy, whereby recombinational breakpoints are used to identify a small subset of individuals that define a set of bins bounded by the breakpoints (Howad et al., 2005). From this analysis, 264 additional SSRs were placed on the 'T' × 'E' map, although exact linkage distances within each bin remain unknown (Howad et al., 2005). Twenty-eight major agronomic genes have been integrated into the 'T' × 'E' map (Dirlewanger et al., 2004a). The current 'T' × 'E' linkage map is available publicly through the Genome Database for Rosaceae (GDR; http://www.rosaceae.org).

Since the adoption of the 'T' × 'E' map as the reference *Prunus* map, all linkage group orientation and terminology have been assigned according to the 'T' × 'E'

nomenclature. This has allowed comparison between the 'T' × 'E' map and several other *Prunus* species (Dirlewanger et al., 2004a; Lambert et al., 2004). For the diploid *Prunus* species, genome synteny appears to be the rule, not the exception (Dirlewanger et al., 2004a). Only one major chromosomal rearrangement has been identified. A reciprocal translocation between LG 6 and LG8 was identified in both an interspecific almond × peach population and an intraspecific peach population (Jauregui et al., 2001; Yamamoto et al., 2001). Although the cultivars used in the development of these populations are different, in each case a red-leaved peach was one of the parents. The gene for red vs. green leaf color (*Gr*) is located close to the translocation breakpoint, and a relationship between the cytogenetic and morphological phenotypes has not been excluded (Dirlewanger et al., 2004a). Unfortunately, it is not known which of the parents in these crosses had the standard or translocated configurations.

QTL Analyses in Prunus

Many vegetative, fruit, and disease resistance genes have now been mapped in *Prunus* using these populations (Abbott et al., 1998; Ballester et al., 1998; Bliss et al., 2002; Chaparro et al., 1994; Dirlewanger et al., 1996, 1998, 2004a; Gillen and Bliss 2005; Hurtado et al., 2002; Joobeur et al., 2000; Lambert et al., 2004; Lu et al., 1998; Vilanova et al., 2003; Yamamoto et al., 2001). However, QTL analyses of *Prunus* species has been documented only recently compared to other agronomic crops. Dirlewanger et al. (1996) identified QTL for powdery mildew resistance in a peach x *P. davidiana* population designed specifically for that purpose. Subsequently, that population was used for identification of fruit quality traits such as bloom, maturity date,

fruit and pit size, dry matter content, soluble solids content, individual sugar fractions, organic acid fractions, titratable acidity, and fruit and flesh color (Quilot et al., 2004). QTL for bloom date, maturity date, productivity, fresh weight, pH, titratable acidity, soluble solid content, malic acid, citric acid, quinic acid, sucrose, glucose, fructose, and sorbitol were identified in a peach intraspecific cross (Dirlewanger et al., 1999) and candidate genes were subsequently identified for several of these sugar and organic acid QTL (Etienne et al., 2002). Bloom date has been a priority for almond; QTL have been identified for the trait (Ballester et al., 2001) and subsequent candidate genes were located in similar positions as the late bloom QTL (Silva et al., 2005). QTL for bloom date, pistil death, pollen germination, maturity date, fruit weight, and soluble solids content were identified in sour cherry (Wang et al., 2000). Although only LG 2, LG 4, LG 6, and LG 7 of sour cherry have been aligned with the same peach linkage groups (Wang et al., 1998), it is possible to establish a degree of synteny between the two species for some of these traits. For example, at least one QTL for bloom date, soluble solids content, and maturity date was identified in both populations on the corresponding LG 2, LG 4, and LG 6, respectively. To date, no QTL analyses have been performed in sweet cherry.

Importance of Prunus LG 6

Prunus LG 6 appears to be of significant importance to fruit size. On this linkage group, Dirlewanger et al. (1999) identified QTL for nearly all the fruit quality characters measured in an intraspecific peach mapping population, including a QTL for fruit weight explaining up to 47% of the total variation. In a different intraspecific peach population,

Yamamoto et al. (2001) identified four QTL for fruit weight. Three of these QTL were located on LG3 in this map. However, shared markers with the 'T' × 'E' map (Dirlewanger et al., 2004a) indicate that LG3 in this map is the same as LG 6 and LG8 in the 'T' × 'E' map. Two of the QTL map near the *Dw* brachytic dwarf locus which also is located on LG 6 in a separate almond × peach population (Bliss et al., 2002). That the linkage group in question consists of markers located on LG 6 and LG 8 of the 'T' × 'E' map is not without precedence. Jauregui et al. (2001) found a similar situation in a cross between 'Garfi' almond and 'Nemared' peach. In this case, a reciprocal translocation was identified as the source of the exhibited linkage between LG 6 and LG8.

Coincidentally, the location of these fruit weight QTL appears to be near the self-incompatibility locus (*S*). Sweet cherries possess a gametophytic self-incompatibility system. Until recently, most sweet cherry cultivars were self-incompatible, requiring co-cultivation of at least two cultivars for adequate pollination. Although a few naturally self-fertile cultivars have been described (Bargioni, 1996), self-fertility was not used commonly until the release of the cultivar 'Stella'. 'Stella' was developed from a cross between 'Lambert' and the self-fertile seedling John Innes 2420. Gamma irradiated 'Napoleon' pollen, presumably creating a loss of function mutation for the pollen recognition component of self-incompatibility, was used to fertilize 'Emperor Francis' to create the John Innes 2420 seedling (Lewis and Crowe, 1954). Since the introduction of 'Stella' as a source of self-fertility (Lapins, 1970), all subsequent self-fertile cultivars released have had 'Stella' in their pedigree. Although peach is self-fertile, almond has the same self-incompatibility system as sweet cherry, and this locus has been mapped in an almond × peach population (Bliss et al., 2002). On this map, the *S* locus is within 1.6

cM of the *Dw* locus. Therefore, several of the QTL described previously for fruit size in peach are located in this area of LG 6. Linkage between QTL for fruit size and the *S* locus may have great implications for future breeding efforts and marker assisted selection. Because of the gametophytic incompatibility system in both almond and sweet cherry, linkage distortion around the *S* locus is often observed in linkage maps (Bliss et al., 2002; Joobeur et al., 1998; Foulongne et al., 2003; Lambert et al., 2004; Vilanova et al., 2003). This occurs in cases where the paternal parent has a common *S*-allele with the maternal parent. In these cases, haploid pollen containing the common *S*-allele is rejected in the style of the flower and cannot complete fertiliztion. This gametophytic selection is observed as distorted marker segregation ratios for those markers linked to the *S* locus.

Utility of Simple Sequence Repeat Markers

Until recently, linkage mapping in *Prunus* was accomplished using morphological, isozyme, RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), and AFLP (Amplified Fragment Length Polymorphism) markers. However, many labs have now developed SSR (Simple Sequence Repeat) markers for use in *Prunus* (Aranzana et al., 2002; Cantini et al., 2001; Cipriani et al., 1999; Clarke and Tobutt, 2003; Dirlewanger et al., 2002; Lopes et al., 2002; Mnejja et al., 2004, 2005; Silva et al., 2005; Sosinski et al., 2000; Struss et al., 2002; Testolin et al., 2000; Vaughan and Russell, 2004; Wang et al., 2002; Yamamoto et al., 2002). SSR markers, short tandem repeats of single, di-, tri-, or tetranucleotide motifs that are common throughout eukaryotic genomes (Ellegren, 2004; Weising et al., 1989),

are attractive because they are codominant, polymerase chain reaction (PCR) based, repeatable, and often show a high degree of polymorphism. Because they are codominant and repeatable, SSR markers are ideally suited for comparative mapping.

SSR markers developed from peach libraries have been added to maps of peach and other *Prunus* species (Aranzana et al, 2003; Bliss et al., 2002; Dirlewanger et al., 2004a; Hurtado et al., 2002; Joobeur et al., 2000; Yamamoto et al., 2001). SSR markers developed from peach libraries have shown amplification in all of the main cultivated *Prunus* species (peach/nectarine, sweet cherry, sour cherry, apricot, Japanese plum, European plum, and almond) as well as wild *Prunus* species (*P. serotina*) (Aranzana et al., 2002; Cantini et al., 2001; Cipriani et al., 1999; Dirlewanger et al., 2002; Downey and Iezzoni, 2000; Hormaza, 2002; Sosinski et al., 2000; Testolin et al., 2000; Wang et al., 2002; Wunsch and Hormaza, 2002;Yamamoto et al., 2002). SSR markers from peach and other *Prunus* species have been used for fingerprinting and genetic diversity analysis of peach (Aranzana et al., 2002; Dirlewanger et al., 2002; Testolin et al., 2000), apricot (Hormaza, 2002), sweet cherry (Wunsch and Hormaza, 2002; Dirlewanger et al., 2002), and sour cherry (Cantini et al., 2001).

The transferability and reproducibility of SSR markers, as well as the extensive collinearity of *Prunus* genomes, warrants their extensive use in any new linkage map development.

OBJECTIVES

To fully exploit the genetic potential to increase fruit size in sweet and sour cherry, a more thorough understanding of the control of this quantitative trait is needed.

For increased efficiency in the breeding process, a reductionist approach can be used, whereby the total phenotypic variation for a quantitative trait such as fruit size is reduced to various components that influence the overall phenotype. In this manner, phenotypic variance for the trait can be partitioned into testable units to determine those with the most genotypic variance. This strategy is attractive for sweet and sour cherry, since the available F_1 population structures limit the ability to identify minor-effect QTL.

Two populations well-suited for the identification of fruit size QTL were recently developed in the Michigan State University sour cherry breeding and genetics program. Reciprocal crosses between the sweet cherries 'New York 54' ('NY 54') and 'Emperor Francis' ('EF') were used to develop a population of 617 individuals. 'NY 54' is a small (1-2 g), acid, dark red, wild forest Mazzard selection, while 'EF' is a larger (6-8 g), sub-acid, blushed yellow cherry cultivar. This intraspecific cross represents the change in fruit size that occurred during domestication of wild sweet cherry. In sour cherry, the phenotypic difference between the parents of the 'Újfehértói Fürtös' ('UF') × 'Surefire' population was not great, but transgressive segregation for fruit size in the progeny from the population suggested that different alleles for fruit size QTL were segregating (A.F. Iezzoni, pers. comm.).

The overall goal of this study was to understand the genetic bases for achieving large fruit size in sweet and sour cherry. Experiments were designed to provide knowledge that would be used to develop future genetic improvement strategies to maximize fruit size in new cultivars. The specific objectives for this project included identifying cherry fruit mesocarp histological differences that are associated with fruit size differences, development of genetic linkage maps suitable for comparative mapping

within *Prunus*, and identification of the loci responsible for fruit size differences through QTL analyses.

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CHAPTER TWO

Genotypic differences in sweet cherry (*Prunus avium* L.) fruit size are primarily a function of cell number

ABSTRACT

Large fruit size is critical for profitable fresh sweet cherry (*Prunus avium* L.) production. However, little is known about histological differences that contribute to fruit size differences in sweet cherry. Although fruit size varies widely between sweet cherry cultivars, significant variation exists among genetically identical fruit from the same cultivar due to cultural and environmental differences. This research examined the difference in mesocarp cell traits between five cultivars ['Selah', 'Emperor Francis' ('EF'), 'New York 54' ('NY 54'), 'Bing', and 'Regina'] that represent a wide range of fruit size in sweet cherry, as well as within genotype differences in fruit size due to environmental variation.

The relative contributions of mesocarp cell number and size to final fresh fruit size were determined by analyzing equatorial sections of 'Selah', 'EF', 'NY 54', 'Bing', and 'Regina'. The cell number count in this dimension, representing the total radial cell division at the widest diameter of cherry fruit, was significantly different ($P < 0.05$) between all cultivars except 'Bing' and 'Regina'. The relationship of cell number with fruit weight and diameter was significantly and positively correlated ($r^2 = 0.72$ and 0.59, respectively), while cell length was not correlated with either measure of fruit size. Cell number was stable during the three years of this study and at two different locations. Differences in cell number due to environmental variation were examined in fruit of 'Bing', 'Regina', and 'Selah' that differed significantly ($P < 0.001$) in fruit size. Within each cultivar, fruit size differences were attributed to a difference in cell size rather than cell number, confirming cell number as the primary genetic component resulting in fruit size differences. Cell division differences were most pronounced during the

developmental period between bloom and endocarp sclerification. This experiment suggests that duration of cell division affects fruit size more than the rate of cell division. Based on these results, fruit mesocarp cell number is controlled genetically and has low environmental variance. Therefore, this trait could be used for selection of improved fruit size through breeding efforts.

INTRODUCTION

The mature cherry fruit is composed of a thin protective exocarp, a fleshy mesocarp, and an inedible stony endocarp or pit surrounding the seed (Esau, 1977). All three tissue types arise from the ovary and the increase in fruit size results from a coordinated series of cell divisions and expansions. Cherry, and other *Prunus* species, exhibit a double sigmoid growth curve, consisting of three distinct growth stages (Coombe, 1976; Chalmers and van den Ende, 1975; Lilleland and Newsome, 1934; Nitsch, 1953). Stage I is characterized by rapid and exponential fruit growth following anthesis, stage II by a lag period of fruit growth coinciding with endocarp (pit) hardening and embryo development, and stage III by a second period of exponential fruit growth ending with either harvest or physiological maturity. During Stage I, mesocarp growth consists of both cell division and cell enlargement, while Stage III mesocarp growth is predominantly due to cell expansion (Coombe, 1976; Gage and Stutte, 1991; Nitsch, 1953; Tukey and Young, 1939).

Sweet cherry fruit exhibit a dramatic range in fruit size. Wild forms of forest sweet cherry, which are generally used for wood or cherry rootstocks, have small (~1 to 2 g) fruit that is ideal for dispersal since it consists predominantly of the pit containing the seed. In contrast, cultivated sweet cherries produce fruit that weigh approximately 6 g (exhibited by the old landrace varieties) to over 13 g for cultivated varieties. A previous study of a diverse range of sweet cherry selections that exhibit differences in fruit size concluded that mesocarp thickness is determined primarily by cell number, however, differences in cell size were also found (Yamaguchi et al., 2004). In peach [*Prunus persica* (L.) Batsch], cell number and cell size were found to vary continuously in

cultivars of different fruit sizes indicating that fruit size is a quantitative character (Scorza et al., 1991; Yamaguchi et al., 2002a).

Fruit diameter is the primary criterion upon which fresh cherries are graded for sale. Fruit averaging over 29 mm in diameter are worth nearly twice as much ($/kg) as fruit less than 24 mm in diameter (Whiting et al., 2005, 2006). Therefore, our long term goal is to determine the quantitative trait loci (QTL) that control fruit size, identify large fruited alleles at these loci, and use marker assisted selection to increase the efficiency of breeding large fruited sweet cherry cultivars. The improved fruit size of old landrace varieties (~6 g) compared to sweet cherry forest trees (~1-2 g) represents a classic example of fruit size increase associated with domestication (Janick, 2004; Tanksley, 2004). This common recurring increase in fruit size that has accompanied the domestication of many fruit and vegetable crops has been studied in most detail in tomato (*Lycopersicon esculentum* Mill.), the fruit of which is a fleshy carpel like in cherry (Doganlar et al., 2002; Frary et al., 2000; Grandillo et al., 1999; Nesbitt and Tanksley, 2002; Tanksley, 2004). These studies suggest that the evolution of fruit size in tomato likely represents the "stacking" of alleles at many loci. However, accumulated QTL evidence for domestication traits in maize (*Zea mays* L.), rice (*Oryza sativa* L.), sorghum (*Sorghum bicolor* L.) and tomato supports the hypothesis that the majority of domestication-associated anatomical changes can be attributed to a few loci with larger effects (Paterson, 2002, Tanksley, 2004). The application of this hypothesis to sweet cherry is consistent with the finding that cultivars with fruit sizes of over 13 g have been obtained from just three generations of breeding among 6 to 8 g landrace selections (Choi and Kappel, 2004).

QTL analysis is a powerful method to identify those chromosomal regions carrying genes contributing to trait variation, as this analysis requires no *a priori* information other than the existence of variation and a means to quantify this variation (Paterson, 2002). However, not all QTL can be detected with statistical significance. In many cases, the ability to quantify trait variation is the limiting factor, as QTL cannot be resolved if significant environmental and sampling variation obscures the resulting phenotype. To increase our ability to identify fruit size QTL, we investigated the components of fruit size among potential parental selections to determine those traits that would be most likely to identify QTL that contribute to an increase in fruit size. The specific objectives were to determine: (1) the relative contribution of mesocarp fruit cell number and size differences to mature fruit weights among five sweet cherry selections, (2) the environmental stability of these measurements, and (3) the relative timing of the cell number increases.

MATERIALS AND METHODS

Plant material

Images in this dissertation are presented in color. Five sweet cherry cultivars were selected to represent a wide range in average fresh fruit size. 'NY 54' is a small-fruited wild cherry selection, used commercially as a seed source for seedling *P. avium* rootstock. 'EF' is a mid-sized old European cultivar of unknown origin, representing the fruit size achieved through domestication. 'Bing' is a large-fruited, 130-year-old selection, while 'Regina' is a large-fruited cultivar released in 1998. Finally, 'Selah' is a very large-fruited cultivar introduced in 2000, which is among the largest current

cultivars. Experimental trees were located at Washington State University's Irrigated Agriculture Research and Extension Center (WSU-IAREC) in Prosser, Wash. (46.29 N, 119.73 W), and Michigan State University's Clarksville Horticultural Station (MSU-CHES) in Clarksville, Mich (42.87 N, 85.26 W). For comparative histology measurements between genotypes, all except 'NY 54', 'Bing', and 'Regina' at MSU-CHES were mature (> 20 yr) trees grafted on *P. avium* seedling rootstock and trained to an open-center. 'NY 54', 'Bing', and 'Regina' at MSU-CHES were younger (3-5 yr), grafted on 'Gisela 6' rootstock and trained to a central leader system. For histology measurements within genotype, young (7 yr) 'Selah', 'Bing', and 'Regina' trees grafted on *P. avium* seedling rootstock were used. Trees at WSU-IAREC were irrigated weekly with under-tree sprinklers, while those at MSU-CHES were provided supplemental irrigation by drip lines from mid-June until August. Standard orchard management practices for each location (irrigation, fertilization, pest control, and dormant pruning) were followed.

Flower and fruit sampling scheme for mesocarp cell number and size comparisons among the five cultivars

To evaluate histological differences among the five cultivars ('Selah', 'EF', 'NY 54', 'Bing', and 'Regina'), well-exposed fruit were sampled randomly from the exterior portion of the canopy. Five fruit from each cultivar were analyzed. At WSU-IAREC, 'Bing' and 'Regina' were not sampled in 2003, and 'Selah' was not sampled from MSU-CHES in 2004 or 2005. In 2003, samples at bloom, endocarp hardening, and harvest maturity were taken at WSU-IAREC. Bloom samples were taken when 50% of the

flowers on a treatment tree were open. Only flowers that had recently opened fully, as judged by non-dehiscent anthers, were sampled. Endocarp hardening was when a complete cut could not be made easily through the fruit. In 2004, samples at each developmental stage were taken from WSU-IAREC and MSU-CHES, as well as weekly samples during the period from bloom to endocarp hardening. In 2005, samples were taken at one to two day intervals for all genotypes except 'EF' at WSU-IAREC. In 2005, samples at the endocarp hardening stage were used to calculate cell numbers for 'EF' and 'NY 54' from WSU-IAREC and for all genotypes from MSU-CHES. To equalize the potential temperature influence on cell division, growing degree day (GDD) accumulation using a 4.4 C base temperature was calculated for periods between sampling dates.

Flower bud thinning treatments to determine the influence of crop load on mesocarp cell number and size

To evaluate different-sized fruits within genotype, three cultivars ('Selah', 'Bing', and 'Regina') were subjected to whole tree pre-bloom thinning treatments at WSU-IAREC in 2004 and 2005. Selah was not sampled in 2004. For the thinning treatments, all spurs on a tree were hand-thinned to one flower bud per spur prior to bloom. Control trees were left unthinned. This thinning treatment had previously resulted in significant fruit size differences (Lang and Ophardt, 2000; Whiting and Lang, 2004). However, for some genotypes, thinning did not result in significant fruit size difference between treatments; in such cases, a large random sample of fruit was harvested at maturity, and

individual fruit were weighed to create pools of small and large size fruit from the same genotype.

Fruit measurement and sectioning

The five fruit per cultivar or treatment were weighed individually and diameters were measured at the widest point of the fruit (Fig. 1) using a digital caliper. The fruit then were placed individually in storage vessels, immersed in a formalin-acetic acid-alcohol solution (10:5:50 FAA; Ruzin, 1999) and stored until sectioning. Radial mesocarp flesh sections were obtained at the widest diameter of the fruit (Fig. 1) by hand sectioning with a double-edged razor blade. Cell division in drupes occurs in a radial direction as the mesocarp develops (Tukey and Young, 1939). In addition, this plane of measurement is equivalent to the dimension that commercially produced sweet cherries are measured for size before sale. From bloom until endocarp hardening, tissue sections were cut through the entire diameter of the fruit; from endocarp hardening on, tissue sections were cut from the skin to the endocarp wall, consisting only of exocarp and mesocarp tissue. After sectioning, mesocarp tissue was rehydrated with distilled water before staining. For sections created after endocarp hardening, the pit weight and diameter were measured.

Microscopic analysis of mesocarp tissue

Following tissue rehydration, the sections were stained for at least 24 h in a dilute 1:20 solution of 1 mg/ml acridine orange. Preliminary tests indicated that acridine orange was suitable for staining mesocarp cell walls at all stages of development. Acridine orange is a metachromatic fluorescent dye that is excited at 500 nm and emits with peaks

in both green (526 nm) and red (650 nm) ranges (Lillie, 1977). After this staining period, tissue sections were briefly rinsed again in distilled water, and fresh slide mounts using distilled water were prepared immediately before microscopic evaluation. Unstained samples of mesocarp tissue from each developmental stage were observed using the same microscope parameters to ensure that the fluorescence signal was not due to autofluorescence.

All microscopy was performed at the MSU Center for Advanced Microscopy, using a Zeiss laser scanning confocal microscope and software (Zeiss LSM Pascal, Jena, Germany). The following microscope parameters were used to collect fluorescent images: 488 nm argon laser line, 505-530 nm band pass filter, and 650 nm long pass filter. Both 10x and 20x objectives were used for different stages of development. Pinhole apertures of 70 μm and 84 μm were used with the 10x and 20x objectives, respectively. For all but the earliest developmental date, multiple field of view images were necessary to scan through the entire mesocarp section. Images were captured digitally as Tagged Image Format files with no compression using the integrated microscope software, and stored on compact disc for later image analysis.

Image analysis

Individual field of view images were first aligned together into one composite image using Adobe Photoshop 6.0 software (Adobe Systems Inc., San Jose, Calif.). Composite images were then calibrated to a defined dimension using Sigma Scan Pro 5.0 software (Systat, Richmond, Calif.). Once calibrated, the trace measurement function in Sigma Scan Pro was used to draw and measure a line the length of the mesocarp section.

For each image, all of the cells touching the line were counted and this measurement was subsequently used in all analyses, similar to that of Yamaguchi et al. (2002a, 2002b, 2004). Cell length in the sections was calculated by dividing the total mesocarp section length by the number of cells counted in the same length.

Statistical analysis

Data were subjected to analysis of variance using SAS general linear model procedure with the variance for subsamples used as the error term (SAS Institute, Inc., Cary, N.C.). SAS correlation procedure was used when appropriate to determine the Pearson correlation coefficient between related measures. All means were separated using Tukey's HSD or Fisher's LSD.

RESULTS

Comparison of fruit and pit measurements among five sweet cherry selections

In 2003, fruit and pit measurements were made from 'NY 54' and 'Selah' as the extremes in the range of fruit size diversity, and 'EF' as a domesticated selection (Table 1, Fig. 2). Mean fruit weights and diameters were significantly different ($P < 0.05$), with 'EF' exhibiting intermediate values. In 2004, fruit from 'Bing' and 'Regina' also were sampled. As in 2003, 'Selah', 'EF', and 'NY 54' exhibited significant ($P < 0.05$) differences in fruit weight and diameter; however, 'Bing' and 'Regina' had similar values that were significantly larger than 'EF' but significantly less than 'Selah' (Table 1). This is consistent with 'Bing' and 'Regina' representing a fruit size improvement over that achieved through domestication.

In both years, the numbers of mesocarp cells counted in radial sections of 'Selah', 'EF', and 'NY 54' (Fig. 3) were significantly different ($P < 0.05$) with 'Selah' having ~3x the number of mesocarp cells as 'NY 54' (Table 1). As with fruit weight and diameter, the number of mesocarp cells for 'EF' was intermediate to that of 'NY 54' and 'Selah'. In 2004, mean radial cell number for 'Bing' and 'Regina' were 48.3 and 43.8, respectively (Table 1). As with fruit weight and diameter, this cell number value for 'Bing' was significantly larger than the value for 'EF', but significantly smaller than the value for 'Selah'. Although the cell number for 'Regina' was statistically similar to that of 'Bing', it was not significantly different from that of 'EF'.

Variation for cell length was less significant than that for cell number, as the selections fell into only two groups (Table 1). In both years, 'EF' had the longest calculated cell lengths, which were similar to those from 'Bing' and 'Regina'. Interestingly, although 'Selah' fruit were the largest overall and had the greatest number of cells, the calculated cell length was not statistically different from 'NY 54' and was shorter than that for 'EF', 'Bing', and 'Regina'.

'NY 54' exhibited a significantly smaller pit weight and diameter compared to the other selections. The mean pit diameters for the four remaining selections were not significantly different. Pit weight did vary; however, this variation is more likely due to seed development as early maturing selections have less developed seeds (Fogle, 1975). The maturity order for these cultivars from earliest to latest, is 'EF', 'Bing' ~ 'Selah', and 'Regina'.

Correlations were calculated for the fruit and cell measurements for the five sweet cherry selections evaluated in 2004. Highly significant positive relationships were

identified between cell number and both fruit diameter and fruit weight (r^2 = 0.59 and 0.72, respectively, $P < 0.001$) (Fig. 4). However, cell length was not significantly correlated with either fruit diameter or fruit weight (Fig. 4). This supports the conclusion that cell number and not cell size is the major cellular component contributing to the genotypic differences in fruit size.

Stability of mesocarp cell number and size for selections subjected to different climatic and cultural conditions

To determine the stability of the cell number measurements for the five selections, data was collected for three years (2003-05) and two locations (WSU-IAREC and MSU-CHES). Analysis of variance indicated no significant year × location interaction between cell numbers for each cultivar. Likewise, no significant within cultivar difference was identified between the two locations. However, a significant ($P < 0.001$) difference was identified within the year main effect of the model. Therefore, the potential interaction and location variance was pooled, and the analysis of variance was performed to identify the year difference by mean separation. A significant cell number difference for 'EF' was identified in 2003 (Table 2). In that year, within cultivar cell number counts averaged higher than other years and locations. However, for all other cultivars, no significant within cultivar difference was found for cell number. This indicates that cell division is under strong genetic control, and in general, is unaffected by different climatic conditions.

To further examine the stability of mesocarp cell number, an analysis of within-cultivar variation was done. Because sweet cherries are clonally propagated and the

measured area of the fruit is solely maternal tissue, a random sample of fruit from the same cultivar will be genetically identical. However, variation in fresh fruit size within the same tree occurs due to physiological variables such as crop load and fixed carbon availability. In 2004, pre-bloom crop load adjustment was performed on 'Bing' and 'Regina' trees at WSU-IAREC to create differences in available carbon allocated to individual fruit. Crop load adjustment was performed by hand-thinning whole trees to one fruit bud per fruiting spur. Similar treatments have been shown to result in significant increases in overall fruit size (Land and Ophardt, 2000; Whiting and Lang, 2004). Un-thinned control trees were used for comparison.

Crop load adjustment resulted in a significant increase ($P > 0.001$) in overall fruit weight and diameter for 'Bing' in 2004. However, due to low initial fruit set, the same treatment on 'Regina' trees did not result in significant fruit size differences in random samples. Therefore, individual fruit from 'Regina' trees were weighed and separate pools of small and large-sized fruit were created. The difference between the pools was at least 2 g, similar to the average weight differential for the 'Bing' treatments. In 2005, bud thinning treatments were applied to 'Bing', 'Regina', and 'Selah' trees. However, spring frost damage resulted in non-significant differences between treatments; therefore, selected pools of different-sized fruit were used again for comparison.

In both 2004 and 2005, the mean fresh fruit weights and diameters for the small versus large fruit within each of the three cultivars were significantly different ($P < 0.001$) (Table 3). Mesocarp cell numbers for a given cultivar were not significantly different for the large or small fruit samples. However, the calculated cell lengths were significantly different ($P < 0.05$) between all comparisons except 'Selah' in 2005. For

'Bing' and 'Regina', the larger fruit had significantly longer mesocarp cell lengths compared to small fruit. These results indicate the differences in fruit weights and diameters between the large and small fruit of 'Bing', 'Regina', and 'Selah' were not due to differences in mesocarp cell number. Instead, fruit size increases within 'Bing' and 'Regina' were due to increases in cell length. Although cell lengths were not significantly different between large and small fruit from 'Selah', the trend of larger fruit size correlated with longer cell lengths was evident. Interestingly, the mean mesocarp cell length for the ~13 g 'Selah' fruit were similar to those of the very small fruit from 'NY 54', and this cell length was unaffected by those environmental or cultural conditions that resulted in larger fruit size. 'Selah' fruit may have been judged to be at harvest maturity prior to the end of the fruit developmental period, resulting in incomplete cell expansion.

Pit weight was significantly different between the 'Bing' comparisons in both 2004 and 2005 ($P < 0.001$, $P < 0.05$, respectively), and 'Regina' in 2005 ($P < 0.001$) (Table 3). Pit diameter was significantly different between treatments for 'Bing' in 2004 ($P < 0.001$), and 'Regina' and 'Selah' in 2005 ($P < 0.05$, $P < 0.0001$, respectively). In these cases, larger fruit had heavier pits with increased diameters. However, when the percentage of total fruit diameter due to pit diameter was analyzed, it was apparent that pits in small sized fruit contributed a greater percentage to the total fruit diameter ($P < 0.01$ for all cultivars), and thus smaller mesocarp flesh diameter. In addition, these differences were not consistent among cultivars and years. For example, the large 'Regina' fruit in 2004 and large 'Selah' fruit exhibited mean diameters of 27.7 mm and 30 mm, respectively, yet their mean pit diameters were nearly equivalent, at 8.3 mm and

8.1 mm, respectively. This suggests that genetic increases in fruit size can occur without an associated increase in pit diameter.

Duration of mesocarp cell division for the five sweet cherry selections

Examination of flesh mesocarp sections of 'Selah', 'EF', 'NY 54', 'Bing' and 'Regina' at different stages of fruit development in 2003 and 2004 confirmed the classic general stone fruit growth pattern (Chalmers and van den Ende, 1975; Coombe, 1976; Gage and Stutte, 1991; Lilleland and Newsome, 1934; Nitsch, 1953; Tukey and Young, 1939). Mesocarp cell division occurred during the period from bloom until endocarp lignification; generally, only cell enlargement occurred after endocarp hardening (Fig. 5, Table 4). In 2003, samples taken from 'EF' flowers just after opening had significantly fewer ($P < 0.05$) mesocarp cells than 'Selah or 'NY 54' sampled at the same stage. In 2004, 'NY 54' and 'EF' had the fewest cells at bloom while 'Selah' had the most (Table 4). In 2004, the ranking of mean cell numbers at bloom was equivalent to the ranking based upon mature fruit size (e.g., large to small, 'Selah', 'Regina' ~ 'Bing', 'EF', 'NY 54'), although this was not the case in 2003.

In 2004, the GDD accumulation at WSU-IAREC until the total number of mesocarp cells was reached ranged from 65 ('Regina') to 237 ('Selah'). 'Bing', 'EF', and 'NY 54' each took slightly less than 95 GDD to reach the total mesocarp cell numbers. At MSU-CHES, the GDD accumulation until total cell numbers were reached ranged from 22 ('NY 54') to 171 ('Regina'). At WSU-IAREC, the GDD accumulation until total cell numbers were reached ranged from 21 ('NY 54') to 139 ('Selah'). More frequent sampling, every three to five days as done in 2005, provided the ability to

calculate a cell division rate for each cultivar (Table 5). Because destructive sampling was needed to measure cell numbers at each date, the differential between the mean numbers of mesocarp cells at bloom was used to estimate the number of new cells added. In contrast to the difference in duration of cell division for each cultivar, the calculated cell division rate was only significantly different ($P < 0.05$) for 'NY 54'. However, the calculated low rate of cell division in 'NY 54' is not biologically relevant since 'NY 54' essentially has its full complement of mesocarp cells at bloom time. Therefore, the increase in mesocarp cell number associated with increased fruit size in the sweet cherry selections was due to an increase in the duration of the cell division period, not more rapid cell division.

DISCUSSION

Fruit size differences among the sweet cherry selections were determined primarily by differences in mesocarp cell numbers. 'NY 54' essentially had its full complement of mesocarp cells at bloom, whereas the larger-fruited cultivars underwent significant cell number increases between bloom and endocarp hardening. This is similar to results reported previously for sweet cherry (Yamaguchi et al., 2004), with the degree of correlation between whole fruit size measurements and mesocarp cell number estimates in similar ranges. However, in the present study, the correlation between cell size and overall fruit size was not significant in a comparison between cultivars, while Yamaguchi et al. (2004) report higher correlations between cell size and fruit size between cultivars. This may be a result of the limited number of cultivars examined in

this study. For example, the small cell size combined with large fruit size of 'Selah', and large cell size in the smaller-fruited 'EF' may be unique among cherry genotypes.

An increase in the number of mesocarp cells corresponding to increased fruit size also has been reported for comparisons between small and large-fruited peach cultivars (Scorza et al., 1991; Yamaguchi et al., 2002a, 2002b). Collectively, these reports and the present study indicate that the gene(s) involved in mesocarp cell number proliferation are keys to understanding the genetic potential for increased fruit size in sweet cherry. Interestingly, these reports for *Prunus* species are similar to that of the model fruit plant, tomato. In tomato, the gene underlying a major QTL (*fw2.2*) contributing to fruit size difference between wild and cultivated species has been cloned and shown to influence cell division and consequently fruit size (Frary et al., 2000; Nesbitt and Tanksley, 2002).

It is noteworthy, however, that 'NY 54' and 'Selah' mean mesocarp cell lengths were similar, but were shorter than those for 'EF', 'Bing', and 'Regina'. This suggests that it might be possible to further genetically increase fruit size in sweet cherry by combining the increased number of cell divisions in 'Selah' with the increased cell size exhibited by 'EF', 'Bing', and 'Regina'.

This study was undertaken as a component of a larger research plan to identify the major QTL and genes involved in sweet cherry fruit size. 'NY 54' and 'EF' were included in these analyses because they are the parents of a genetic linkage mapping population developed at MSU. Therefore, histological differences between 'NY 54' and 'EF' mesocarp cells have the potential to segregate among progeny from the linkage mapping population and can be used in a future QTL analysis. Furthermore, 'NY 54' is a true wild example of *P. avium* (R.L. Andersen, pers. comm.) and 'EF' can be considered

an early domesticate of sweet cherry in Europe. Hence, differences in cellular development identified between the two are a direct result of early selection and domestication by farmers. Because larger fruit size is considered one of the hallmarks of early domestication (Janick, 2004), these differences are important to document from a plant breeding standpoint. With many potentially valuable genes for traits such as pest, disease, and stress tolerance available in wild members of the species and relatives, an understanding of the traits originally selected during domestication will certainly speed the recovery of suitable fruit size following gene introgression.

'Selah' was included in the study because it falls at the opposite end of the fruit size spectrum from 'NY 54'. 'Selah' is one of the largest-sized sweet cherry cultivars released from the Washington State University stone fruit breeding program. 'Bing' and 'Regina' were also included in these experiments as production cultivars because they either currently dominate U.S. production ('Bing'), or provide valuable niche-market alternatives ('Regina'). Together with the goal of identifying useful genetic variation for fruit size, an unanticipated but potentially valuable result of these experiments was to clarify key stages of developmental activity relating to sweet cherry fruit growth that horticulturists and physiologists may exploit to better maximize fruit size of current cultivars.

For a quantitative trait, such as fruit size, to be efficiently selected in a breeding program, there must be a high level of genetic variation coupled with low environmental and sampling variation. In our study, mesocarp cell number exhibited an extraordinary stability when subjected to different climatic conditions and cultural practices. During the experimental time period, no differences for mature cell number were identified

between Washington and Michigan. The only within-cultivar difference in cell number was identified for 'EF' in 2003. In that year, 'EF' had significantly more cells than in later samples.

In this study, although fruit size differed significantly between pools of fruit from the same cultivar, cell number was not different (Table 3). Consequently, calculated cell size differences were apparent. Environmental effects on fruit size appear to be manifested through an increase or decrease in mean cell size. The magnitude of size difference between pools of fruit from the same cultivar was similar to differences in mean fruit size between cultivars. Both cell number and cell length were not significantly different between 'Selah' fruit that averaged nearly five mm different in diameter. However, these results are based on one year of data, and additional samples will be necessary to determine whether the trend of increased cell size among larger 'Selah' fruit is significant. It is possible that Selah fruit were judged to be at harvest maturity based on color and taste before they were actually close to physiological maturity. Pit diameter differences commonly were measured between different sized fruit from the same cultivar. The data suggest that larger diameter fruit have larger diameter pits. However, when the percentage of total fruit diameter due to pit diameter was analyzed, it was apparent that pits in small sized fruit contributed a greater percentage to the total fruit diameter.

Differences in fruit size within the same cultivar without a corresponding increase in cell number have been reported for other fruit species. In olive (*Olea europaea* L.), development of fruit under water deficit conditions, resulted in overall fresh fruit size differences but no significant difference in cell number in the fruit (Costagli et al., 2003;

Rapoport et al., 2004). However, Jackson and Coombe (1966) report that within and between tree variation in fruit size for a single apricot (*Prunus armeniaca* L.) cultivar was due to both cell number and size differences. Although a general tendency was observed toward smaller cell sizes in small peach fruit (Bradley, 1959), the correlation between cell size and mesocarp size was not significant, and the author concluded that cell number differences must be involved.

In apple (*Malus domestica* Borkh.), conflicting evidence has been reported concerning potential environmental effects on cell size and number in the same genotype. Temperature differences applied to potted apple trees during the cell division period after bloom resulted in larger cells but no increase in the number of cells (Atkinson, et al., 2001). In contrast, Warrington et al. (1999) found that early season temperature differences affect cell division in apple cultivars. Bain and Robertson (1951) report that, from a single cultivar, different fruit sizes were related to increased cell numbers in the cortex. The biennial bearing nature of certain cultivars has been shown to decrease fruit cell number in the year after heavy cropping (Bergh, 1985). Similarly, a higher chilling unit accumulation resulted in a greater number of cells (Grebeye and Bergh, 2000). Thinning of flowers or fruit on apple trees of a single cultivar has repeatedly been shown to increase cell number and/or cell size (Bain and Robertson, 1951; Bergh, 1990; Denne, 1960; Goffinet et al., 1995; Westwood et al., 1967). The rootstock effect on apple cell size or number in a single cultivar has been attributed either to cell size difference (Al-Hinai and Roper, 2004) or number (Hirst and Flowers, 2000) differences in the fruit. Although on the surface, experiments with different rootstocks can be considered as potential environmental variation, the fact remains that the rootstock and scion are two

genetically different entities, and the extent to which rootstock-produced proteins may influence scion growth and development has yet to be explored thoroughly. Apple fruit flesh results from cortical or accessory tissue in the flower, while sweet cherry flesh begins as the true ovary wall. It is possible that the cortical region that comprises apple fruit flesh is under different genetic control than sweet cherry and *Prunus* in general.

The data from the present study indicate that cell number and not cell size in sweet cherry has the greatest genotypic influence on fruit size. Within a genotype, cell number was constant while variations in fruit size resulted from increasing mesocarp cell size. Together, these results indicate that mesocarp cell number is not greatly affected by environmental variation, and is therefore an ideal trait for which to select in sweet cherry breeding programs to increase fruit size.

In *Prunus*, mesocarp cell division occurs during the period between anthesis and endocarp hardening (Coombe, 1976; Gage and Stutte, 1991; Nitsch, 1953; Tukey and Young, 1939). In this study, fruit from 'Selah', 'EF', 'NY 54', 'Bing', and 'Regina' followed this general growth pattern (Fig. 5, Table 4). Once endocarp hardening began, the final mesocarp cell number had nearly been reached, and difference in cell number between cultivars was significant (Table 4). At bloom, the endocarp cells are included in radial sections through the fruit diameter (Fig. 5 A), but they are distinguishable from the mesocarp parenchyma, as has been noted for peach (Masia et al., 1992), and were not included in the cell number count at this developmental stage. The number of mesocarp cells at bloom varied by year, and there was no clear relationship between the number of cells at bloom and the final cell number at harvest maturity (Table 4). This could be due to the timing of sample collection at bloom. Samples from each different cultivar were

collected when the entire tree was judged to be at 50% full bloom by visual estimation. At that point, only flowers that were fully open but with anthers not yet dehiscent were collected. Although this was deemed the most efficient way to synchronize samples from cultivars with divergent bloom times, the phenology estimate may not have been accurate. Alternatively, the difference in cell numbers at bloom may be a cultivar-specific trait that reflects differences in time or extent of floral differentiation. Difference in cell number between small and large fruit size peach cultivars, before and at bloom, has been previously documented (Scorza et al., 1991). The extent to which cell numbers can be increased prior to mesocarp cell division after bloom warrants further investigation in sweet cherry. However, the fact remains that to reach the final cell numbers observed in this study, mesocarp cells in Selah had to divide nearly twice as often in the period between bloom and harvest as the other cultivars examined.

CONCLUSIONS

These experiments indicate mesocarp cell number is the major genetic determinant of fruit size in sweet cherry. The number of mesocarp cell layers present (from the endocarp to the exocarp) was remarkably stable in the three years of this study and at two different locations with disparate environmental conditions. Cell number was not affected by environmental or cultural variation, as illustrated by analyzing fruit of different sizes from the same genotype. Collectively, these data suggest that cell number difference would be an ideal trait to identify using QTL analysis. The low environmental variance also would be advantageous for selection in a breeding program.

The variation in cell number between genotypes at bloom remains to be fully analyzed. Carbohydrate reserves are important for early season growth in sweet cherry (Ayala, 2004), and reduction in stored carbohydrates has been shown to reduce cell division in the following season in Japanese pear (*Pyrus serotina*) (Toyama and Hayashi, 1956). Although the majority of cell division occurs in the period between bloom and endocarp sclerification, horticultural treatments applied in the same season that flower buds are initiated and differentiate may increase the number of mesocarp cells prior to the onset of cell division at bloom.

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TABLES AND FIGURES

Figure 1. Diagram illustrating the area of sweet cherry fruit samples sectioned for microscopic analysis. Radial sections were prepared from the thickest part of the fruit mesocarp, halfway between the point of stem attachment and the stylar scar, and 90 degrees from the suture line.

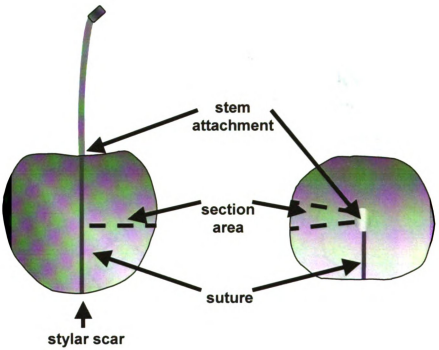

Figure 2. Images of fruit from 'Selah' (A), 'Emperor Francis' (B), and 'NY 54' (C) sweet cherries, illustrating the variation in fruit size and mesocarp thickness.

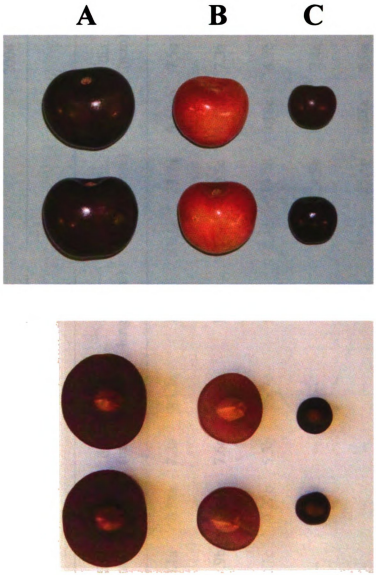

Table 1. Comparison of mean whole fresh fruit size, pit size, and mesocarp cell number per radial section and size measurements for 'Selah', 'Emperor Francis' ('EF'), 'NY 54', 'Bing', and 'Regina' sweet cherries from WSU-IAREC at harvest maturity.

	2003						2004					
	Fruit		Pit		Mesocarp		Fruit		Pit		Mesocarp	
Cultivar	wt. (g)	dia. (mm)	wt. (g)	dia. (mm)	cell no.	cell length (µm)	wt. (g)	dia. (mm)	wt. (g)	dia. (mm)	cell no.	cell length (µm)
Selah	12.8a[z]	30.1a	0.38a	7.8a	83.2a	148a	10.5a	27.7a	0.50ab	7.5a	78.8a	140a
EF	6.0b	21.9b	0.41a	7.6a	47.4b	168b	6.5c	22.5c	0.43b	7.3a	41.4c	205b
NY54	1.4c	12.1c	0.22b	5.3b	26.7c	136a	1.4d	13.0d	0.18c	4.9b	28.6d	155a
Bing	--	--	--	--	--	--	7.6b	24.8b	0.50ab	7.4a	48.3b	188b
Regina	--	--	--	--	--	--	7.7b	25.1b	0.58a	8.0a	43.8bc	199b

[z]Mean separation within columns by Tukey's HSD at $P < 0.05$.

Figure 3. Examples of 'Selah' (A), 'Bing' (B), 'Regina' (C), 'Emperor Francis' ('EF') (D), and 'NY54' (E) composite mesocarp images at fruit maturity (20x). Images were created by aligning adjoining microscope field-width images (n = 19, 15, 13, 13, and 5 for 'Selah', 'Bing', 'Regina', 'EF', and 'NY 54', respectively), and scaled relative to each other for presentation. Scale bar = 200 μm.

Figure 4. Linear correlation between mesocarp cell number and both fresh fruit diameter and weight (A), and mesocarp cell length and both fresh fruit diameter and weight (B) for all sweet cherry cultivars measured in 2004. The *P*-values calculated for the Pearson correlation coefficient (r) and R-square value for each comparison is indicated on the plot.

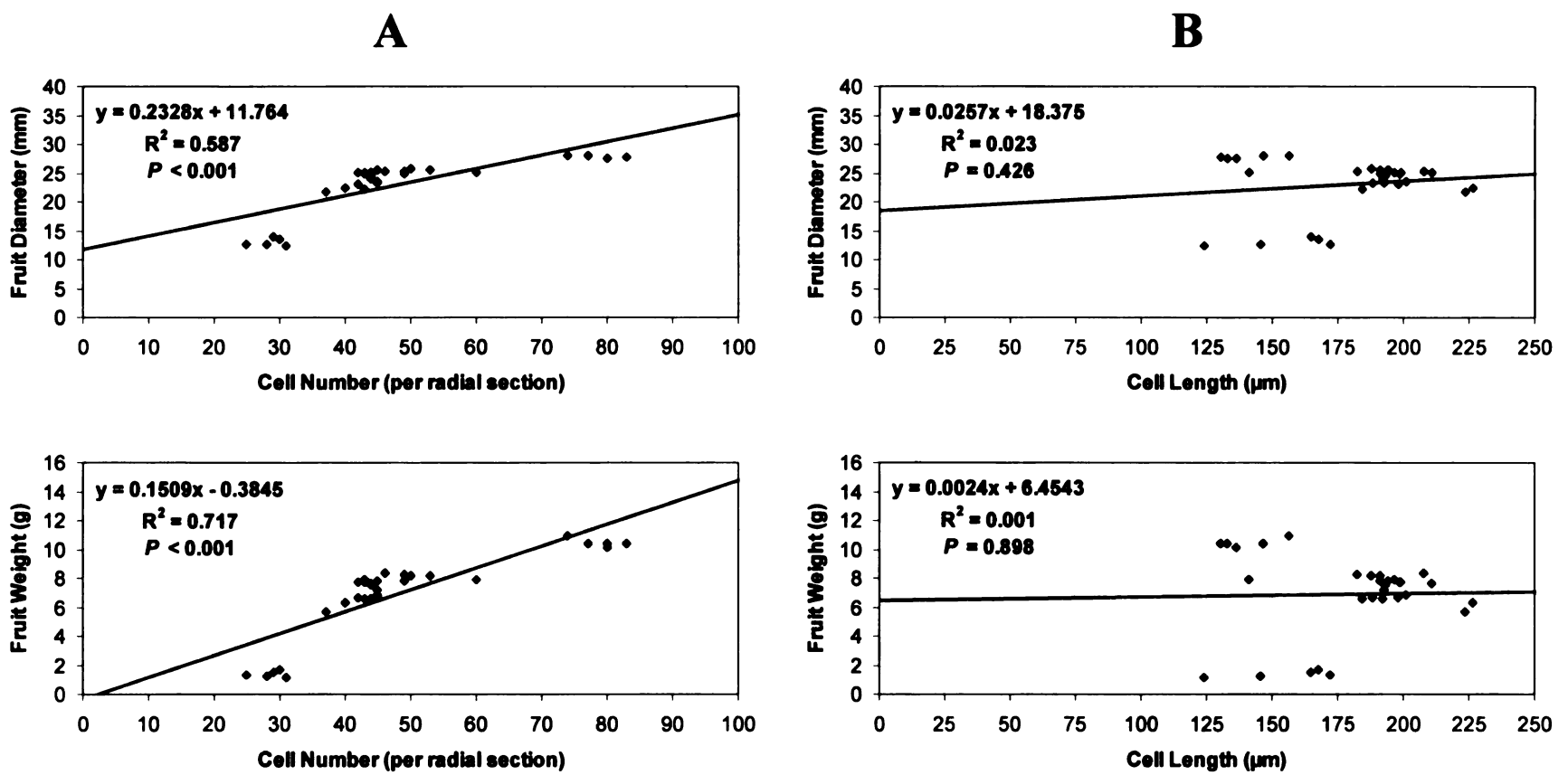

Table 2. Comparison of mean cell numbers at maturity for 'Selah', 'Emperor Francis' ('EF'), 'NY 54', 'Bing', and 'Regina' sweet cherry fruit from 2003-2005 and at two locations (WSU-IAREC and MSU-CHES).

	Cell no. per radial section				
Year and Location	Selah	EF[z]	NY54	Bing	Regina
WA 2003	83.2 a	47.4 a	26.7 a	--	--
WA 2004	78.8 a	41.4 b	28.6 a	48.3 a	43.8 a
WA 2005[y]	78.6 a	38.4 b	28.0 a	47.4 a	45.2 a
MI 2004	--	40.0 b	28.6 a	44.8 a	47.6 a
MI 2005[y]	--	40.6 b	29.0 a	46.8 a	43.8 a

[z]Mean separation within columns by Tukey's HSD at $P < 0.05$.
[y]Cell number for EF and NY54 in 2005 at WSU-IAREC and all samples from 2005 at MSU-CHES were determined after endocarp hardening.

Table 3. Comparison of mean whole fresh fruit size, pit size, and mesocarp cell number (per radial section) and size measurements for populations of large and small fruit from 'Bing', 'Regina', and 'Selah' sweet cherries at harvest maturity.

	2004						2005					
	Fruit		Pit		Mesocarp		Fruit		Pit		Mesocarp	
Cultivar	wt.[z] (g)	dia. (mm)	wt. (g)	dia. (mm)	cell no.	cell length (μm)	wt. (g)	dia. (mm)	wt. (g)	dia. (mm)	cell no.	cell length (μm)
Bing high wt.	9.4***	26.7***	0.57**	7.9**	48.5ns	196*	11.3***	27.6***	0.56*	7.6ns	49.0ns	208*
Bing low wt.	7.6***	24.8***	0.50**	7.4**	48.3ns	181*	7.5***	24.0***	0.48*	7.3ns	48.0ns	185*
Regina high wt.	10.3***	27.7***	0.64ns	8.3ns	45.6ns	214*	12.4***	28.8***	0.64**	8.2*	46.8ns	219*
Regina low wt.	7.7***	25.1***	0.58ns	8.0ns	43.8ns	195*	8.3***	24.3***	0.48**	7.5*	47.0ns	176*
Selah high wt.	--	--	--	--	--	--	13.7***	30.0***	0.55ns	8.1***	78.8ns	137ns
Selah low wt.	--	--	--	--	--	--	8.8***	25.0***	0.49ns	7.2***	78.2ns	125ns

[z]Mean separation for paired treatments in columns by Fisher's LSD; ns=non significant, *,**,*** significance at $P < 0.05$, 0.01, 0.001, respectively.

Figure 5. Examples of mesocarp cell development at different stages of fruit development for 'NY 54' sweet cherry (45x). (A) bloom, (B) endocarp hardening, and (C) harvest maturity. Images from endocarp hardening and harvest are composite images created by aligning adjoining microscope field-width images (n = 3 and 5, respectively), and are scaled relative to each other for presentation. Scale bar = 200 μm.

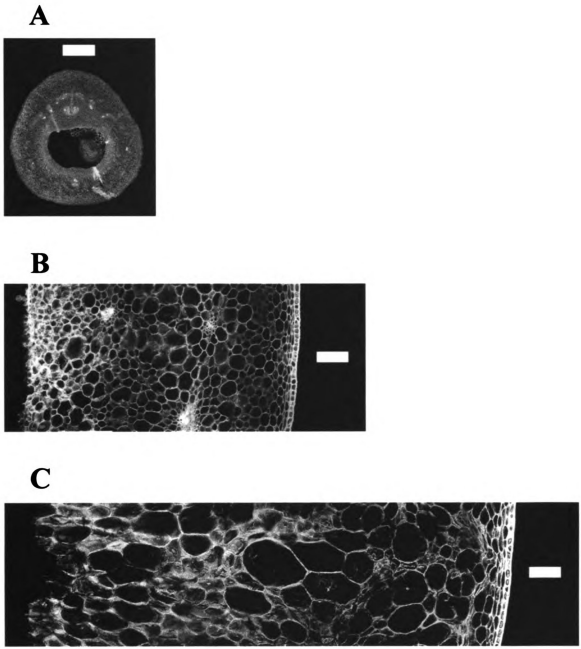

Table 4. Comparison of mean cell numbers at bloom, start of endocarp hardening, and maturity for 'Selah', 'Emperor Francis' ('EF'), 'NY 54', 'Bing', and 'Regina' sweet cherry fruit.

	Cell no. per radial section					
	2003			2004		
Cultivar	Bloom[z]	Pit harden	Harvest	Bloom	Pit harden	Harvest
Selah	23.5a	70.3a	83.2a	28.4a	76.2a	78.8a
EF	17.0b	40.2b	47.4b	24.0bc	38.2c	41.4c
NY54	24.7a	26.0c	26.7c	23.4c	29.6d	28.6d
Bing	--	--	--	25.8abc	45.8b	48.3b
Regina	--	--	--	27.6ab	43.4b	43.8bc

zMean separation within columns by Tukey's HSD at $P < 0.05$.

Figure 6. Comparison of mesocarp cell number increase for 'NY 54', 'Emperor Francis' ('EF'), 'Bing', 'Regina', and 'Selah' sweet cherry fruit from WSU-IAREC from bloom to endocarp hardening. Sampling in 2004 (A) was on a weekly basis, while 2005 (B) at 1-2 day intervals. Growing degree day accumulation (4.4 C base) from the beginning of the sampling period is indicated on the lower x-axis. Analysis was discontinued once the measured number of cells equaled that of the harvest sample. Due to late spring freeze in 2005, no 'EF' fruit were available for sampling.

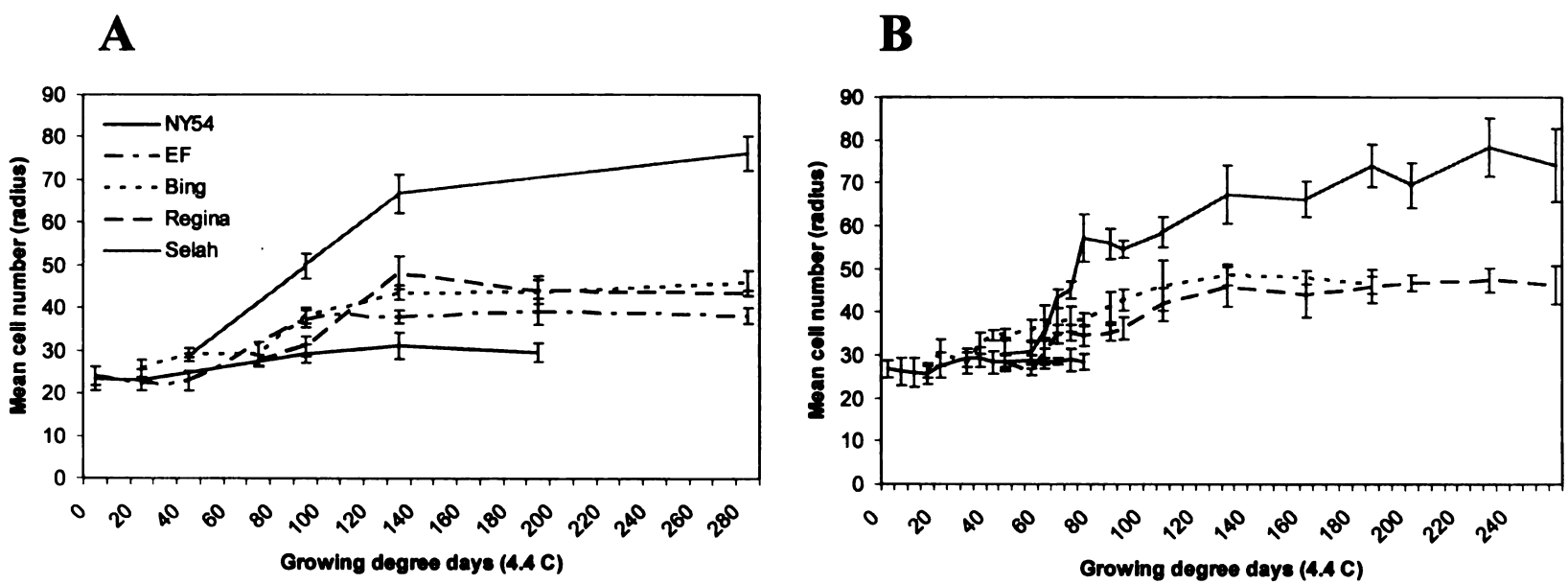

Figure 7. Comparison of mesocarp cell number increase for 'NY 54', 'Emperor Francis' ('EF'), 'Bing' and 'Regina' sweet cherry fruit from MSU-CHES from bloom to endocarp hardening. Sampling in 2004 (A) and 2005 (B) was at 3-5 day intervals. Growing degree day accumulation (4.4 C base) from the beginning of the sampling period is indicated on the lower x-axis. Analysis was discontinued once the measured number of cells equaled that of the harvest sample.

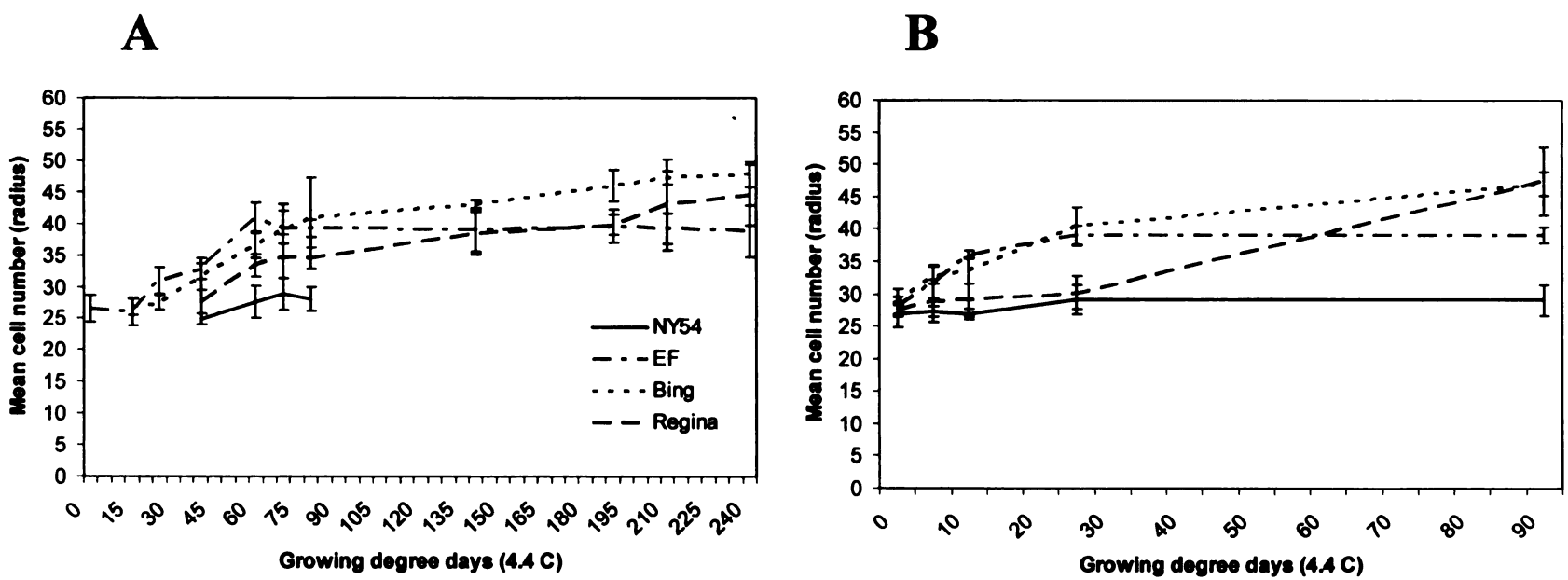

Table 5. Comparison of the duration and rate of cell division between 'Bing', 'Regina', and 'Selah' fruit from the period between bloom and endocarp hardening at WSU-IAREC in 2005. The rate of cell division was calculated by dividing the increase in the number of cells from bloom by the total accumulation of growing degree days [GDD (4.4 C base)] from the point when bloom for that cultivar occurred.

Cultivar	Mean initial radial cell no. at bloom	Mean radial cell no. increase after bloom	GDD accumulation between bloom and max. radial cell no.	Post-bloom cell division rate[z] (no./GDD)
Bing	26.2	21.2	75	0.28a
Regina	28.4	16.8	59	0.29a
Selah	30.4	48.2	139	0.35a

[z]Mean separation within column by Tukey's HSD at $P < 0.05$.

CHAPTER THREE

Construction of a genetic linkage map for the 'NY 54' × 'Emperor Francis' sweet cherry (*Prunus avium* L.) population

ABSTRACT

Genetic linkage maps were constructed from reciprocal crosses between the sweet cherry (*Prunus avium* L.) (*2n* = 16) cultivars New York 54 ('NY 54') and Emperor Francis ('EF'). The linkage maps consist of 8 linkage groups (LG) for the 'EF' parent and 10 LG for the 'NY 54' parent. The linkage maps for the two parents are 479.1 cM and 308.9 cM for 'EF' and 'NY 54', respectively, and consist of 40 SSR, 47 AFLP, 3 SRAP, and 1 morphological marker. The average distance between marker loci is 7 cM for 'EF' and 8 cM for 'NY 54'. The largest gaps in the maps are 29 cM for 'EF' and 34 cM for 'NY 54'. A total of 24% of the 1:1 markers exhibited distorted segregation ratios ($P < 0.05$), many of which were linked together on the 'EF' LG 6. A comparison of the 'NY 54' × 'EF' reciprocal crosses revealed that distorted marker segregation occurred only when 'EF' was used as the paternal parent, presumably resulting from gametophytic selection. Fourteen of the sweet cherry linkage groups could be aligned with the reference *Prunus* map, 'Texas' ('T') almond × 'Earlygold' ('E') peach, based on shared SSR markers.

INTRODUCTION

Sweet cherry is a diploid ($2n = 16$) member of the genus *Prunus*, which contains many of the economically important tree fruit and nut crops including peach [*Prunus persica* (L.) Batsch], almond [*P. dulcis* (Miller) D.A. Webb], sour cherry (*P. cerasus* L.), plum (*P. salicina* Lindl.), and apricot (*P. armeniaca* L.). A gametophytic self-incompatiblity (GSI) system is present in sweet cherry, typically preventing self-fertilization and promoting outcrossing (de Nettancourt, 1971). The GSI system combined with long juvenility periods and large space requirements significantly reduce progeny numbers that can be evaluated in a given time period in sweet cherry breeding programs. Therefore, marker-assisted selection (MAS) for both qualitative and quantitative traits, particularly those involved in fruit characteristics, holds great promise for increasing the efficiency of sweet cherry breeding programs.

Currently, self-compatibility, controlled by a self-fertile allele at the *S*-locus, is the only sweet cherry trait for which selection is routine using MAS (Dirlewanger et al., 2004a). PCR-based primers that amplify multiple or specific *S-RNase* and *SFB* alleles at this locus (Sonneveld et al., 2001; Tao et al., 1999; Yamane et al., 2001) provide an efficient method of compatible parental selection and identification of progeny allelic constitution years prior to, and at considerably less expense than, controlled crossing studies in field situations. The paucity of additional candidates for MAS results in part from the lack of suitable populations segregating for traits of interest that can be used for either genetic linkage map development or bulked segregant analysis (Michelmore, 1991). Additionally, GSI and long generation times of sweet cherry necessitate F_1 mapping populations between presumably heterozygous individuals, a mapping

configuration that theoretically limits the ability to identify quantitative trait loci (QTL) because of the heterozygous background (Conner et al., 1998; Grattapaglia and Sederoff, 1994; Wang et al., 2000).

The status of genetic linkage map development in sweet cherry currently lags behind other important *Prunus* crops. Stockinger et al. (1996) developed a randomly amplified polymorphic DNA (RAPD) marker-based linkage map of a microspore-derived callus culture population. However, because of the marker system, this map is not comparable with other *Prunus* linkage maps, and phenotypic analysis of horticulturally important traits was not possible. Isozyme-based interspecific maps of sweet cherry × *Prunus incisa* Thunb. ex Murr. and sweet cherry × *Prunus nipponica* Matsum. were reported by Boskovic et al. (1997, 1998), but only 27 markers were placed on the combined map, a marker density far below that needed for QTL studies. Dirlewanger et al. (2004a) constructed a linkage map from a 'Regina' × 'Lapins' sweet cherry cross consisting of *Prunus* simple sequence repeat (SSR) markers suitable for comparative mapping within *Prunus* species. However, the marker density and coverage was insufficient for QTL analyses.

In *Prunus*, interspecific hybridization between peach and almond has been used effectively to generate marker diversity to facilitate the construction of highly saturated linkage maps. The reference *Prunus* map is from the 'Texas' ('T') almond × 'Earlygold' ('E') peach cross, and consists of 562 markers covering 519 cM over the expected 8 linkage groups with a 0.92 cM average marker density (Dirlewanger et al., 2004a). However, the use of interspecific crosses in this and other *Prunus* linkage maps has resulted in up to 46% of the markers exhibiting distorted segregation ratios (Bliss et al.,

2002; Foolad et al., 1995; Joobeur et al., 1998). A common region of linkage distortion is around the GSI locus (*S*), resulting from gametophytic selection (Bliss et al., 2002; Foulongne et al., 2003; Joobeur et al., 1998; Lambert et al., 2004; Vilanova et al., 2003). Similarly, the 'Regina' × 'Lapins' cross is partially incompatible, $S_1 S_3 \times S_1 S_4'$, thereby limiting the analysis for the *S*-locus region to meiotic products from 'Regina' as all the progeny would have the S_4' allele from 'Lapins'. Therefore, the ideal sweet cherry mapping population would be an intraspecific, fully compatible cross where the progeny segregate for many fruit and tree traits of interest. SSR markers already placed on existing *Prunus* linkage maps would be the most informative marker system, taking advantage of potential codominance, and collinearity of the *Prunus* genome (Dirlewanger et al., 2004a).

The objective of this study was to construct a sweet cherry genetic linkage map from the reciprocal crosses between 'NY 54' and 'EF' which would be comparable to previously generated *Prunus* linkage maps as well as suitable for future QTL mapping experiments.

MATERIALS AND METHODS

Plant material

Images in this dissertation are presented in color. The sweet cherry population used for this study was developed from reciprocal crosses of 'NY 54' and 'EF' (Fig. 1). 'NY 54' was selected from wild *P. avium* forests in Germany and introduced at the New York State Agricultural Experiment Station, Cornell University (R.L. Andersen, pers. comm.). 'EF' is a cultivated sweet cherry of unknown origin, grown primarily for

processed cherry products. In 2001, pollen was collected from 'NY 54' and 'EF' trees in the National Research Support Project 5 (NRSP5) collection in Prosser, Wash. 'NY 54' was used as a maternal parent in Washington State, and pollen was transported to Michigan for use in reciprocal crosses with 'EF' as the maternal parent. From the crosses, 617 F_1 individuals were planted at Michigan State University's Clarksville Horticultural Experiment Station (MSU-CHES) in Clarksville, Michigan in the spring of 2002. The seedlings were planted at 1.5 m and 6.1 m within and between row spacing, respectively. Standard orchard management practices (irrigation, fertilization, and pest and disease control) for MSU-CHES were followed.

From the total population, a linkage mapping subset of 190 individuals was selected. This subset consisted of 86 individuals from the 'NY 54' × 'EF' reciprocal cross, 103 individuals from the 'EF' × 'NY 54' reciprocal cross, and one individual with no reciprocal cross information. Approximately equal numbers of progeny from each of the four *S*-haplotype groups (48, S_2S_3; 49, S_2S_4; 47, S_3S_6; 46, S_4S_6) were included in the mapping population. These four *S*-haplotype groups were shown previously to segregate according to the expected 1:1:1:1 ratio (Ikeda et al., 2005).

DNA isolation and marker analyses

For DNA extraction, young, unfolded leaves from the parents and each progeny individual were collected, placed immediately on dry ice, transported to the laboratory, and placed directly in a -80°C freezer for at least 24 h. Leaves from each individual were then lyophilized for 48 h and stored long-term at -20°C. DNA isolation was done using the CTAB method described by Stockinger et al. (1996).

Genotypic data for *S*-allele segregation was published previously by Ikeda et al. (2005). Briefly, 'EF', 'NY54', and all progeny were genotyped for their *S-RNase* alleles using the *S-RNase* gene specific PCR primer pair, Pru-C2 and PCE-R (Tao et al., 1999; Yamane et al., 2001). Because the S_2-*RNase*-specific fragment is not clearly amplified with the PruC2/PCE-R combination, the S_2 allele specific PCR primer pair, PaS2-F and PaS2-R was used for confirmation of S_2 presence (Sonneveld et al., 2001). Reaction mixtures, PCR conditions, and PCR product visualization were as described by Ikeda et al. (2005).

SSR markers developed from several *Prunus* species were used in the development of the 'NY 54' × 'EF' linkage map (Table 1). The SSR markers used in these analyses were derived from peach ("BPPCT", Dirlewanger et al., 2002; "CPPCT", Aranzana et al., 2002; "UDP", Cipriani et al., 1999; "MA", Yamamoto et al., 2002; and "Prp", Silva et al., 2005), sweet cherry ("EMPA", Clarke and Tobutt, 2003; "EMPaS", Vaughan and Russell, 2004; and "PMS", Struss et al., 2002), sour cherry ("Pce", Struss et al., 2002; and "PS", Sosinski et al., 2000), almond ("CPDCT", Mnejja et al., 2005; and "EPDCU", P. Arus, pers. comm.), and plum ("CPSCT", Mnejja et al., 2004). A similar temperature profile, other than annealing temperature, was used for all PCR reactions: 94°C for 5 min, 35 cycles of 94°C (45 sec), X°C (45 sec), 72°C (90 sec), and a final extension step of 72°C for 5 min, where X = the published optimum annealing temperature for each primer. For "EMPA" and "EMPaS" primers, a touchdown PCR temperature profile was used as described by Clarke and Tobutt (2003). The reaction mixture contained 1× PCR buffer, 2.5 mM $MgCl_2$, 120 μM of each dNTP, 2.5 pmol of each primer, 50 ng of genomic DNA and 0.3 U *Taq* polymerase (Invitrogen Corporation,

Carlsbad, Calif.) in a 12.5 µl reaction. PCR reactions were run in a MJ Research PTC 100 Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, Calif.). PCR reactions were stored at 4°C until use.

Amplified fragment length polymorphism (AFLP) analysis consisting of genomic DNA digestion with *Eco*RI and *Mse*I restriction enzymes, adapter ligation, pre-amplification, and selective amplification were similar to Vos et al. (1995), with the following modifications described by Hazen et al. (2002). Pre-amplification of 2 µl of restriction ligation genomic DNA product was combined with 25 ng each of *Eco*RI + A and *Mse*I + C oligonucleotides, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM of each dNTP, and 0.5 U *Taq* polymerase (Promega Corporation, Madison, Wisc.) in 20 µl total volume and amplified in a MJ Research PTC 100 Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, Calif.). The temperature profile used for pre-amplification was 94°C for 2 min, 26 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and a final extension step of 72°C for 5 min. The pre-amplification PCR product was diluted 6× with sterile water. Selective amplification used 1µl of the diluted pre-amplification product with 25 ng of *Eco*RI + AN primer, 30 ng of *Mse*I + CNN primer, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.4 U *Taq* polymerase (Promega Corporation, Madison, Wisc.) in 20 µl total volume and amplified in a MJ Research PTC 100 Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, Calif.). The temperature profile used for selective amplification was 94°C for 2 min, 12 cycles of 94°C for 30 sec, 65°C for 30 sec, 72°C for 1 min, 23 cycles of 94°C for 30 sec, 56°C for 30 sec, 72°C for 1 min, and a final extension step of 72°C for 2 min. Dinucleotide *Eco*RI + AN, and trinucleotide *Mse*I + CNN selective amplification primers were used as the best compromise between number

of polymorphic bands per primer combination and ease and reliability of scoring (Lu et al., 1998).

Sequence related amplified polymorphism (SRAP) primer combinations me1/em and me1/em2 were used as reported by Li and Quiros (2001) with the following PCR modifications. The reaction mixture contained 1× PCR buffer, 2.5 mM $MgCl_2$, 120 μM of each dNTP, 2.5 pmol of each primer, 50 ng of genomic DNA and 0.3 U *Taq* polymerase (Invitrogen Corporation, Carlsbad, Calif.) in a 12.5 μl reaction. The temperature profile used was 94°C for 2 min 5 cycles of 94°C for 45 sec, 35°C for 45 sec, 72°C for 1 min, 35 cycles of 94°C for 45 sec, 50°C for 45 sec, 72°C for 1 min, and a final extension step of 72°C for 7 min. PCR reactions were run in a MJ Research PTC 100 Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, Calif.).

Fragment visualization was the same for SSR, AFLP, and SRAP markers. After the addition of 4 μl formamide/dye solution, the PCR products were denatured at 94°C for five min. The PCR products were visualized by electrophoresis on a 6% denaturing polyacrylamide gel in a 50 cm Sequi-Gen GT vertical sequencing apparatus (Bio-Rad Laboratories, Hercules, Calif.) for 2.5 h at 70 W with 1× TBE buffer. Following electrophoresis, the gels were stained with the Silver Sequence DNA Sequencing System (Promega Corporation, Madison, Wisc.) and dried for 24 h. DNA fragment sizes were scored visually using 10 and 50 base pair ladders (Invitrogen Corporation, Carlsbad, Calif.).

Chi square analysis and linkage map construction

'NY 54', 'EF', and a total of 190 progeny were genotyped using 50 SSR markers, 8 AFLP primer combinations, and 2 SRAP primer combinations. Both dominant and codominant SSR markers were genotyped in the 'NY 54' × 'EF' population. All AFLP and SRAP fragments scored were dominant. For ease and reproducibility of scoring, all markers were scored initially as dominant fragments whereby individual alleles for codominant SSR markers were scored separately. Segregating fragments present in one parent and absent in the other parent were tested to fit a 1:1 ratio, while segregating fragments present in both parents were tested to fit a 3:1 ratio. Chi square goodness-of-fit tests were performed using functions in Excel 2002 (Microsoft Corp., Redmond, Wash.). Linkage analysis was performed with JoinMap 3.0 (Van Ooijen, and Voorrips, 2001), using a minimum LOD score of 3.0 and a maximum recombination fraction of 0.4. Linkage groups were constructed using MapChart 2.1 (Voorrips, 2002), with distances presented in cM calculated by the Kosambi (1944) function.

RESULTS

Marker segregation

A total of 116 SSRs from various sources were screened for amplification and segregation in 190 progeny from the 'NY 54' × 'EF' reciprocal populations (Table 1). Of these SSRs, 66 (57%) either did not amplify or did not segregate in this population. Six of the surveyed SSR markers (5%) resulted in no amplification product, while 60 (52%) did not segregate in the 'NY 54' × 'EF' population. However, this high number of monomorphic markers cannot be attributed solely to the use of primers developed in

other *Prunus* species, as a similar percentage (45%) of the SSR markers derived from *P. avium* genomic libraries did not segregate. The remaining 50 (43%) SSRs were used to genotype the progeny populations (Fig. 2). One of these SSRs (BPPCT021) was removed from the analysis because the complex banding pattern was difficult to interpret. For AFLP markers, *Eco*RI dinucleotide and *Mse*I trinucleotide selective primers were used. Results from Lu et al. (1998) suggested that this configuration offered the best compromise between the number of polymorphic fragments produced and ease of scoring. From eight different *Eco*RI dinucleotide and *Mse*I trinucleotide AFLP selective primer combinations, a total of 61 polymorphic fragments were generated (Table 2). The number of fragments identified varied from one (*Eco*RI + AA, *Mse*I +CAA) to 17 (*Eco*RI + AT, *Mse*I +CTC), with an average of eight polymorphic fragments per primer combination (Fig. 3). This average fragment number is similar to the average (6.8) reported for similar combinations in peach (Lu et al., 1998). Two SRAP primer combinations were used during the development of the linkage map, generating seven polymorphic loci. Fifteen (31%), 13 (21%), and four (57%) of the SSR, AFLP, and SRAP markers, respectively, deviated significantly ($P < 0.05$) from the expected F_1 segregation ratio. However, these markers were used in the initial linkage analysis.

'NY 54' exhibited less heterozygosity than 'EF' for SSR, AFLP, and SRAP markers. Only 42% of SSR and AFLP markers used to genotype the population segregated for alleles from 'NY 54' (Table 3).

Linkage map construction

An F_1 pseudo-testcross mapping strategy was used to develop linkage maps for both parents, 'NY 54' × 'EF'. A total of 8 LG were constructed for 'EF' and 10 LG were constructed for 'NY 54' (Fig. 4). Forty (82%) SSRs were placed on the linkage map, while nine (18%) remained unlinked (Table 1). Forty-seven AFLP fragments (76%) were placed on the linkage map, while 14 (24%) remained unlinked (Table 2). Three of the SRAP markers (43%) were placed on the linkage map. The 'NY 54' × 'EF' linkage map consists of a total of 91 markers; 40 SSR, 47 AFLP, and 3 SRAP markers and one morphological (*S*) locus. Fifty-three dominant markers segregate on the 'EF' parental map, while 23 segregate on the 'NY 54' parental map (Tables 3 and 4). Fifteen codominant markers appear on both parental maps. Ten SSR, 10 AFLP, and 2 SRAP markers placed on the linkage map deviated significantly from the expected segregation ratio. The total cM coverage is 479.1 cM and 308.9 cM for the 'EF' and 'NY 54' parents, respectively (Tables 3 and 4). The average distance between markers is 7 and 8 cM for 'EF' and 'NY 54', respectively. The largest gaps in the linkage map are 29 cM for 'EF' and 34 cM for 'NY 54'.

Based on shared markers, homology between 12 of the 18 'NY 54' and 'EF' linkage groups could be assigned. The use of SSR markers previously placed on the 'T' × 'E' reference *Prunus* map allowed tentative homology between the maps to be established. At least one SSR marker on 14 of the linkage groups generated in this study was located on the 'T' × 'E' map. These 14 linkage groups have been assigned group numbers according to the 'T' × 'E' terminology (Fig. 4). Four linkage groups consisting entirely of AFLP markers currently cannot be compared with the 'T' × 'E' map.

A large region (~ 50 cM) of LG 6 from the 'EF' parent consisted of linked markers exhibiting distorted segregation ratios (Fig. 4). When chi-square goodness-of-fit tests were used to test for deviation from the expected segregation ratios within each reciprocal cross, segregation distortion was only evident when 'EF' was used as the pollen donor (Table 5).

DISCUSSION

The parents for this cross were selected for several reasons. 'NY 54' is a wild *P. avium* selection, cultivated only for seedling rootstock production, while 'EF' is a domesticated and cultivated variety. Therefore, although this cross is intraspecific, it is a cross between a wild relative and a cultivated variety, presumably maximizing the available heterozygosity in *P. avium*. Since many potentially desirable traits are often found in wild relatives (Tanksley and McCouch, 1997), future QTL studies using this population may identify alleles not currently represented in the cultivated germplasm of sweet cherry. Furthermore, 'NY 54' and 'EF' differed in many important fruit size and quality characters, and identification of QTL for these traits may provide insight into the mechanism of domestication of sweet cherry. Finally, the presumed heterozygosity of the two parents was predicted to maximize the SSR loci available for linkage mapping. The other published intraspecific sweet cherry linkage map, a cross of 'Regina' × 'Lapins' (Dirlewanger et al., 2004a), two cultivated varieties, was presumed to have lower overall heterozygosity.

'EF' is an extremely important cultivar in the history of sweet cherry improvement. It was the maternal parent in crosses with irradiated 'Napoleon' pollen

that resulted in the self-fertile selection John Innes 2420 (Lewis and Crowe, 1954). Because self-fertility is a highly desirable production trait, progeny from this original cross have been heavily used in sweet cherry breeding programs. Therefore, both 'EF' and 'Napoleon' have contributed to all current self-compatible cultivars, and 'EF' has contributed to 20% of the self-incompatible cultivars grown in North America (Choi and Kappel, 2004). Because of the long juvenility period, only four to five generations have been developed in the most advanced breeding programs since the introduction of self-fertility (Kappel and Lay, 1997). The frequent appearance of 'EF' in modern sweet cherry pedigrees, and the potential for continued marker-trait linkages due to a limited number of meioses, suggest that marker and QTL alleles identified in this mapping population would be informative even for current cultivars.

The size of the diploid *Prunus* reference map is 519 cM (Dirlewanger et al., 2004a). The cM length for the 'EF' map was 479.1 cM, therefore approximating the expected size. Despite the ~ 500 cM length for the 'EF' linkage map, it is incomplete, with only six of the eight potential linkage groups identified. Although incomplete, the total cM distance of the 'EF' map is only 40 cM less than the coverage of the 'T' × 'E' map (519 cM), and in the lower end of the 393 to 1,144 cM range of map distances reported for diploid *Prunus* (Bliss et al., 2002; Chaparro et al., 1994; Dettori et al., 2001; Dirlewanger et al., 1998, 2004a; Foolad et al., 1995; Hurtado et al., 2002; Joobeur et al., 1998; Lambert et al., 2004; Vilanova et al., 2003; Viruel et al., 1995). The current distance is consistent with the predicted genome size of sweet cherry, which is slightly larger than peach (6.6×10^8 vs. 5.3×10^8) (Dickson et al., 1992), and two linkage groups have yet to be identified. Unfortunately, the 'NY 54' map was only 309.8 cM, as it

exhibited less heterozygosity than 'EF' for the SSR loci. With a framework map constructed, future marker selection will be targeted to reduce current gaps in the linkage maps.

SSR markers were chosen as a framework for the 'NY 54' × 'EF' map, and higher throughput marker systems, such as AFLP, were used to increase the map marker density. The transferability of markers and collinearity of genomes between *Prunus* species suggested that the use of SSR markers developed for other *Prunus* species would be successful in sweet cherry (Cantini et al., 2001; Cipriani et al., 1999; Clarke and Tobutt, 2003; Dirlewanger et al., 2002, 2004a; Downey and Iezzoni, 2000; Hormaza, 2002; Messina et al., 2004; Mnejja et al., 2004, 2005; Schueler et al., 2003; Sosinski et al., 2000; Struss et al., 2003 Vilanova et al., 2003; Wang et al., 2002; Wunsch and Hormaza, 2002; Yamamoto et al., 2002). As predicted, only 5% of the surveyed SSR markers resulted in no amplification product and a high number of polymorphic fragments per AFLP primer combination were generated by using *Eco*RI dinucleotide and *Mse*I trinucleotide selective primers.

A high percentage of distorted segregation ratios have been reported previously in *Prunus* genetic linkage maps. This is particularly apparent in the linkage maps developed from interspecific peach × almond crosses (Bliss et al., 2002; Foolad et al., 1995; Joobeur et al., 1998), where the percent of loci exhibiting distorted segregation ratios range from 37% to 46%. Similarly, interspecific hybrids of peach × *P. davidiana* (Foulongne et al., 2003) and the three-way cross of Myrobalan plum (*P. cerasifera* Ehrh) × (almond × peach) (Dirlewanger et al., 2004b) exhibited high percentages of distorted marker segregation ratios, 30% and 42%, respectively. Reported distorted segregation ratios

have been lower in intraspecific crosses; 15% to 18.5% in peach (Lu et al., 1998; Dettori et al., 2001), 10% in almond (Joobeur et al., 2000), and 11% to 14% in apricot (Hurtado et al., 2002; Vilanova et al., 2003). In the present study, 24% of all markers deviated from the expected segregation ratio (Fig. 4). Both SSR and AFLP marker types had a similar percentage of markers exhibiting distorted segregation.

The high percentage of markers exhibiting distorted segregation ratios has been attributed to the interspecific nature of many of the *Prunus* crosses, and the presence of gametophytic self-incompatibility (Bliss et al., 2002; Foulongne et al., 2003; Joobeur et al., 1998; Lambert et al., 2004; Vilanova et al., 2003). Therefore, an intraspecific, fully compatible population for sweet cherry linkage mapping was a priority, as the importance of the *S*-locus region for fruit quality traits has been highlighted in several QTL studies in *Prunus* (Dirlewanger et al., 1999; Etienne et al., 2002; Quilot et al., 2005; Yamamoto et al., 2001). 'NY 54' (S_2S_6) and 'EF' (S_3S_4) are both self-incompatible cultivars, but the cross between the two cultivars is fully compatible. Conversely, the 'Regina' × 'Lapins' sweet cherry mapping population (Dirlewanger et al., 2004a) is only partially compatible, resulting in the potential loss of genomic regions linked to the S_1 haplotype of the 'Lapins' *S* locus. Analysis of progeny segregation for the four potential *S*-haplotype genotypic classes in the 'NY 54' × 'EF' population by Ikeda et al. (2005) confirmed the cross-compatibility. However, when 'NY 54' was used as the pollen parent there were a significant excess of individuals with the S_2 allele from 'NY 54', and a deficit of individuals with the S_6 allele from 'NY 54' (Ikeda et al., 2005). The apparent competitive advantage of the S_2 was not indicated by distorted segregation for the allele-

specific marker for that allele in this mapping population because the subset of progeny used for mapping were comprised of equal number from each *S*-haplotype group.

Although the 'NY 54' × 'EF' population reported here did not show distorted segregation ratios at or around the *S* locus, a significant portion (~ 50 cM) of LG 6 from the 'EF' parent consisted of linked markers exhibiting distorted segregation ratios (Fig. 4). Interestingly, a similar location of aberrant segregation ratios has been documented in two previous studies. Dirlewanger et al. (2004b) identified a group of linked markers exhibiting distorted segregation ratios in F_1 progeny of the three-way cross of Myrobalan plum × (almond × peach). In the interspecific parent, distorted markers were located throughout the linkage group corresponding to LG 6 of the 'T' × 'E' map. Map coverage was not as complete for the Myrobalan plum parent, but distorted loci corresponding to the central region of the homologous linkage group in that cross were also present. In that study, distorted segregation was attributed to meiotic problems due to the interspecific nature of the parent. Foulongne et al. (2003) identified loci in the same location on LG 6 with distorted segregation ratios in an F_2 population generated from a peach × *P. davidiana* interspecific cross. An excess of the peach alleles were present around two loci, UDP98-412 and UDP96-001. Both of these SSR loci also were identified as distorted in the Myrobalan plum × (almond × peach) map reported by Dirlewanger et al. (2004b). In the 'NY 54' × 'EF' sweet cherry population, UDP98-412 was not polymorphic, but it is located one to three cM from the *S* locus on the 'T' × 'E' linkage map (Dirlewanger et al., 2004a). Gametophytic selection near the UDP98-412 locus was attributed to self-incompatibility in the *P. davidiana* parent in the peach × *P. davidiana* interspecific cross (Foulongne et al., 2003). As expected, no distorted loci

were linked to the *S* locus in the 'NY 54' × 'EF' sweet cherry population because it is fully compatible. UDP96-001 segregates in both 'NY 54' and 'EF' and was located on the opposite end of the linkage group as the *S* locus in each of these linkage maps. The male sterility locus for peach was located 5.5 cM from UDP96-001 in the 'T' × 'E' linkage map (Dirlewanger et al., 2004a). Segregation at this locus may contribute to distorted segregation ratios in an F_2 population as reported by Foulongne et al. (2003), but this would not explain the distorted segregation ratios that occurred in the F_1 'NY 54' × 'EF' population reported in this study.

Because the 'NY 54' × 'EF' population consists of reciprocal crosses, we were able to examine the influence of gamete sources from each parent on the observed marker distortion. Segregation distortion was only evident when 'EF' was used as the pollen donor (Table 5). This observation is similar to that described by Foulongne et al. (2003), in which gametophytic selection causing distorted segregation in a peach × *P. davidiana* F_2 population was assumed to occur only among male gametes. Since the 'NY 54' × 'EF' cross is intraspecific, the cause of pollen gametophytic selection cannot be attributed to homologous chromosome pairing inconsistencies during interspecific hybridization. These data, and the repeated observation of distorted segregation in this area of the *Prunus* genome, suggests a genetic influence for the reduced fitness of pollen gametes. The linkage of the male-sterility (*Ps*) locus to markers exhibiting distorted segregation is compelling. Although male sterility has not been documented in sweet cherry and cannot fully explain the segregation distortion observed in the 'NY 54' × 'EF' cross, allele combinations at the locus may influence pollen fitness. Alternatively, this genomic

region may be important in meiosis and gamete formation, and other dysfunctional and sub-lethal factors may be present.

CONCLUSIONS

This first-generation genetic linkage map developed from the reciprocal cross between 'NY 54' and 'EF' in this study provides a good starting point for future QTL analyses. The parents were selected to maximize available *P. avium* heterozygosity for important traits such as fruit size and color, while avoiding linkage distortion problems identified in previous *Prunus* maps. The use of SSR markers common to other *Prunus* maps allow for between species comparisons previously unavailable for sweet cherry. Placement of additional SSR markers, ongoing at this time, will continue to refine and establish collinearity between sweet cherry and other *Prunus* species.

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TABLES AND FIGURES

Figure 1. (A) Image of mature fruit from 'Emperor Francis' ('EF') (left) and 'NY 54' (right) sweet cherry, illustrating size variation between the two cultivars. (B) Selected progeny from the 'EF' × 'NY 54' sweet cherry linkage mapping population illustrating variation for fruit characteristics.

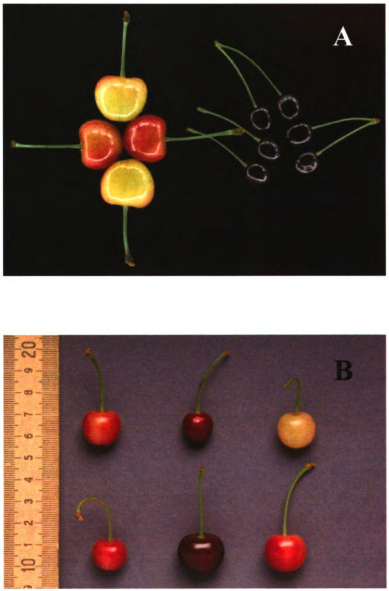


Table 1. Origins of simple sequence repeat (SSR) markers used in the development of the 'NY 54' × 'Emperor Francis' sweet cherry genetic linkage map.

Marker terminology	*Prunus* species	Number of SSRs tested	Number of SSRs mapped	Reference
BPPCT	*P. persica*	17	6	Dirlewanger et al., 2002
CPDCT	*P. dulcis*	9	4	Mnejja et al., 2005
CPPCT	*P. persica*	18	5	Aranzana et al., 2002
CPSCT	*P. salicina*	3	1	Mnejja et al., 2004
EMPA	*P. avium*	7	3	Clarke and Tobutt, 2003
EMPaS	*P. avium*	7	4	Vaughan and Russell, 2004
EPDCU	*P. dulcis*	3	2	P. Arus (pers. comm.)
MA	*P. persica*	2	1	Yamamoto et al., 2002
Pce	*P. cerasus*	6	2	Struss et al., 2002
Pch	*P. persica*	5	0	Sosinski et al., 2000
PMS	*P. avium*	8	4	Struss et al., 2002
Prp	*P. persica*	2	1	Silva et al., 2005
PS	*P. cerasus*	6	2	Sosinski et al., 2000
UDP	*P. persica*	23	5	Cipriani et al., 1999

Figure 2. Co-dominant simple sequence repeat (SSR) fragments obtained with primers for the CPDCT022 marker. From left to right: (1) 50 base pair sizing ladder, (2) 10 base pair sizing ladder, (3) 'NY 54', (4) 'Emperor Francis', (5-24) progeny. Arrows indicate segregating fragments.

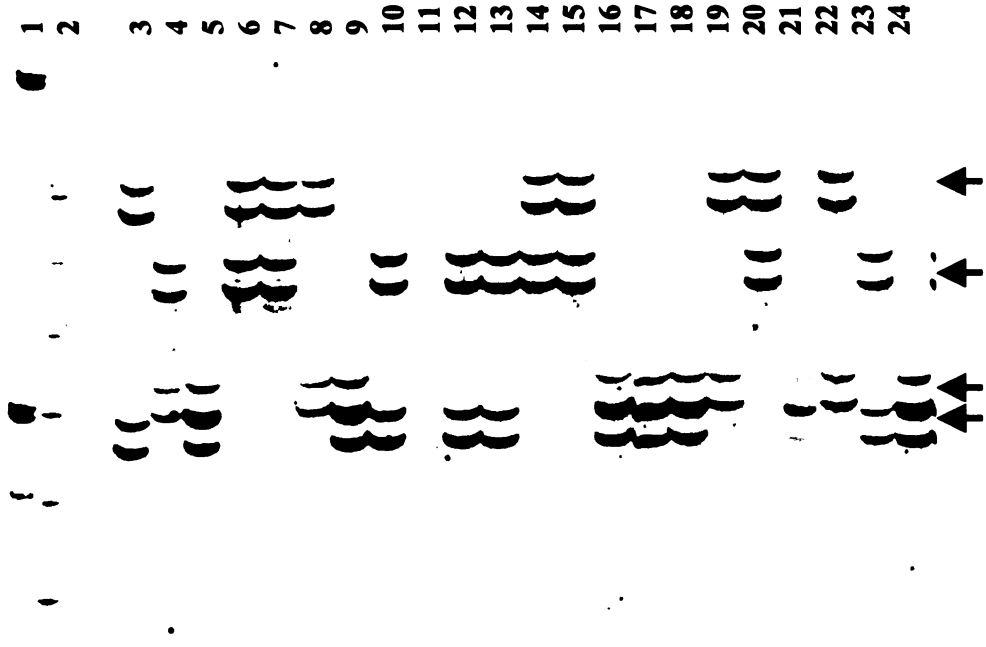


Table 2. Enzymes used for digest, selective nucleotide combinations used as primers, number of polymorphic fragments, and number of mapped fragments generated by amplified fragment length polymorphism (AFLP) analysis in the development of the 'NY 54' × 'Emperor Francis' sweet cherry genetic linkage map.

*Eco*RI	*Mse*I	Number of polymorphic fragments	Number of mapped fragments
EAA	CTT	6	5
EAA	CAC	8	7
EAA	CCC	6	4
EAA	CCT	3	1
EAA	CAA	1	0
EAT	CTC	17	12
EAT	CCC	9	7
EAC	CTA	11	11

Figure 3. Amplified fragment length polymorphism (AFLP) fragments obtained with the selective primers *Eco*RI+AC and *Mse*I +CTA. From left to right: (1) 10 base pair sizing ladder, (2) 50 base pair sizing ladder, (3) 'NY 54', (4) 'Emperor Francis', (5-29) progeny. Arrows indicate segregating fragments.

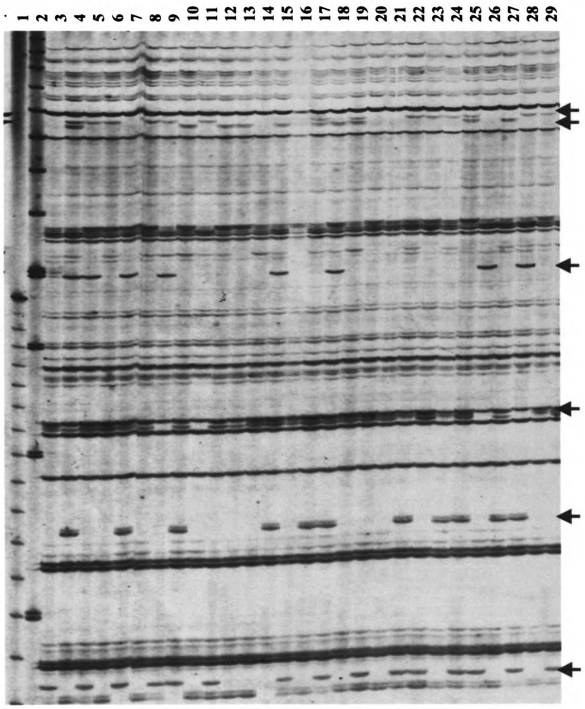


Figure 4. 'Emperor Francis' ('EF') and 'NY 54' sweet cherry genetic linkage maps. Distance in cM is given to the left of each linkage group. Marker names are on the right, with the scored fragment size after the dash. Amplified fragment length polymorphism (AFLP) combinations are named using the *Eco*RI / *Mse*I selective primers. Linkage groups corresponding to the 'T' × 'E' reference *Prunus* linkage map are numbered; letters designate groups not aligned with the reference map. Anchor loci between the parental linkage groups are connected by a solid line. Distorted loci are indicated by * and ** at $P < 0.05$ and $P < 0.01$, respectively.

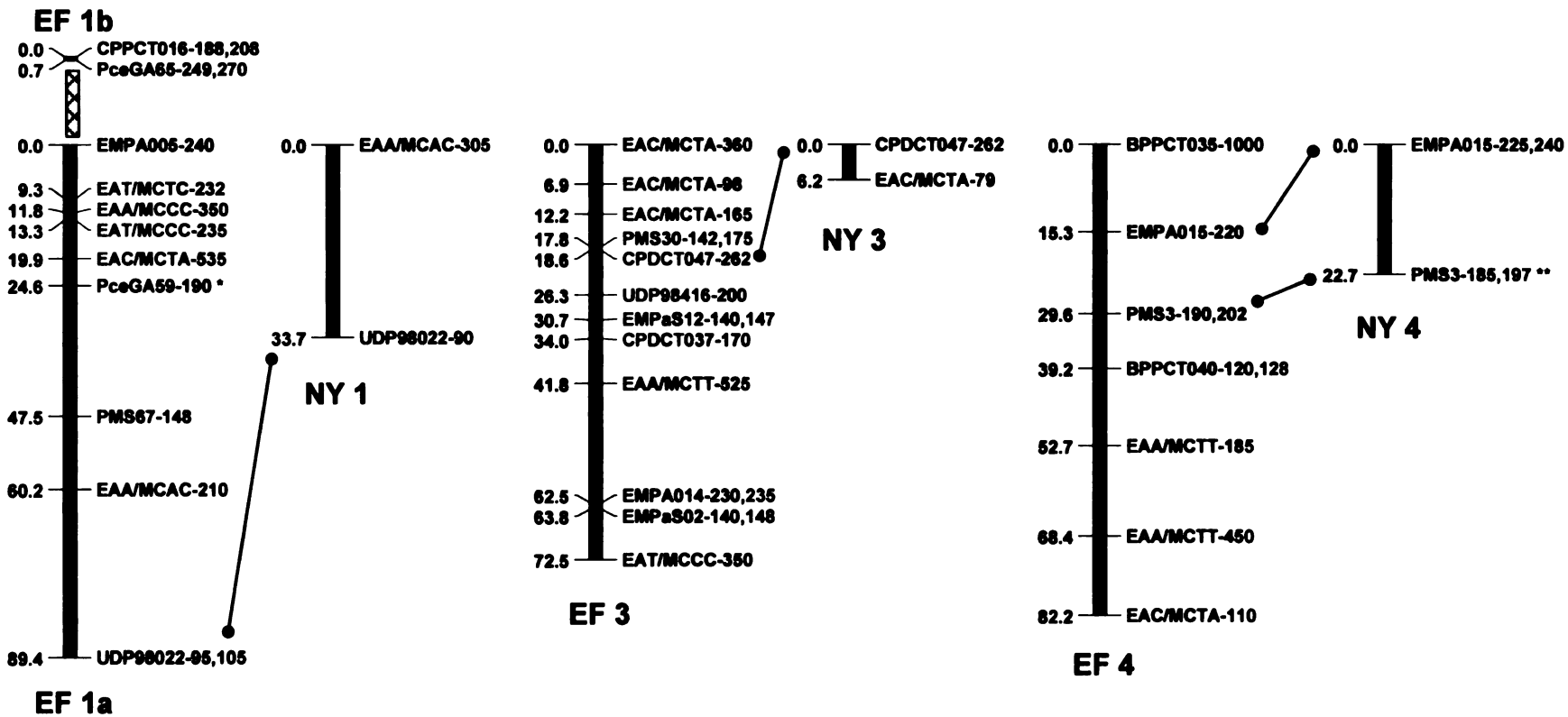
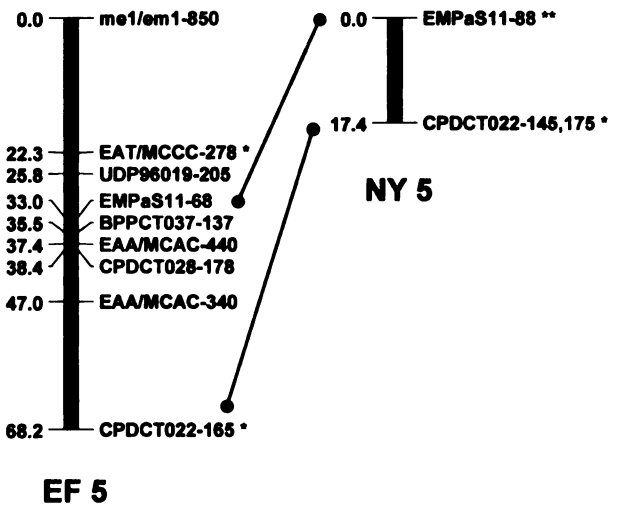


Figure 2. (cont'd)



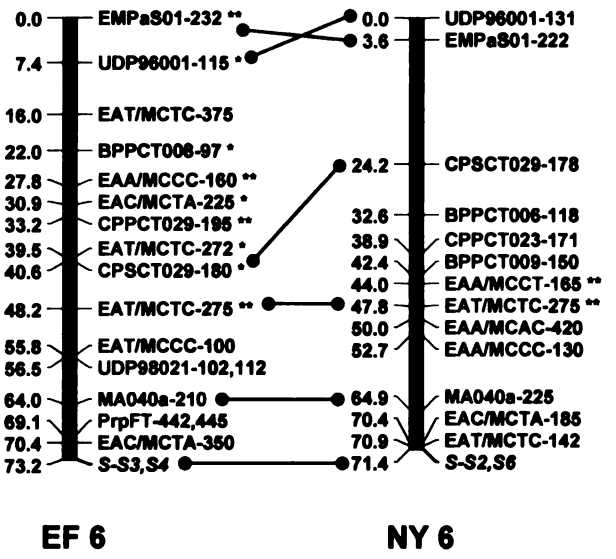


Figure 2. (cont'd)

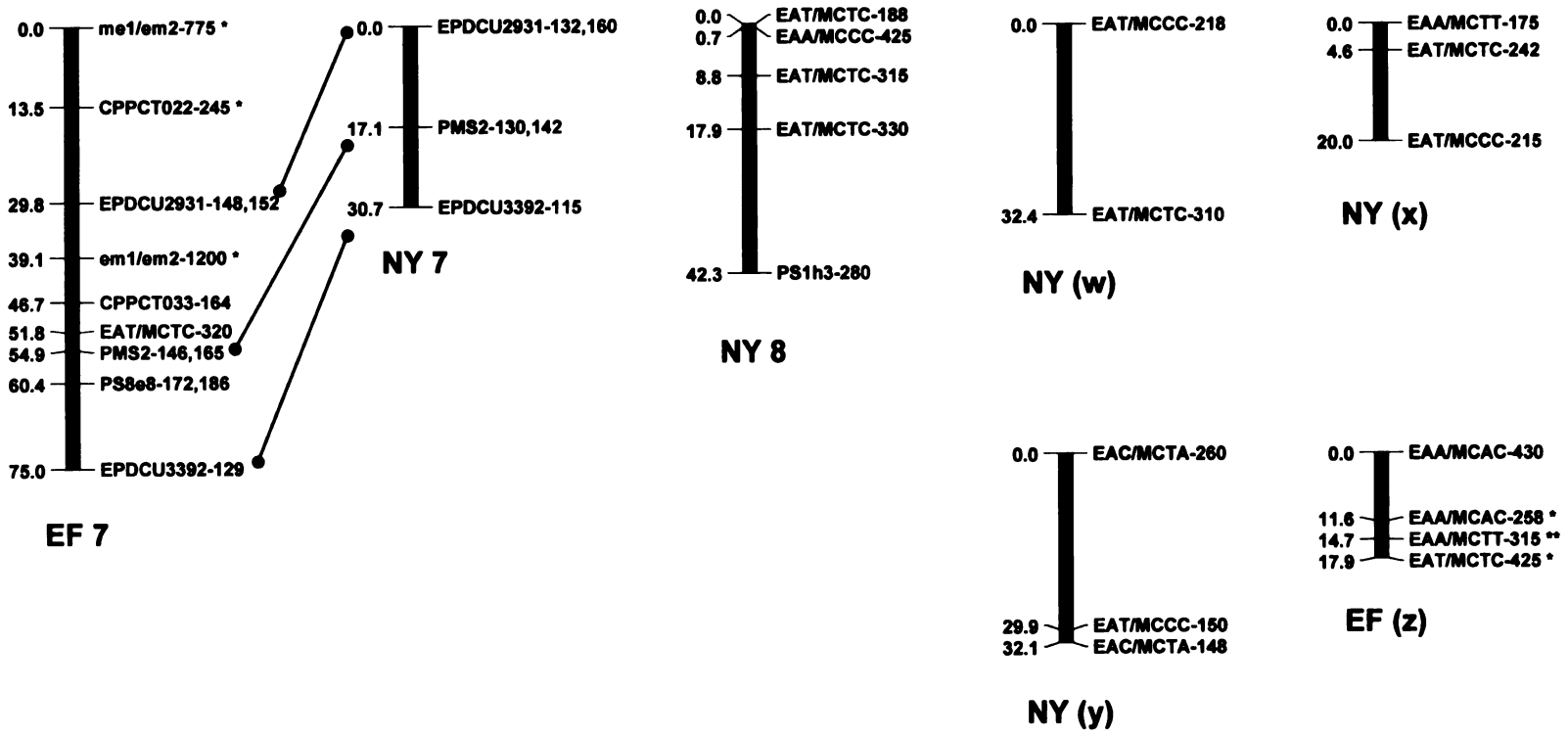


Table 3. Number and type of markers for 'Emperor Francis' ('EF') and 'NY 54' parental maps, map length, target map length from the 'T' × 'E' *Prunus* reference map (Dirlewanger et al., 2004a), marker density, marker gap length, average linkage group length, and average number of markers per linkage group.

		Parental maps	
Marker type	Total	EF	NY 54
SSR	40	36	17
AFLP	47	28	20
SRAP	3	3	0
Total	90	67	37
Map statistics			
Length in cM		479.1	308.9
Target map length (T × E cM)		519	519
Marker density (markers/cM)		0.14	0.12
Average distance between markers (cM)		7.1	8.3
Largest gap between markers (cM)		29.0	34.0
Average cM/linkage group		59.9	30.9
Average markers/ linkage group		8.4	3.7

Table 4. Number and type of marker, length, target length from the 'T' × 'E' *Prunus* reference map (Dirlewanger et al., 2004a), marker density and marker gap length for 'Emperor Francis' ('EF') and 'NY 54' parental linkage groups.

	Linkage groups									
Marker type	EF1	NY1	EF2	NY2	EF3	NY3	EF4	NY4	EF5	NY5
SSR	6	1	0	0	7	1	4	2	5	2
AFLP	5	1	0	0	5	1	3	0	3	0
SRAP	0	0	0	0	0	0	0	0	1	0
Total	11	2	0	0	12	2	7	2	9	2
Map statistics										
Length (cM)	89.4	33.7	-	-	72.5	6.2	82.2	22.7	68.2	17.4
Target length (T × E cM)	87.0	87.0	50.3	50.3	48.4	48.4	62.5	62.5	49.1	49.1
Marker density (markers/cM)	0.12	0.06	-	-	0.17	0.32	0.09	0.09	0.13	0.11
Average marker distance (cM)	8.1	16.9	-	-	6.0	3.1	11.7	11.4	7.6	8.7
Largest gap (cM)	29.2	33.7	-	-	20.7	6.2	15.7	22.7	22.3	17.4

Table 4. (cont'd)

Marker type	Linkage groups									
	EF6	NY6	EF7	NY7	EF8	NY8	NY(w)	NY(x)	NY(y)	EF(z)
SSR	8	7	6	3	0	1	0	0	0	0
AFLP	7	6	1	0	0	4	2	3	3	4
SRAP	0	0	2	0	0	0	0	0	0	0
Total	15	13	9	3	0	5	2	3	3	4
Map statistics										
Length (cM)	73.2	71.4	75.0	30.7	-	42.3	32.4	20.0	32.1	17.9
Target length (T × E cM)	83.7	83.7	70.6	70.6	55.9	55.9	-	-	-	-
Marker density (markers/cM)	0.20	0.18	0.12	0.10	-	0.12	0.06	0.15	0.09	0.22
Average marker distance (cM)	4.9	5.5	8.3	10.2	-	8.5	16.2	6.7	10.7	4.5
Largest gap (cM)	8.6	20.6	16.3	17.1	-	24.4	32.4	15.4	29.9	11.6

Table 5. Comparison of reciprocal differences in segregation of distorted markers present in 'Emperor Francis' that map to LG 6.

	NY 54 × EF					EF × NY 54				
	Observed	Expected				Observed	Expected			
		1:1		2:1			1:1		2:1	
Marker	Presence: absence ratio	χ^2 - value	*P*- value	χ^2 - value	*P*- value	Presence: absence ratio	χ^2 - value	*P*- value	χ^2 - value	*P*- value
EMPaS01-232	57 : 27	10.71	0.001	0.03	0.863	58 : 45	1.64	0.200	5.32	0.021
UDP96001-115	57 : 29	9.12	0.003	0.02	0.888	51 : 47	0.16	0.689	9.92	0.002
BPPCT008-97	25 : 51	8.89	0.003	0.01	0.920	49 : 53	0.16	0.689	10.43	0.001
EAA/MCCC-160	59 : 26	12.81	0.001	0.22	0.639	52 : 46	0.37	0.543	8.61	0.003
EAC/MCTA-225	28 : 57	9.89	0.002	0.01	0.920	48 : 49	0.01	0.920	11.92	0.001
CPPCT029-195	27 : 59	11.91	0.001	0.10	0.752	45 : 54	0.82	0.365	6.95	0.008
EAT/MCTC-272	28 : 55	8.78	0.003	0.02	0.888	50 : 50	0.00	1.000	13.07	0.001
CPSCT029-180	27 : 56	10.13	0.002	0.01	0.920	46 : 50	0.17	0.680	9.66	0.002
EAT/MCTC-275	53 : 30[z]	5.50	0.019	0.37	0.543	69 : 31	1.92	0.166	0.18	0.671

[z]The fragment for marker EAT/MCTC-275 is present in both parents; expected segregation is 3:1 rather than 1:1.

CHAPTER FOUR

Targeted mapping of fruit size and shape QTL in sour cherry (*Prunus cerasus* L.)

ABSTRACT

Fruit size and shape are important production traits in sour cherry (*Prunus cerasus* L.), and certain size and shape parameters must be met for a new sour cherry cultivar to be successful. Identification of the genomic regions involved in variation for fruit size and shape by quantitative trait loci (QTL) analysis would provide an early selection method to increase the efficiency of sour cherry breeding. Previous QTL analyses in both sour cherry and peach [*P. persica* (L.) Batsch] described fruit size QTL. In many cases, these QTL were located on linkage group 6 (LG 6) in the same region as the *Prunus* gametophytic self-incompatibility locus (*S*) and the peach flat/round fruit shape locus (*S**). The objective of this study was to conduct a targeted mapping and QTL analysis for fruit size and shape traits using progeny from the cross between the sour cherry cultivars Újfehértói Fürtös ('UF') and 'Surefire'. Both homeologous LG 6 from 'UF' were developed using previously mapped SSR markers from other *Prunus* species and aligned with the reference *Prunus* linkage map. The homeologous LG 6 were 49.1 cM (LG 6a) and 68.2 cM (LG 6b), respectively. Population distributions for progeny fruit weight, diameter, and length/width percentage approximated normal distributions with transgressive segregation. Mean values for all three traits were significantly different ($P < 0.05$) among progeny *S*-allele groups. This indicates that the fruit traits did not vary independently of *S*-genotype, suggesting that QTL for each trait may be linked to the *S* locus. Using all the LG 6 markers, QTL were identified for fruit weight and fruit length/width percentage, but not fruit diameter. The fruit weight QTL was only significant in 2004, but in that year it explained 26.4% of the phenotypic variation. As predicted by the analysis of variance, the nearest marker was the S_d allele marker for the

S locus at a map distance of three cM on 'UF' LG 6a. The fruit length/width QTL was significant for all three years of the study, and explained between 10.5 and 22.6% of the phenotypic variation. When all three years were combined, the QTL was co-located with the CPSCT012 marker on 'UF' LG 6a.

INTRODUCTION

Sour cherry (*Prunus cerasus* L.) is a tetraploid (*2n* = 4x = 32) member of the predominantly diploid (*2n* = 16) cultivated *Prunus* genus. Sour cherry is believed to have arisen multiple times through natural hybridization between ground cherry (*P. fruticosa* Pall.; *2n* = 4x = 32) and unreduced gametes from sweet cherry (*P. avium* L.; *2n* = 16) (Beaver and Iezzoni, 1993; Brettin et al., 2000; Olden and Nybom, 1968). Currently, the sour cherry industry in the United States is based almost entirely on production of one cultivar, 'Montmorency', a 400-year-old selection from France (Iezzoni, 1988, 2005). Primary utilization of fruit from 'Montmorency' is for processed cherry products. Thus, adoption of 'Montmorency' as the major sour cherry cultivar was likely due to consistent production and suitability for mechanical harvesting (Iezzoni, 2005), and not necessarily for superior fruit quality characteristics.

Breeding for genotypes with fruit quality superior to 'Montmorency' is an important goal of the Michigan State University (MSU) sour cherry breeding program. One of the parents used in the breeding program is Újfehértói Fürtös ('UF'), due to its excellent fruit quality (Iezzoni, 2005). Fruit of 'UF' is larger, firmer, and sweeter than 'Montmorency' fruit, and has been used for fresh market production (Lang et al., 2003). As this market expands, a premium will also likely be placed on large sour cherries, similar to sweet cherry fresh market production (Whiting et al., 2005, 2006). Therefore, future breeding efforts may be directed toward selection of larger sour cherry varieties for fresh market production.

In the MSU sour cherry breeding program, development of improved cultivars for processing use is complicated by factors other than fruit quality. Existing harvest and

processing equipment designed for 'Montmorency' sour cherries requires any new cultivar to be adaptable to existing technologies. For example, to avoid potential breakage of endocarp (pit) ends during the pitting process, a small, round pit is desired (A.F. Iezzoni, pers. comm.). Although pit shape was not correlated with fruit shape in peach (Quilot et al., 2004), observation of the MSU sour cherry breeding program germplasm indicated that fruit shape may be a good predictor of pit shape.

Because of the long juvenility period and extensive land use requirements associated with breeding perennial tree crops such as sour cherry (Fogle, 1975), the MSU sour cherry breeding program has a continued interest in development of molecular tools useful for marker-assisted selection (MAS). Identification of molecular markers for fruit characteristics could have tremendous impact on speeding up the breeding and evaluation cycle. However, many fruit quality characteristics are presumed to be quantitatively inherited, resulting from the coordinated action of many potential genes affecting the phenotypic expression of the trait. Currently, the primary method of evaluating the number and relative significance of the potential genes influencing a given trait is by quantitative trait loci (QTL) analysis. In this type of analysis, phenotypic trait data are combined with a genetic linkage map to identify regions of the genome significantly associated with mean differences in trait values.

For sour cherry, one QTL analysis has been reported for the 'Rheinische Schattenmorelle' ('RS') × 'Erdi Botermo' ('EB') sour cherry population (Wang et al., 2000). However, the map used for that analysis (Wang et al., 1998) was incomplete, and the number of common markers with the *Prunus* reference map {'Texas' almond [*Prunus dulcis* (Miller) D.A. Webb] × 'Earlygold' peach ('T' × 'E') (Dirlewanger et al., 2004)} is

low, preventing more general conclusions. Two fruit weight QTL accounting for over 29% of the phenotypic variation in that population were identified (Wang et al., 2000). Few additional QTL analyses have been performed in peach (Dirlewanger et al., 1999; Etienne et al., 2002; Quilot et al., 2005; Yamamoto et al., 2001). However, among these few populations, there has been some consistency observed for fruit weight QTL. For example, in three different peach populations, one or more fruit size QTL have been identified on linkage group 6 (LG 6) in the same region as the *Prunus S* locus (Dirlewanger et al., 1999; Etienne et al., 2002; Quilot et al., 2005; Yamamoto et al., 2001). The *S* locus has been an area of continued importance in sour cherry, as even though most sour cherry cultivars are self-compatible, progeny from crosses can segregate for self-incompatibility, an undesirable production trait (Lansari and Iezzoni, 1990). Interestingly, the peach *S** is also located ~ 10 cM from the *S* locus (Dirlewanger et al., 2004). Thus, three traits mapped in peach, but also important for sour cherry production, are located in the same genomic region. The transferability of markers and collinearity of genomes between *Prunus* species suggest that targeted mapping of this region for the purpose of identifying QTL for fruit weight and shape in sour cherry could be successful (Cantini et al., 2001; Cipriani et al., 1999; Clarke and Tobutt, 2003; Dirlewanger et al., 2002, 2004; Downey and Iezzoni, 2000; Hormaza, 2002; Messina et al., 2004; Mnejja et al., 2004, 2005; Schueler et al., 2003; Sosinski et al., 2000; Struss et al., 2003 Vilanova et al., 2003; Wang et al., 2002; Wunsch and Hormaza, 2002; Yamamoto et al., 2002). The objective of this study was to determine whether previously identified QTL in peach, that are putatively co-located with the *S* and *S** loci on LG 6, are also present in sour cherry.

MATERIALS AND METHODS

Plant material and phenotypic analysis

Images in this dissertation are presented in color. The sour cherry population used for this study was developed from the cross 'UF' × 'Surefire' (Fig. 1). 'UF' was selected from a Hungarian landrace and is sold in the U.S. as Balaton® (Iezzoni, 2005). 'Surefire' was released from the New York State Agricultural Experiment Station, Cornell University, and results from a cross between 'Borchert Black Sour' × 'NY 6935' (Cummins, 1994). From the cross, 197 F_1 individuals were planted at Michigan State University's Clarksville Horticultural Experiment Station (MSU-CHES) in Clarksville, Michigan in spring 1998. Parental trees of 'UF' and 'Surefire' were also located at MSU-CHES. The seedlings were planted at 1.5 m and 6.1 m within and between row spacing, respectively. The number of individuals used in this study was reduced to 126, as 71 individuals either died, were identified as resulting from out-crossing or self-crossing, or did not have any fruit, and were eliminated from the analyses. The seedling trees were not pruned since establishment. Standard orchard management practices (irrigation, fertilization, and pest and disease control) for MSU-CHES were followed.

Phenotypic measurements were performed for 'UF', 'Surefire', and the 126 individuals from the population. Fruit measurements were made on five replicate fruit from each individual at least twice during estimated harvest maturity. The harvest date with the largest mean fruit weight was used for QTL analyses. Progeny were sampled for three years (2002-2004). Individual fruit weight, length (polar diameter), and width (cheek diameter) were measured. The length/width percentage, an indication of the overall shape of the fruit, was calculated from the measured fruit length and width. In

2004, a crop load rating was made for each progeny individual based on a 1-10 scale (1 = low crop load, 10 = high crop load).

DNA isolation and marker analysis

For DNA extraction, young, unfolded leaves from the parents and each progeny individual were collected, placed immediately on dry ice, transported to the laboratory, and placed directly in a -80°C freezer for at least 24 h. Leaves from each individual were then lyophilized for 48 h and stored long-term at -20°C. DNA isolation was done using the CTAB method described by Stockinger et al. (1996).

Restriction fragment length polylmorphism (RFLP) analysis was used to genotype parents and progeny for their *S*-allele haplotype. For RFLP analysis, six µg of DNA was digested with *Hin*dIII (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), run on a 1.0 % agarose gel for 36 h at 30 V, and transferred to a nylon membrane (Hybond-N+, Amersham Biosciences, Piscataway, N.J.) according to Wang et al. (1998). PCR amplified fragments of the S_6-RNase cDNA from sweet cherry (Tao et al., 1999) were used as the probe. Probes were radiolabelled with ^{32}P-dCTP (Amersham Pharmacia Biotech, N.J.) using the random primer hexamer-priming method described by Feinberg and Vogelstein (1983). After hybridization at 60°C for 16 h and high stringency washes (2 × 20 min with 2x SSC and 1% SDS followed by 2 × 30 min with 0.2x SSC and 0.5% SDS at 60°C), radioactive signal was detected on X-ray films.

Simple sequence repeat (SSR) markers developed from several *Prunus* species that have been mapped to LG 6 of the 'T' × 'E' reference *Prunus* map (Dirlewanger, et al., 2004) were selected for mapping in the 'UF' × 'Surefire' population. Subsequently,

other SSR markers that were not mapped on the 'T' × 'E' map, but had been placed on linkage groups in other *Prunus* maps that aligned to the 'T' × 'E' LG 6, were added to the analyses. The SSR markers used in these analyses were derived from peach ("BPPCT", Dirlewanger et al., 2002; "CPPCT", Aranzana et al., 2002; "UDP", Cipriani et al., 1999; and "MA", Yamamoto et al., 2002; "Prp", Silva et al., 2005), sweet cherry ("EMPA", Clarke and Tobutt, 2003; and "PS", Sosinski et al., 2000), and plum (*P. salicina* Lindl.) ("CPSCT", Mnejja et al., 2004).

A similar temperature profile, other than annealing temperature, was used for all PCR reactions: 94°C for 5 min, 35 cycles of 94°C (45 sec), X°C (45 sec), 72°C (90 sec), and a final extension step of 72°C for 5 min, where X = the published optimum annealing temperature for each primer. For "EMPA" primers, a touchdown PCR temperature profile was used as described by Clarke and Tobutt (2003). The reaction mixture contained 1× PCR buffer, 2.5 mM $MgCl_2$, 120 μM of each dNTP, 2.5 pmol of each primer, 50 ng of genomic DNA and 0.3 U *Taq* polymerase (Invitrogen Corporation, Carlsbad, Calif.) in a 12.5 μl reaction. PCR reactions were run in a MJ Research PTC 100 Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, Calif.). PCR reactions were stored at 4°C until use.

After the addition of 4 μl formamide/dye solution, the PCR products were denatured at 94°C for five min. The PCR products were visualized by electrophoresis on a 6% denaturing polyacrylamide gel in a 50 cm Sequi-Gen GT vertical sequencing apparatus (Bio-Rad Laboratories, Hercules, Calif.) for 2.5 h at 70 W with 1× TBE buffer. Following electrophoresis, the gels were stained with the Silver Sequence DNA Sequencing System (Promega Corporation, Madison, Wisc.) and dried for 24 h. DNA

fragment sizes were scored visually and fragment sizes were estimated relative to 10 and 50 base pair ladders (Invitrogen Corporation, Carlsbad, Calif.).

Single marker analysis of variance

Based on RFLP profiles, all progeny could be placed in six *S*-allele groups ($S_4\ S_a\ S_{13}'\ S_d$, $S_a\ S_{13}'\ S_d\ S_1'$, $S_a\ S_{13}'\ S_1'\ S_{null}$, $S_4\ S_a\ S_{13}'\ S_{null}$, $S_4\ S_a\ S_{13}'\ S_1'$, and $S_a\ S_{13}'\ S_d\ S_{null}$). Two of these groups ($S_4\ S_a\ S_{13}'\ S_1'$, and $S_a\ S_{13}'\ S_d\ S_{null}$) result from non-disomic inheritance and occurred in only 10% of the progeny. Analysis of variance for progeny fruit weight, diameter, and length/width percentage within each disomically-inherited *S*-allele group was performed using the general linear model procedure in SAS (SAS Institute, Cary, N.C.) to determine potential linkage of each trait with the *S*-locus.

Linkage analysis and map construction

Due to the tetraploid genome and F_1 pseudo-testcross population structure, all SSR markers were scored as single-dose restriction fragments (Wang et al., 1998; Wu et al., 1992), where the fragment was present in one but not both parents and fit a 1:1 (presence:absence) segregation ratio, or present in both parents and fit a 3:1 segregation ratio. 'UF', 'Surefire', and a total of 126 progeny were genotyped using 17 SSR markers. Linkage analysis was performed with JoinMap 3.0 (Van Ooijen, and Voorrips, 2001), using a minimum LOD score of 3.0 and a maximum recombination fraction of 0.4. Linkage groups were constructed using MapChart 2.1 (Voorrips, 2002), with distances presented in cM calculated by the Kosambi (1944) function.

QTL and statistical analysis

QTL analyses were performed using Windows QTL Cartographer 2.0 (Wang et al., 2005) using composite interval mapping (CIM). CIM was run with model 6 of the program with the background markers selected using the forward and backward regression method. The LOD threshold for declaring a QTL was determined by 1000 permutations for each trait at a significance level of $P < 0.05$, *a priori*. Estimates of the R-squared value indicating the explained phenotypic variance for each QTL and the additive effect of the QTL were obtained from the QTL Cartographer output. Graphical representations of the QTL were made using output from QTL Cartographer and MapChart 2.1 (Voorrips, 2002). Analysis of variance, correlations, t-tests, and heritability estimates were performed using the appropriate function in SAS statistical analysis software (SAS Institute, Cary, N.C.).

RESULTS

Marker analysis and LG 6 construction

A total of 27 SSR markers placed on the 'T' × 'E' reference *Prunus* LG 6, or other *Prunus* LG 6 aligned with the 'T' × 'E' map, were tested for amplification and segregation in the 'UF' x 'Surefire' population. Of those markers, 37% either did not amplify a corresponding locus in the 'UF' x 'Surefire' population or were monomorphic. Of the 17 remaining markers, 14 fit the expected 1:1 or 3:1 segregation ratios for single dose restriction fragments (Wang et al., 1998; Wu et al., 1992), while three markers that did not fit the expected segregation ratio were not included in the analysis.

The *S*-allele genotype of 'UF' is S_4 S_1' S_d S_{null} and 'UF' produces four gamete types from regular pairing between homologous chromosomes (S_4 S_d , S_4 S_{null}', S_1' S_d , S_1' S_{null}) and two gamete types from pairings between non-homologous chromosomes (S_4 S_1', S_d S_{null}) (Hauck et al., submitted). For our analysis, only those progeny that resulted from normal homologous pairing were included, and not the 10 % produced by non-homologous pairing. As predicted, the segregation of the *S*-alleles (S_4, S_1', and S_d) in the progeny all fit the expected 1:1 segregation ratio. The *S*-allele phenotype of 'Surefire is S_4 S_a S_{13}' (Hauck et al. 2006) Since 'UF' has a functional S_4 allele, all 'Surefire' pollen gametes containing an S_4 allele will be incompatible in the 'UF' style and pollen tube growth will be arrested. As expected, the only 'Surefire' pollen gamete type that successfully fertilized 'UF' was S_a S_{13}', resulting in four progeny types from regular pairing between homologous chromosomes (S_4 S_a S_{13}' S_d, S_a S_{13}' S_d S_1', S_a S_{13}' S_1' S_{null}, S_4 S_a S_{13}' S_{null}). Therefore, it was not possible to place the *S*-locus on the 'Surefire' linkage map and only the meiotic products from 'UF' could be used to test the association of trait variation with *S*-locus genotype.

Linkage analysis was performed with the 14 SSR markers and *S*-allele data for the S_4, S_1', and S_d self-incompatibility alleles segregating in the population generated from RFLP analysis. Two homeologous linkage groups (LG 6a and LG 6b), consisting entirely of SSR markers and the respective *S*-alleles at a 10 to 25 cM distance for 'UF' were generated and aligned with the 'T' × 'E' LG 6 (Fig. 2). The S_d *S*-allele was located on LG 6a, while the S_4 and S_1' *S*-alleles were located on LG 6b. Only one LG 6 from 'Surefire' was identified, consisting of two markers; however, the *S* locus could not be mapped because no *S*-alleles segregated from 'Surefire'. LG 6a and LG 6b had lengths

of 49.1 cM and 68.9 cM, respectively, less than the 83.7 cM distance for LG 6 of the 'T' × 'E' reference map. Seven additional SSR markers remained unlinked. Approximately 10% of the progeny in this population had *S*-allele haplotypes indicating non-disomic inheritance, an observation that has previously been made in sour cherry (Wang et al., 1998). Due to the small number of individuals in these groups, the difficulty in determining segregation ratios, and the potential for linkage map distance inflation, these individuals were not included in linkage map development or QTL analysis. However, the S_d allele marker and the flanking marker distal to it on 'UF' LG 6a still appeared distorted, having a slightly skewed segregation ratio ($P < 0.1$) according to chi square tests (Fig. 2). Distorted loci have often been identified in the *S* locus region, presumably due to self-incompatibility (Bliss et al., 2002; Joobeur et al., 1998; Lambert et al., 2004; Vilanova et al., 2003).

Fruit size and shape

Fruit weight, diameter, and length/width percentage were measured for three consecutive years (2002-2004). To reduce variation in shape measurements, the percentage of fruit length (polar diameter) divided by width (cheek diameter) was calculated. Thus, the more flat-oblate shape the fruit is, the lower the length/width percentage. From 2002-2004, fruit weight, diameter, and shape measurements were made for five fruit per individual. The 'UF' × 'Surefire' population first began fruiting in year 2002. Because of variability in precocity, only 76 individuals in the population had fruit during 2002. In 2003 and 2004, 118 and 126 individuals were measured, respectively.

All traits exhibited continuous variation typical of a quantitative trait with polygenic inheritance (Fig. 3). Broad sense heritability (H^2) for each trait was high (Table 1) indicating consistency over the years of the study and a low genotype × environment interaction. The parental means of 'UF' and 'Surefire' were significantly different ($P < 0.001$) for fruit weight and diameter, but not for fruit length/width percentage. The average value of the parents was significantly different than the progeny mean value for fruit weight and fruit diameter ($P < 0.0001$) but not significantly different for the length/width percentage (Table 1). Transgressive segregation occurred for all traits (Fig. 3). For fruit weight, the distribution of the progeny was skewed toward smaller fruit, with 82% of the progeny averaging smaller fruit weight than the mid-parent value. As expected, there was a strong positive linear correlation ($P < 0.0001$) between fruit weight and fruit diameter (Fig. 4). Fruit weight and fruit length/width percentage were not significantly correlated ($P= 0.324$), but fruit diameter and fruit length/width percentage were ($P < 0.0001$). However, the correlation was weak, with an R-squared value of 0.042 (Fig. 4). Because fruit weight can be influenced by crop load level on individual trees, linear correlation between mean fruit weight and tree crop load after fruit set for each individual in the population was analyzed in 2004. Although the relationship was significant ($P < 0.001$), the low R-square value (0.163) and positive relationship between increasing fruit weight and crop load indicated that small fruit size was not a result of high crop load in this population (Fig. 5).

Single marker analysis to test the association of fruit traits with the S locus

To determine whether potential QTL for fruit weight, diameter, and length/width percentage were linked to the *S*-locus, analysis of variance was used to compare trait phenotypic values of progeny within each potential disomically-inherited *S*-allele group. In essence, this process is similar to single marker QTL analysis. Significant differences between trait mean values for *S*-haplotype groups ($P < 0.001$) indicated linkage between the measured phenotype and the *S* locus (Table 2). However, this type of analysis does not provide a linkage distance estimate from known markers, knowledge that is essential for potential MAS strategies.

QTL analysis

Two QTL were identified on the 'UF' LG 6a, one for fruit weight (*wt*), and one for fruit length/diameter percentage (*shape*) (Table 3, Fig. 6). The significant QTL for fruit weight (*wt*) was only identified in 2004, although there was a peak in the same location for both 2002 and 2003 that did not reach the LOD significance level. Similarly, a QTL peak for fruit diameter was observed at the same map location as fruit weight for all three years; however, the fruit diameter peak failed to reach the LOD significance level in any year. In 2004, the fruit weight QTL explained 26.4% of the phenotypic variation. The QTL had an effect in the opposite direction predicted by the paternal phenotype, reducing fruit weight by 1.55 g. The QTL identified for fruit length/width percentage (*shape*) was consistently identified in all three years of the study (Table 3, Fig. 6). In years 2002-2004, the QTL explained 22.6, 17.5, and 10.5 % of the phenotypic variation, respectively. Again, the QTL had an effect opposite as predicted by the

parental phenotype, increasing the fruit length/width percentage an average of 5%. The S_d allele specific marker for the *S* locus was the closest marker to the fruit weight QTL, 3.6 cM from the QTL peak. CPSCT012, an SSR marker derived from a genomic library of Japanese plum (Mnejja et al., 2004), was the closest marker to the fruit length/width percentage QTL, 0 to 4.9cM from the QTL peak depending on the year.

DISCUSSION

The objective of this study was to determine whether QTL identified in peach that were co-located with the *S* and *S** loci were also present in sour cherry. A targeted mapping approach was used, whereby only SSR markers that have been mapped to the linkage group containing the above loci in the 'T' × 'E' reference *Prunus* map (LG 6) and homologous linkage groups from other *Prunus* populations were used.

A LG 6 totaling 34.4 cM containing the *S* locus had previously been constructed from the 'RS' × 'EB' sour cherry population (Hauck et al., 2002). However, the 'RS' × 'EB' map is currently not comparable with the reference *Prunus* map because few markers are common. In that study, the S_b allele, later named S_{26}, (Hauck et al., 2006) from the 'RS' parent was placed on LG 6. The only other *S*-alleles able to be mapped in that population, S_{13}' and S_6, both also from 'RS', were linked to each other but not to any other marker on the 'RS' × 'EB' linkage map. In a previous QTL study using the 'RS' × 'EB' population, no fruit size QTL were located on this linkage group and fruit shape was not measured (Wang et al., 2000).

Both 'UF' and 'Surefire' are self-compatible sour cherries. However, self-incompatible progeny can result from crosses between two self-compatible sour cherries

(Lansari and Iezzoni, 1990). If self-compatible and self-incompatible individuals segregate in the population, the possibility exists that fruit weight could be influenced by the level of crop load on the tree, with self-compatible individuals presumably having a higher crop load. Because of this possibility, each individual in the population was rated for crop level after fruit set in 2004. The correlation between fruit weight and crop load was statistically significant, but the R-squared value was only 0.163 (Fig. 5). Furthermore, the relationship between fruit weight and crop load was positive, with fruit size increasing with crop load. Therefore, any putative QTL linked to the *S* locus are likely true QTL, not simply artifacts of higher crop load.

From this study, a single QTL for both fruit weight and fruit length/width percentage were identified on the 'UF' LG 6a (Table 3, Fig. 6). The QTL for fruit weight (*wt*) was only significant in year 2004. As predicted by analysis of variance, the QTL peak was 3.6 cM from the nearest marker, S_d, an allele specific marker for the *S* locus. Although only significant for one year of the study, this QTL explained 26.4% of the phenotypic variation. More importantly, the effect was opposite of what was expected from the 'UF' parental phenotype, reducing fruit weight an average of 1.55 g. Interestingly, no significant QTL for fruit diameter was identified, although there was a strong positive correlation between fruit weight and diameter (Fig. 4). In each year of the study, a QTL peak for fruit diameter at the same map location as that of the fruit weight QTL was observed, but it failed to reach LOD significance level in any year. Given the strong correlation between fruit weight and diameter, the fruit weight QTL identified may influence both fruit weight and diameter. Although fruit weight and diameter were the only measured traits used for QTL analysis, the 'UF' × 'Surefire' population appears to

segregate for mesocarp cell number (see Chapters 2 and 5). 'UF' and 'Surefire' have statistically similar numbers of mesocarp cells, but progeny from the tails of the fruit weight distribution are significantly different ($P < 0.05$) for mesocarp cell number (Table 4). Further examination of this trait as a component of total fruit size in this population is warranted.

The *shape* QTL for fruit length/width percentage was significant in all years of the study and explained between 10.5 and 22.6% of the phenotypic variation in a given year. The peak for this QTL was nearest to the CPSCT012 marker, but varied slightly from year to year. When phenotypic measurements from all three years were averaged, the peak of the QTL co-located with the CPSCT012 marker. The peak for the *shape* QTL was 26.2 cM from the *S* locus, within the 50% recombination range and explaining the significant association with the *S* locus when analysis of variance was performed. Like the *wt* QTL, the effect of the *shape* QTL was opposite of the predicted parental phenotype, increasing the fruit length/width percentage by 5 %. The fruit *S** locus is located ~ 10 cM from the *S* locus on the *Prunus* reference map (Dirlewanger et al., 2004). Furthermore, the *S* locus is ~ 33 cM from the CPSCT012 marker on the *Prunus* reference map, similar to the 26.2 cM distance observed in the present study (Fig. 2). In peach, the *S** gene is dominant for flat-oblate fruit (Lesley, 1940). Although flat-oblate fruited progeny are present in the 'UF' × 'Surefire' population, segregation for that character did not indicate that it was controlled by a single locus. Because the *shape* QTL identified in the 'UF' × 'Surefire' population is further away from the *S* locus than the *S** gene and only explains 10.5 to 22.6% of the phenotypic variation, it is likely not the same locus. However, the use of length/width percentage for phenotypic data presumably accounts

for variation that may be present even if the phenotype is measured as a dominant agronomic character. Therefore, we cannot exclude the possibility that a modifier gene for the *S** locus underlies the QTL discovered in the 'UF' × 'Surefire' population.

Allelic effects opposite to those expected from the parental phenotype are not uncommon in sour cherry. Wang et al. (2000) reported 50% of the QTL identified in the 'RS' × 'EB' cross to have effects opposite as predicted by the parental phenotype. As in that study, this phenomenon likely explains the transgressive segregation seen for fruit weight and fruit length/width percentage. Each parent likely contributed both favorable and unfavorable alleles for QTL affecting the same trait, and since both the 'RS' × 'EB' and 'UF' × 'Surefire' populations consist of F_1 individuals, recombination of these alleles in the progeny generated transgressive phenotypes (Wang et al., 2000).

Although few QTL studies have been performed to date in *Prunus*, QTL for fruit size have consistently been identified in the region where the *S* locus is presumably located in peach, at the bottom of LG 6 (Dirlewanger et al., 1999; Etienne et al., 2002; Quilot et al., 2005; Yamamoto et al., 2001). Because peach is self-compatible, this locus is not included in many peach linkage maps. However, almond is self-incompatible, and this locus has been placed on interspecific peach × almond maps. The peach *S** locus is located ~ 10 cM from the *S* locus, nearer to the center of LG 6 (Dirlewanger et al., 2004). However, because this is a dominant locus in peach, and QTL analysis for fruit shape has not been previously published. In the 'Ferjalou Jalousia' ('J') × 'Fantasia' ('F') peach population, a QTL explaining between 22.6% and 51% (depending on the year) of the phenotypic variance for fruit weight was identified (Dirlewanger et al., 1999; Etienne et al., 2002). This QTL was co-located with the *S** locus and had an effect in the same

direction of the parental phenotype. In the 'Akame' ('A') × 'Juseitou' ('J') peach cross, two QTL were identified for fruit weight, near the *Dw* dwarf locus at the top of LG 6 and the *Gr* red/green leaf locus in the central portion of LG 6 (Yamamoto et al., 2001). R-square values indicating the phenotypic variance explained by these QTL were not provided, but both QTL effect fruit weight in the opposite direction as suggested by the parental phenotype. In the backcross population developed from *P. davidiana* × 'Summergrand' peach, a QTL for pit diameter was identified at the PC60 marker locus, 4 cM from the *S** locus. This QTL explained 41% of the phenotypic variation and had an effect in the same direction as the parental phenotype.

CONCLUSIONS

This analysis demonstrates the utility of a targeted mapping approach for identifying useful QTL. The targeted mapping approach for sour cherry takes advantage of the transportability of SSR markers across and collinearity between *Prunus* genomes (Dirlewanger et al., 2004). Identification of the QTL for fruit weight and fruit length/diameter percentage in this study should have immediate impact in sour cherry breeding programs. For example, self-compatibility is one of the few characters selected for using MAS (Dirlewanger et al., 2004). Identification of the QTL in this study with a strong negative effect linked to the S_d allele of the *S* locus suggests selection against this allele could have a positive impact on fruit size. Alternatively, the breeder would need to create larger populations for the potential to break the linkage if the S_d allele itself was desired. Similarly, the close association of the fruit length/width percentage QTL with the nearest marker may allow for efficient marker-assisted selection. Since fruit and pit

shape were observed to be associated in sour cherry (A.F. Iezzoni, pers. comm.), the utility of this marker-trait association may be best utilized for early selection of desirable pit shapes.

As with any QTL analysis, the true utility of the marker-trait associations described in this study will be determined by the stability and repeatability of the association in other populations. However, for at least the fruit weight QTL, the fact that similar QTL have been documented in other *Prunus* species is encouraging, particularly given the low density of the linkage group mapped in this study and the difficulty in identification of QTL in polyploid populations (Wang et al., 1998, 2000).

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TABLES AND FIGURES

Figure 1. Selected progeny from the 'Újfehértói Fürtös' × 'Surefire' sour cherry population illustrating variation for fruit size and shape.

Figure 2. Alignment of the mapped homeologous linkage groups from 'Újfehértói Fürtös' ('UF') sour cherry corresponding to LG 6 from the 'T' × 'E' reference *Prunus* map. Only SSR markers from the 'T' × 'E' map are shown. Map distances in cM are indicated to the left and marker names to the right of each vertical bar. Distorted loci at the level of 0.1% level are denoted with a * following the name. Anchor loci between 'UF' and 'T' × 'E' are connected by a dotted line.

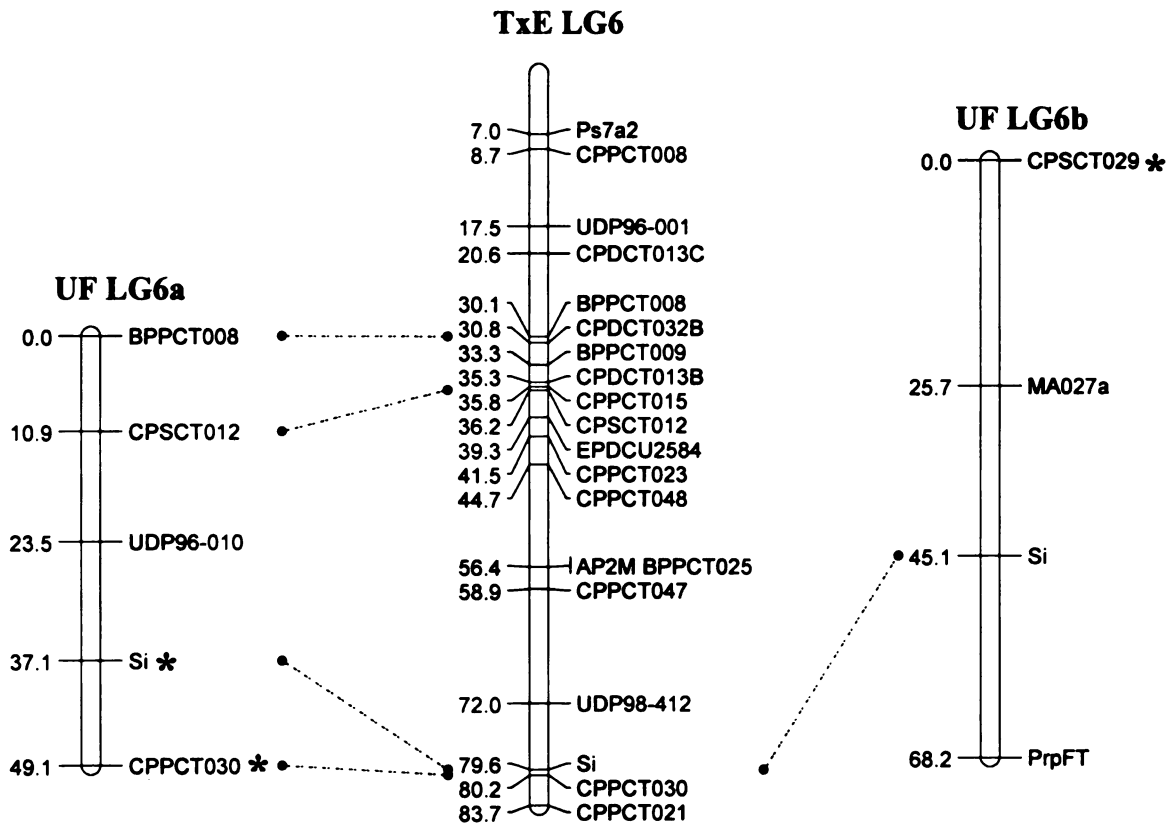
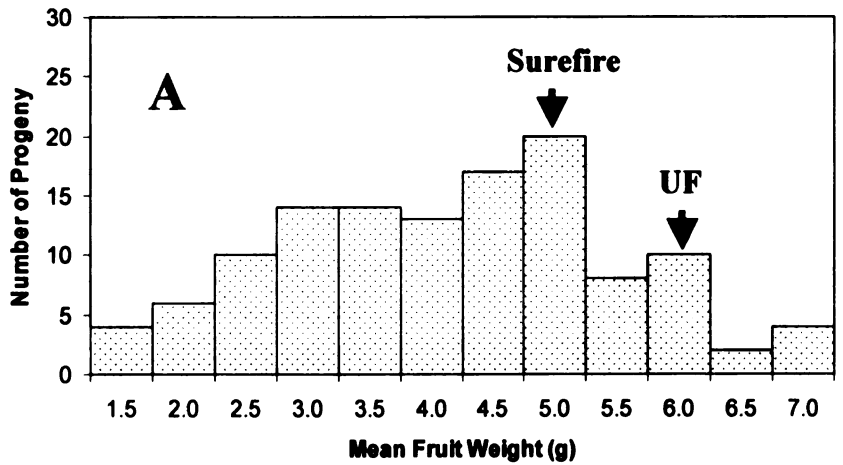
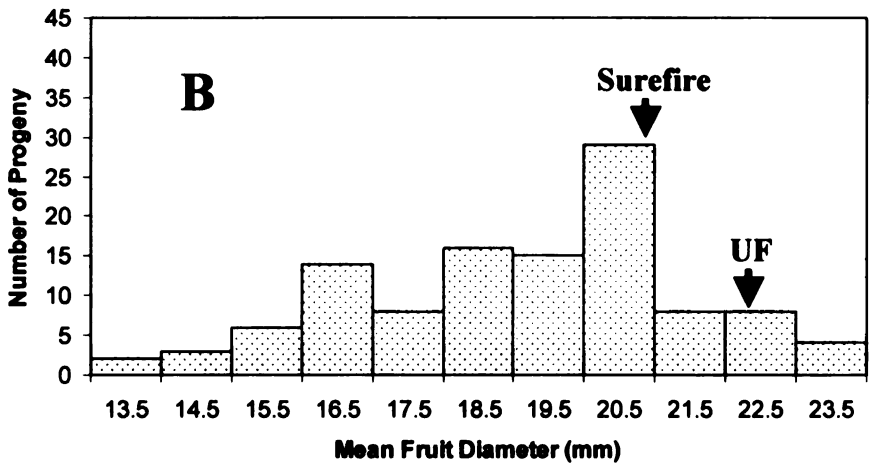


Figure 3. Frequency distribution of sour cherry fruit weight (A), diameter (B), and length/width percentage (C) traits measured on 126 progeny in the 'Újfehértói Fürtös' ('UF') × 'Surefire' population. The distribution is based on the mean value of each genotype in years 2002-2004. Means for the parents 'UF' and 'Surefire' are shown by arrows.





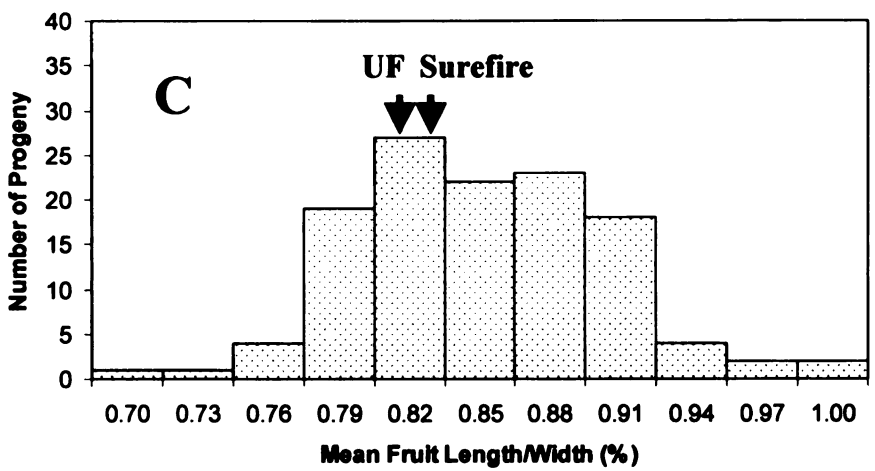
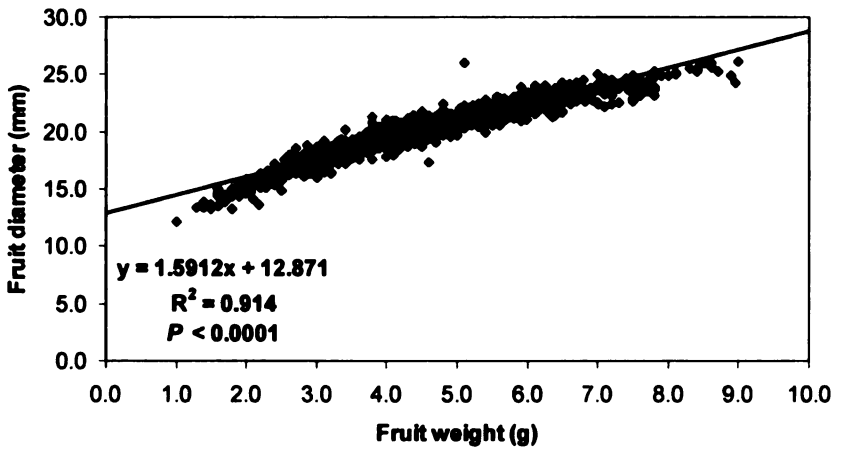
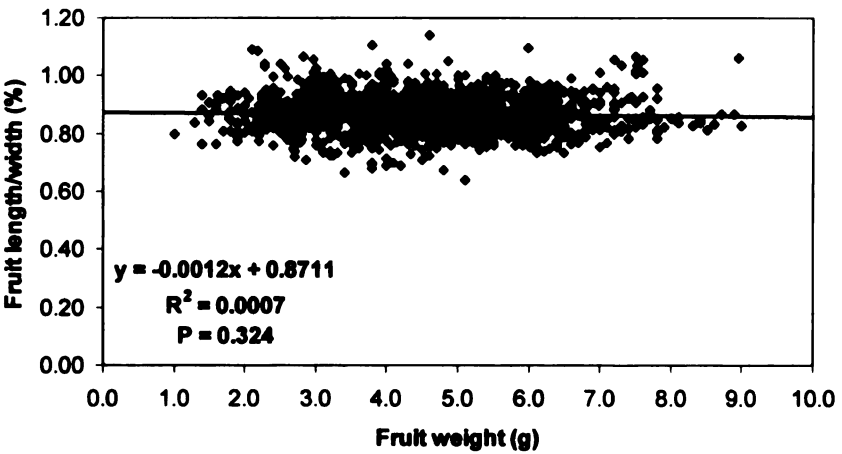


Table 1. Broad sense heritability (H^2), mean phenotypic values and standard deviations, and progeny value range for sour cherry fruit weight, diameter, and length/width percentage.

Trait	Broad-sense heritability (H^2)	Mean ± SD			Progeny range	
		UF	Surefire	Progeny	Minimum	Maximum
Fruit weight (g)	0.94	6.3 ± 0.6	5.2 ± 0.8	4.6 ± 1.4	1.7	7.5
Fruit diameter (mm)	0.93	22.6 ± 0.8	21.1 ± 1.4	20.3 ± 2.4	13.6	24.3
Fruit length/width (%)	0.93	0.844 ± 0.05	0.854 ± 0.02	0.866 ± 0.07	0.723	1.018

Figure 4. Linear correlation between sour cherry progeny mean fruit weight and diameter (A), fruit weight and length/width percentage (B), and fruit diameter and length/width percentage (C). The Pearson correlation coefficient and R-square values for each comparison are indicated on the plot.





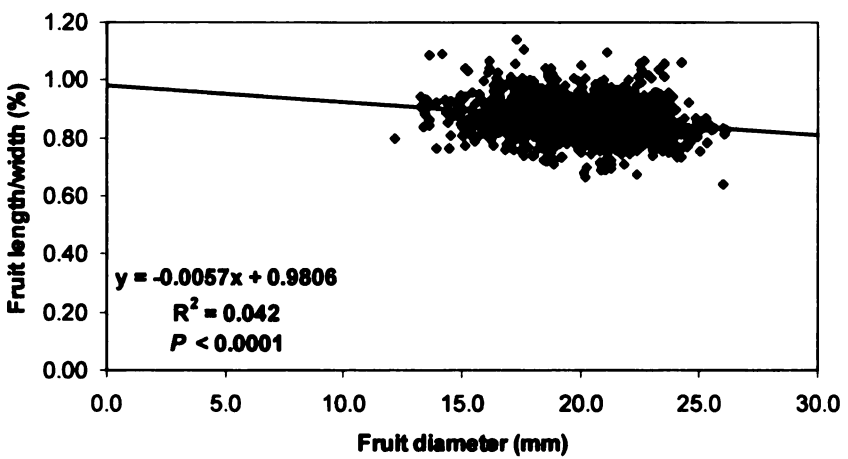
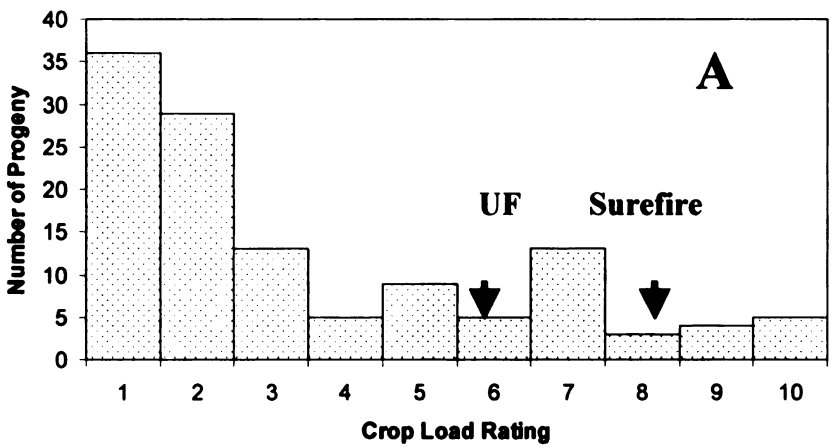


Figure 5. Frequency distribution of crop load rating (A), and linear correlation between sour cherry progeny mean fruit weight and crop load rating (B) for 2004. Means for the parents 'Újfehértói Fürtös' ('UF') and 'Surefire' are shown by arrows on the histogram. Individual progeny crop load was rated on a 1-10 scale, 1 being the lowest crop load. The *P*-value calculated for the Spearman correlation coefficient and R-square value is indicated on the plot.



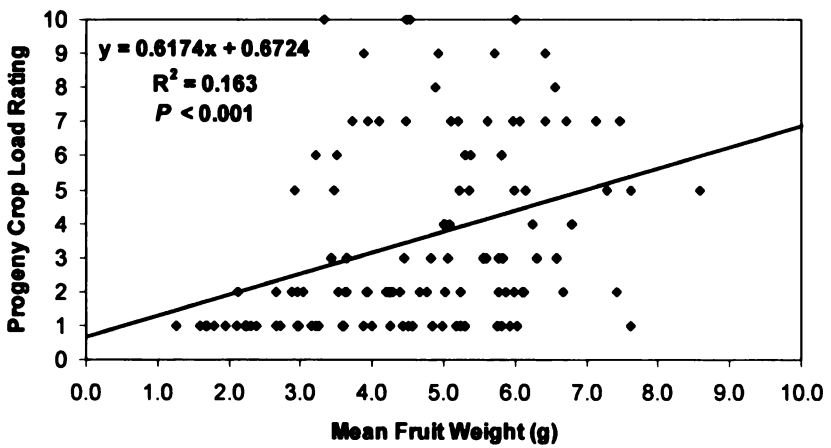


Table 2. Mean fruit weight, diameter, and length/width percentage for 'Újfehértói Fürtös' ('UF') × 'Surefire' sour cherry progeny from each possible *S*-allele group resulting from disomic inheritance for the years 2002-2004.

	Fruit weight (g)			Fruit diameter (mm)			Fruit length/width (%)		
S-allele group	2002	2003	2004	2002	2003	2004	2002	2003	2004
$S_4 S_a S_{13}' S_d$	3.7 a	4.0 a	4.1 a	19.0 a	19.6 a	19.3 a	0.860 a	0.852 a	0.818 a
$S_a S_{13}' S_d S_1'$	4.6 bc	4.4 b	4.5 b	20.6 c	20.0 a	20.0 b	0.860 a	0.860 a	0.830 a
$S_a S_{13}' S_1' S_{null}$	4.8 c	5.1 c	5.2 c	20.4 c	20.9 b	21.0 c	0.898 b	0.885 b	0.848 b
$S_4 S_a S_{13}' S_{null}$	4.3 b	4.8 c	4.9 c	19.8 b	20.5 ab	20.5 bc	0.916 c	0.889 b	0.860 c

[z]Mean separation within columns by Fisher's LSD at $P < 0.05$.

Table 3. QTL detected for sour cherry fruit weight and length/width ratio. All detected QTL were located on the 'Újfehértói Fürtös' linkage group (LG) 6a, corresponding to LG 6 of the 'T' × 'E' reference *Prunus* map.

	QTL year	Interval length (cM)	LOD peak position (cM)	Maximum LOD	Nearest marker	R^2 (%)	Genetic effect: a^z
Fruit weight (g)	*wt-04*	3.0	33.5	2.4	S_d	26.4	- 1.55
Fruit length/width (mm)	*shape-02*	11.9	14.9	2.6	CPSCT012	22.6	- 0.06
	shape-03	> 15.5	12.9	3.0	CPSCT012	17.5	- 0.05
	shape-04	15.0	10.9	3.0	CPSCT012	10.5	- 0.04

[z] a, Additive value of the QTL.

Figure 6. Significant QTL for sour cherry fruit shape (A) and fruit weight (B) on linkage group (LG) 6a of 'Újfehértói Fürtös' ('UF') for years 2002-2004. Curves on the graphs represent results from different years and correspond to the legend. Vertical lines on the graph indicate LOD significance scores for each year based on 1000 permutations. Bars between the graph and linkage group indicate 1-LOD (filled) and 2-LOD (bars) interval for the QTL peak on the graph.

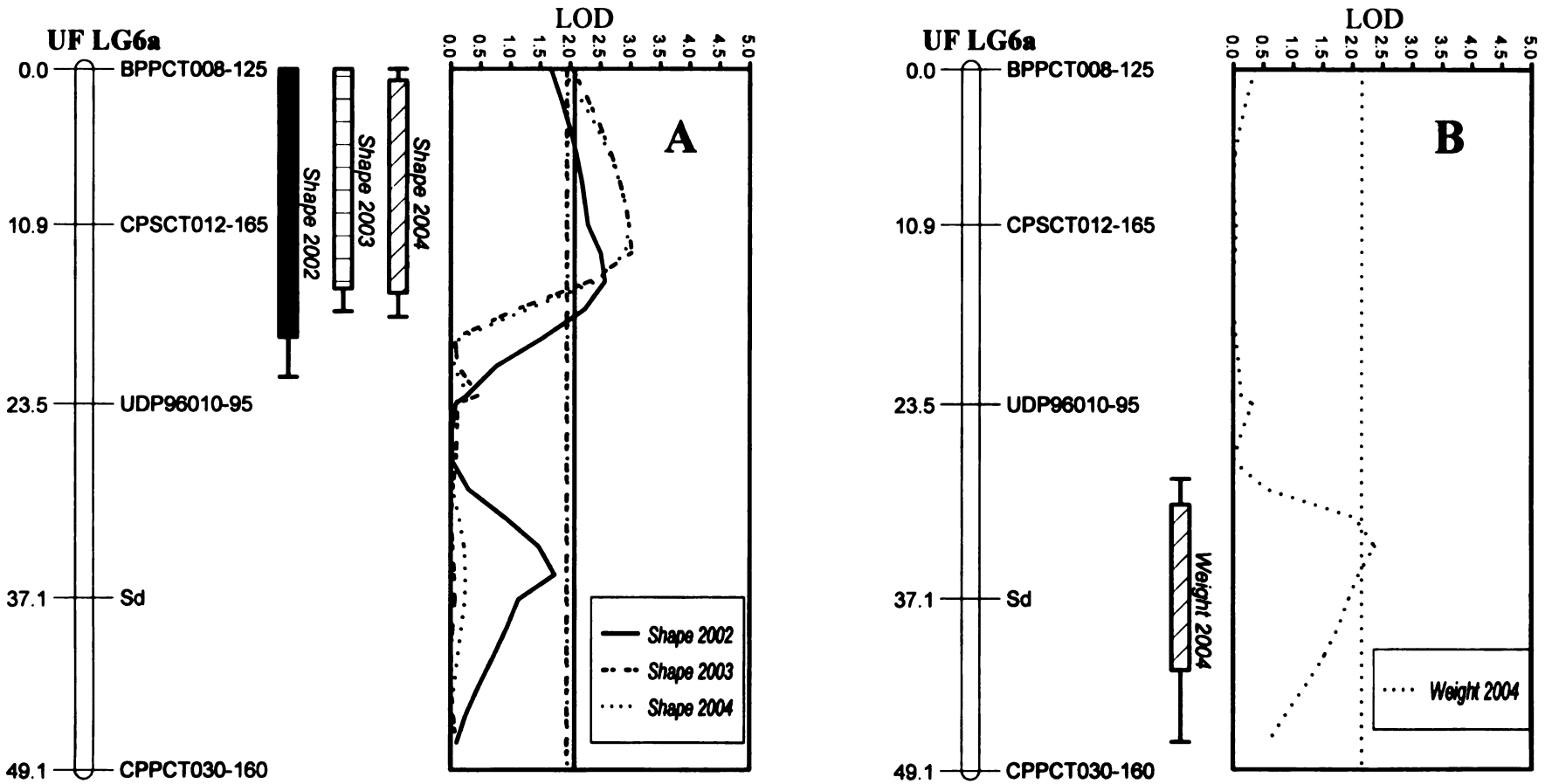


Table 4. Comparison of sour cherry mean fruit weight (2002-2004) and mesocarp cell numbers (2004) for 'Újfehértói Fürtös' ('UF'), 'Surefire', and small and large progeny individuals from the 'UF' × 'Surefire' population. Five fruit were measured for each year and trait.

Genotype	3-yr average weight (g)	Mesocarp cell number (per radial section)[z]
2 (19)	1.72	28.8 a
2 (62)	1.96	32.0 a
Surefire	5.12	37.6 b
UF	5.86	38.2 bc
2 (43)	7.25	41.6 c

[z]Mean separation within column by Tukey's HSD at $P < 0.05$.

CHAPTER FIVE

QTL analysis of fruit size traits for the 'NY 54' × 'Emperor Francis' sweet cherry (*Prunus avium* L.) population

ABSTRACT

Large fruit size is an essential production trait in sweet cherry (*Prunus avium* L.) and an important goal of sweet cherry breeding programs. Identification of the genomic regions involved in variation for fruit size by quantitative trait loci (QTL) analysis would provide an efficient early selection method to increase the efficiency of sweet cherry breeding. QTL analysis for fruit size traits was performed using the 'New York 54' ('NY 54') × 'Emperor Francis' ('EF') sweet cherry reciprocal populations. Fruit mesocarp cell number and cell length, and mesocarp length were measured for 67 individuals in the population. The parental means of 'NY 54' and 'EF' were significantly different ($P <$ 0.01) for all traits measured. Continuous variation was observed for all traits, although the distribution was skewed toward the small fruit size exhibited by 'NY 54'. No QTL was identified for mesocarp cell number. However, five significant QTL were identified for mesocarp length and mesocarp cell length. All identified QTL affected the phenotypic variance in the same direction as predicted by the parent.

For mesocarp length, one QTL (*mlength1*) was identified on 'EF' linkage group 6 (LG 6) and one on 'NY 54' LG (y) (*mlength2*). The QTL *mlength1* explained 18.3% of the total phenotypic variance. The closest marker to *mlength1* was the AFLP marker, EAT/MCCC-100, that was 0.1 cM from the LOD peak. The QTL *mlength2* explained 37.4% of the phenotypic variation, and was 3.9 cM from the nearest marker, EAT/MCCC-150. Three QTL were identified for mesocarp cell length, on 'EF' LG 6 (*clength1*) and 'NY 54' LG 6 (*clength2*) and LG (y) (*clength3*). The QTL explained 17.4, 16.8, and 16.8% of the phenotypic variation, respectively. The closest marker to *clength1* was CPPCT029-195, 0.1 cM from the LOD peak. The QTL *clength2* on 'NY

54' linkage group 6 was 0.1 cM from MA040a-225, while *clength3*, on 'NY54' LG (y) was co-located with EAT/MCCC-150. These QTL were identified with only one year of phenotypic data on just 67 of the 190 progeny individuals genotyped for linkage map construction, and therefore need to be verified in future years based on evaluation of all 190 progeny.

INTRODUCTION

Large fruit size is an essential component of fresh market sweet cherry (*Prunus avium* L.) production as fruit averaging over 29 mm in diameter worth nearly twice as much ($/kg) as fruit less than 24 mm in diameter (Whiting et al., 2005, 2006). Sweet cherry fruit size is a quantitative trait, presumed to be controlled by many separate loci working in concert to produce the fruit size phenotype exhibited in a given cultivar. Although the response to selection for increased fruit size has been relatively high in sweet cherry breeding programs (Fogle, 1961; Hansche, 1966; Lamb, 1953; Matthews, 1973), little is known about the genetic control of fruit size.

Because of the long juvenility period and extensive land use requirements associated with breeding perennial tree crops such as sweet cherry (Fogle, 1975), many of the current cultivars are only a few generations removed from landraces from which original domesticates were selected (Iezzoni et al., 1990). Marker-assisted selection (MAS) could significantly increase the efficiency of sweet cherry breeding, particularly for fruit traits, where selection of favorable alleles based on DNA sequence rather than phenotype would reduce the land use requirement and expense of maintaining seedling individuals until the juvenility period has passed. Currently, the only trait for which MAS is routine in sweet cherry breeding programs is self-compatibility conferred by the mutated S_4' allele at the *S*-locus (Dirlewanger et al., 2004; Lewis and Crowe, 1954).

To implement marker-assisted selection for a quantitative trait, the relative contribution of all the genes involved in expression of the phenotype must be described. Quantitative trait loci (QTL) analysis is the method used to evaluate the number and relative significance of the potential genes influencing a given trait. In this type of

analysis, phenotypic trait data are combined with a genetic linkage map to identify regions of the genome significantly associated with mean differences in trait values. In the 'Rheinische Schattenmorelle' ('RS') × 'Erdi Botermo' ('EB') sour cherry (*P. cerasus* L.) population, two fruit weight QTL accounting for over 29% of the phenotypic variation have been identified (Wang et al., 2000). An additional QTL for fruit weight was identified in the 'Újfehértói Fürtös' ('UF') × 'Surefire' sour cherry population (see Chapter 4). More progress in QTL analysis has been made in peach [*P. persica* (L.) Batsch], but there still have been relatively few analyses performed (Dirlewanger et al., 1999; Etienne et al., 2002; Quilot et al., 2005; Yamamoto et al., 2001). However, among these few populations, significant QTL for fruit size measurements have consistently been identified.

The phenotypic variability for fruit size exhibited by 'New York 54' ('NY 54') and 'Emperor Francis' ('EF') (see Chapter 2) suggested that progeny from this population would segregate for this trait. With the development of a genetic linkage map for the 'NY 54' × 'EF' population (see Chapter 3), the ability to identify QTL for fruit size traits was possible. Therefore, the objective of this study was to identify potential QTL for fruit size traits using the 'NY 54' × 'EF' sweet cherry population.

MATERIALS AND METHODS

Plant material

The sweet cherry population used for this study was developed from the reciprocal crosses between 'NY 54' and 'EF'. 'NY 54' was selected from wild *P. avium* forests in Germany and introduced at the New York State Agricultural Experiment

Station, Cornell University (R.L. Andersen, pers. comm.). 'EF' is a cultivated sweet cherry of unknown origin, grown primarily for processed cherry products. From the reciprocal crosses, 617 F_1 individuals were planted at Michigan State University's Clarksville Horticultural Experiment Station (MSU-CHES). Details concerning population development are provided in Chapter three.

Linkage map construction

Genotypic analyses and genetic linkage map development for the 'NY 54' × 'EF' population was described previously (see Chapter 3). Briefly, simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), sequence related amplified polymorphism (SRAP), and *S-RNase* specific primers for alleles segregating at the self-incompatibility locus were used to develop a 788 cM total distance F_1 pseudo-testcross linkage map for both 'EF' and 'NY54'. SSR markers developed in several *Prunus* species were used in the development of the 'NY 54' × 'EF' linkage map to facilitate alignment and comparison with the *Prunus* reference map (Dirlewanger et al., 2004). A total of 18 linkage groups were identified, with 12 of the 18 labeled according to the reference map nomenclature based on shared markers.

Phenotypic analysis

A total of 67 individuals from the 'NY 54' × 'EF' population flowered in 2005. To ensure fruit set, all available flowers were hand-pollinated with compatible pollen. Whenever possible, at least five fruit from each individual were harvested after endocarp hardening had occurred. The fruit from each individual were placed in storage vessels,

immersed in a formalin-acetic acid-alcohol solution (10:5:50 FAA; Ruzin, 1999) and stored until sectioning. Radial mesocarp flesh sections were created at the widest diameter of the fruit as described previously (see Chapter 2). Microscopic analyses and image analysis were used to determine the number of mesocarp cells per radial section, mesocarp radial length, and mesocarp cell length (see Chapter 2).

QTL and statistical analysis

QTL analyses were performed using Windows QTL Cartographer 2.0 (Wang et al., 2005) using composite interval mapping (CIM). CIM was run with model 6 of the program using the forward and backward regression method. The LOD threshold for declaring a QTL was determined by 1000 permutations for each trait at a significance level of $P < 0.05$, *a priori*. Estimates of the R-squared value indicating the explained phenotypic variance for each QTL and the additive effect of the QTL were obtained from the QTL Cartographer output. Graphical representations of the QTL were made using output from QTL Cartographer and MapChart 2.1 (Voorrips, 2002). Analysis of variance, correlations, and t-tests were performed using the appropriate function in SAS statistical analysis software (SAS Institute, Cary, N.C.). Broad-sense heritability for each trait was calculated using mean square values from analysis of variance (Fehr, 1987).

RESULTS AND DISCUSSION

The objective of this study was to identify potential QTL for fruit size traits using the 'NY 54' × 'EF' sweet cherry population. This population was planted at MSU-CHES in the spring of 2002, but the seedlings did not begin to fruit until 2005. In this year, only

67 individuals from the linkage mapping population had at least one fruit available for phenotypic measurements. Because of the lack of adequate fruit number in the first bearing year, the potential for animal predation, and the importance of mesocarp cell number and mesocarp cell size to final fruit size in *Prunus* (Chapter 2; also, Scorza et al., 1991; Yamaguchi et al., 2002a, 2002b, 2004), emphasis was placed on the collection of phenotypic data for these mesocarp cell number and cell size, and not final fruit weight and diameter. Thus, available fruit were harvested just after endocarp hardening had occurred, prior to harvest maturity.

The parental means of 'NY 54' and 'EF' were significantly different ($P < 0.01$) for all traits measured (Table 1). However, the average value of the parents was only significantly different from the progeny mean value for mesocarp length ($P < 0.05$). All traits exhibited continuous variation typical of a quantitatively inherited polygenic trait (Fig. 1). For each trait, the distributions from the 67 progeny were skewed toward the small values exhibited by 'NY 54'. For mesocarp cell number, mesocarp length, and mesocarp cell length, 87%, 94%, and 87% of the progeny averaged smaller than the mid-parent value for the trait, respectively. Transgressive segregation occurred for all traits, although progeny averaging greater than 'EF' were only measured for mesocarp cell length (Figure 1). The broad sense heritability was high for each trait, although measurements were made only in the year 2005 (Table 1). A positive linear correlation existed between mesocarp cell number and mesocarp length ($P < 0.0001$) and between mesocarp cell length and mesocarp length ($P < 0.0001$). A significant negative correlation was calculated between mesocarp cell number and mesocarp cell length ($P < 0.05$).

Significant QTL were identified only for mesocarp length and mesocarp cell length (Table 2). For mesocarp length, one QTL was identified on the bottom portion of 'EF' LG 6 (Fig. 2) and one on the bottom portion of 'NY 54' LG (y) (Fig. 4). The QTL on 'EF' LG 6, *mlength1* explained 18.3% of the total phenotypic variance and had an effect as predicted by the parent, increasing mesocarp length by 0.31 mm. The closest marker to *mlength1* was the AFLP marker, EAT/MCCC-100, 0.1 cM from the LOD peak. The QTL on 'NY 54' LG (y), *mlength2*, explained 37.4% of the phenotypic variation, reduced mesocarp length by 0.41 mm, and was 3.9 cM from the nearest marker, EAT/MCCC-150. Three QTL were identified for mesocarp cell length; on the central portion of 'EF' LG 6 (Fig. 2), and at the bottom of 'NY 54' LG 6 (Fig. 3) and LG (y) (Fig. 4). The QTL on 'EF' LG 6, *clength1*, explained 17.4% of the phenotypic variation and had an effect similar to that predicted by the parent, increasing cell length by 7.62 μm. The closest marker to *clength1* was CPPCT029-195, 0.1 cM from the LOD peak. The QTL on both 'NY 54' LG 6 and LG (y) explained 16.8% of the phenotypic variation and reduced cell length by 7 μm. The QTL *clength2* on 'NY 54' LG 6 was 0.1 cM from MA040a-225, while *clength3*, on 'NY54' LG (y) was co-located with EAT/MCCC-150.

Fruit size QTL have been identified in other *Prunus* species. In sour cherry, two fruit weight QTL accounting for over 29% of the phenotypic variation were identified in the 'RS' × 'EB' population (Wang et al., 2000). However, there are no common markers between the 'RS × 'EB' and 'NY 54' × 'EF' linkage maps and comparison of the QTL is not possible. In the 'UF' × 'Surefire' sour cherry population, a QTL for fruit weight was identified on the bottom of LG 6 (see Chapter 4), the same linkage group that QTL for

mean cell size and mesocarp length were identified in 'EF' and 'NY 54'. Furthermore, fruit weight QTL have been identified on both the bottom and central portions of LG 6 in peach populations (Dirlewanger et al., 1999; Etienne et al., 2002; Quilot et al., 2005; Yamamoto et al., 2001), similar to the location of mean cell length and mesocarp length QTL identified on 'EF' LG 6 and 'NY 54' LG 6. This suggests potential conservation of fruit size QTL in *Prunus*.

For all QTL identified in this study, the effect of the QTL was in the same direction as predicted by the parental phenotype. However, this may not be an accurate representation of fruit size QTL present, as the use of an F_1 population limits the ability to identify QTL in a heterozygous background (Conner et al., 1998; Grattapaglia and Sederoff, 1994; Wang et al., 2000). In this case, for a QTL to be significant, the effect has to be sufficiently large enough to outweigh the variance of other potential loci influencing the trait. If the parents are heterozygous for alleles at these loci, as they are presumed to be in sweet cherry, both large and small fruit size QTL from both 'NY 54' and 'EF' are likely to segregate in the population (Wang et al., 2000).

The transgressive segregation and skewed population distribution toward small fruit size suggests that small fruit size alleles are also present in 'EF'. However, given that only 35% of the 'NY 54' × 'EF' mapping population had fruit for evaluation in the first bearing year, it is not possible to draw conclusions relative to the inheritance of these traits. For example, if small fruit size is linked to precocious flowering, it would significantly bias the phenotype of those seedlings available for analysis. Nonetheless, the ability to identify QTL associated with fruit size with such a small population size is encouraging. The stability of the QTL identified in this study is yet to be determined.

For example, because of the abbreviated fruit development period before the fruit were sampled, the QTL identified for mesocarp length and mesocarp cell length may actually be indicative of ripening date rather than overall fruit size. The larger fruit from certain individuals may simply have been closer to maturity. These questions will be answered in the coming years when the full complement of fruit traits can be analyzed in this population.

CONCLUSIONS

In this preliminary analysis of fruit size traits for the 'NY 54' × 'EF' sweet cherry population, QTL were identified in both parents for mesocarp length and mesocarp cell length. Unfortunately, no QTL were identified for mesocarp cell number, a trait that has been documented to influence fruit size in *Prunus* (Scorza et al., 1991; Yamaguchi et al., 2002a, 2002b, 2004). This may be due to the limited number of individuals available for QTL analysis in the first bearing year of the 'NY 54' × 'EF' sweet cherry population and the skewed distribution toward the parent with fewer mesocarp cell numbers. However, it is encouraging that QTL for both mesocarp length and mesocarp cell length were identified on LG 6 of the parents in this population, given that fruit size QTL have previously been located on this linkage group in other *Prunus* species (see Chapter 4; Dirlewanger et al., 1999; Etienne et al., 2002; Quilot et al., 2005; Yamamoto et al., 2001).

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TABLES AND FIGURES

Figure 1. Frequency distribution of fruit mesocarp radial cell number (A), cell length (B), and mesocarp radial length (C) measured at endocarp hardening for 67 progeny in the 'NY 54' × 'Emperor Francis' ('EF') sweet cherry population in 2005. Means for the parents 'NY 54' and 'EF' are shown by arrows.

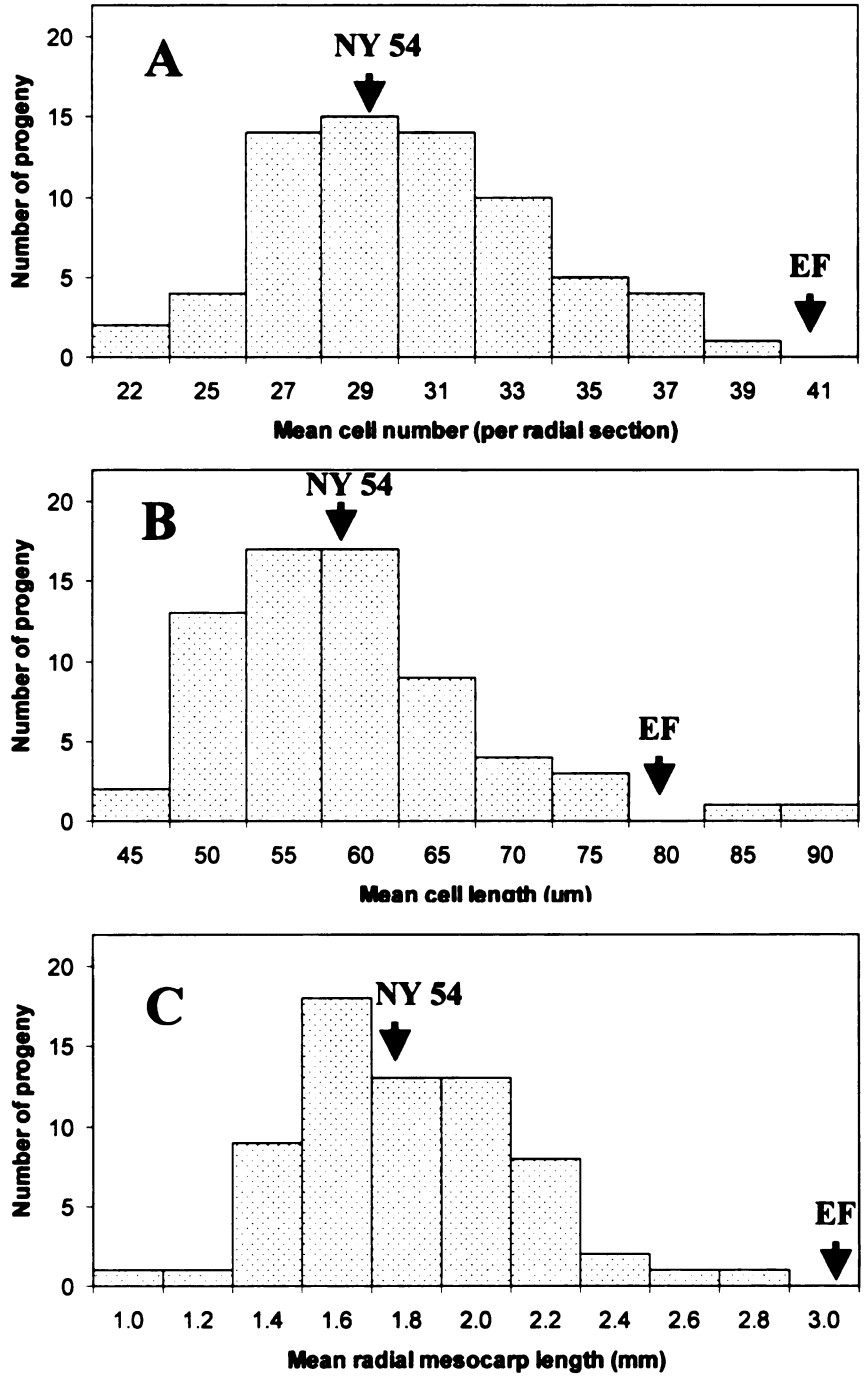


Table 1. Broad sense heritability (H^2), mean phenotypic values and standard deviations, and progeny value range for sweet cherry fruit mesocarp radial cell number, mesocarp radial length, and mesocarp mean cell length at endocarp hardening.

Trait	Broad-sense heritability (H^2)	Mean ± SD			Progeny range	
		NY 54	EF	Progeny	Minimum	Maximum
Mesocarp cell no. (radial)	0.88	29.0 ± 2.12	40.6 ± 5.64	30.9 ± 0.90	22.0	38.6
Mesocarp length (mm)	0.90	1.71 ± 0.08	3.18 ± 0.30	1.89 ± 0.09	1.19	2.85
Mean cell length (μm)	0.87	59.2 ± 7.14	79.0 ± 7.54	61.5 ± 2.70	45.8	95.3

Table 2. QTL detected for mesocarp radial length and mean cell length at endocarp hardening in 'NY 54' and 'Emperor Francis' ('EF') fruit for the year 2005.

	QTL	Linkage group	Interval length (cM)	LOD peak position (cM)	Maximum LOD	Nearest marker	R^2 (%)	Genetic effect: a[z]
Mesocarp length (mm)	*mlength1*	EF 6	11.8	55.7	3.3	EAT/MCCC-100	18.3	0.31
	mlength2	NY(y)	> 13.9	26.0	5.0	EAT/MCCC-150	37.4	- 0.41
Mean cell length (μm)	*clength1*	EF 6	23.7	33.1	3.8	CPPCT029-195	17.4	7.62
	clength2	NY 6	2.3	65.3	3.2	MA040a-225	16.8	- 7.00
	clength3	NY(y)	> 7.92	29.9	3.2	EAT/MCCC-150	16.8	- 7.07

[z] a, Additive value of the QTL.

Figure 2. Significant QTL for fruit mesocarp cell length (A) and mesocarp radial length (B) at endocarp hardening on 'Emperor Francis' ('EF') LG 6. Vertical lines on the graph indicate the LOD significance threshold based on 1000 permutations. Bars between the graph and linkage group indicate 1-LOD (filled) and 2-LOD (bars) interval for the QTL peak on the graph.

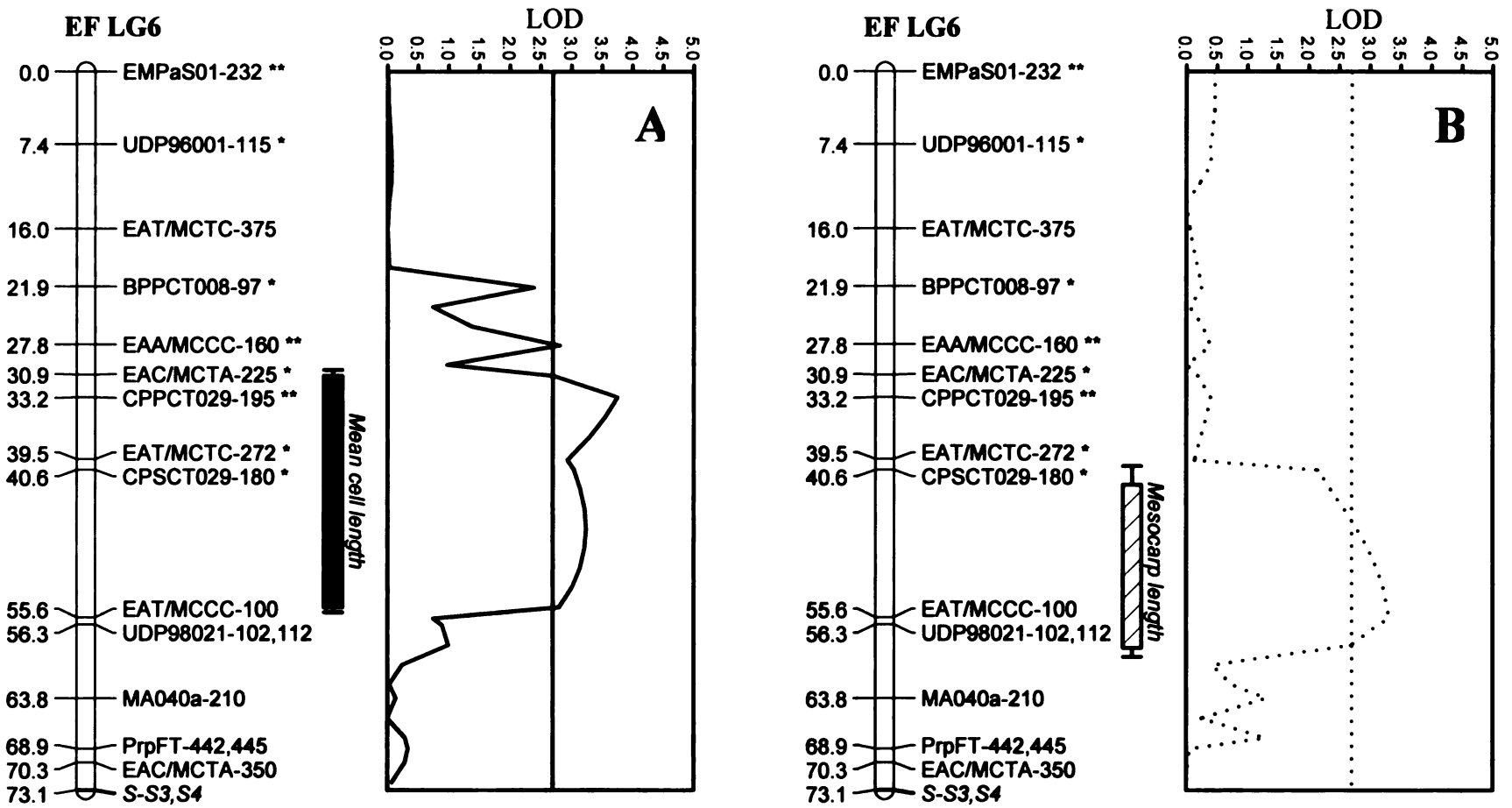


Figure 3. Significant QTL for fruit mesocarp cell length at endocarp hardening on 'NY 54' LG 6. Vertical lines on the graph indicate the LOD significance threshold based on 1000 permutations. Bars between the graph and linkage group indicate 1-LOD (filled) and 2-LOD (bars) interval for the QTL peak on the graph.

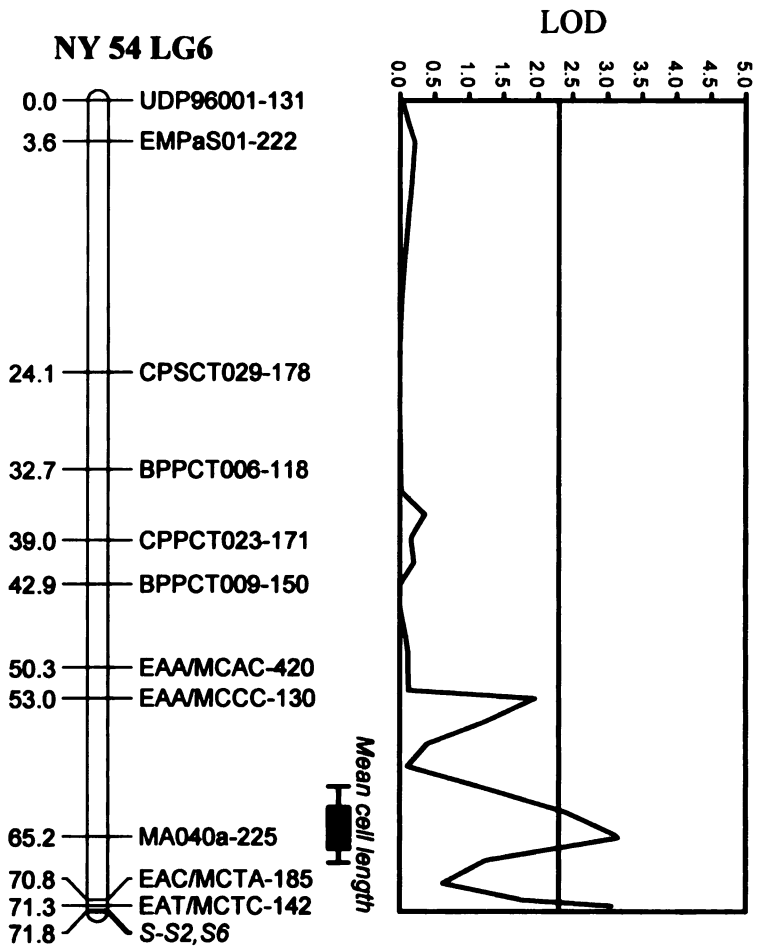
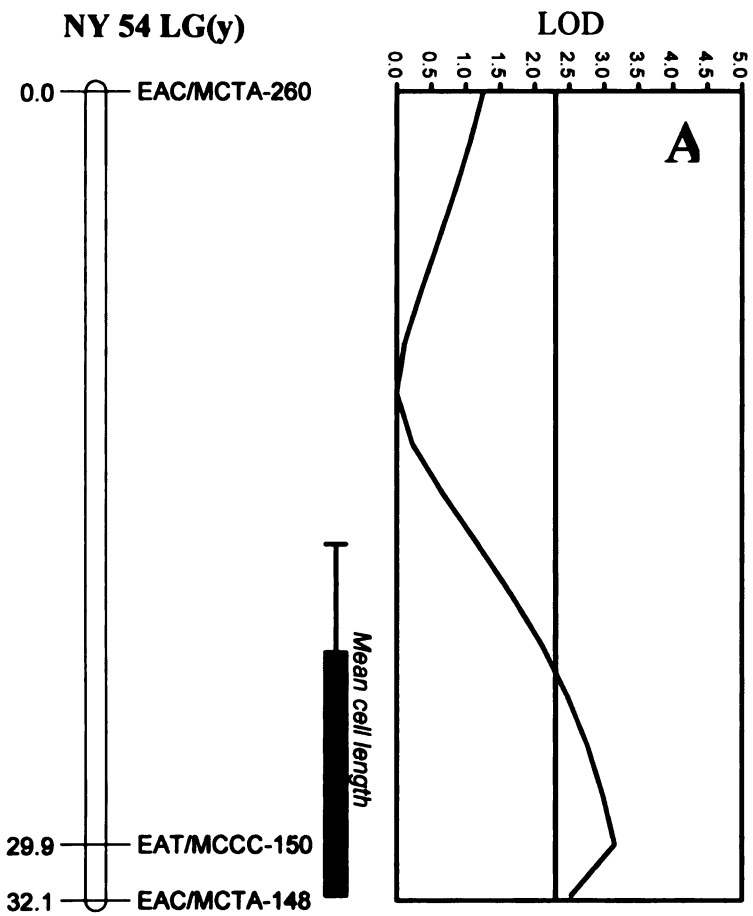
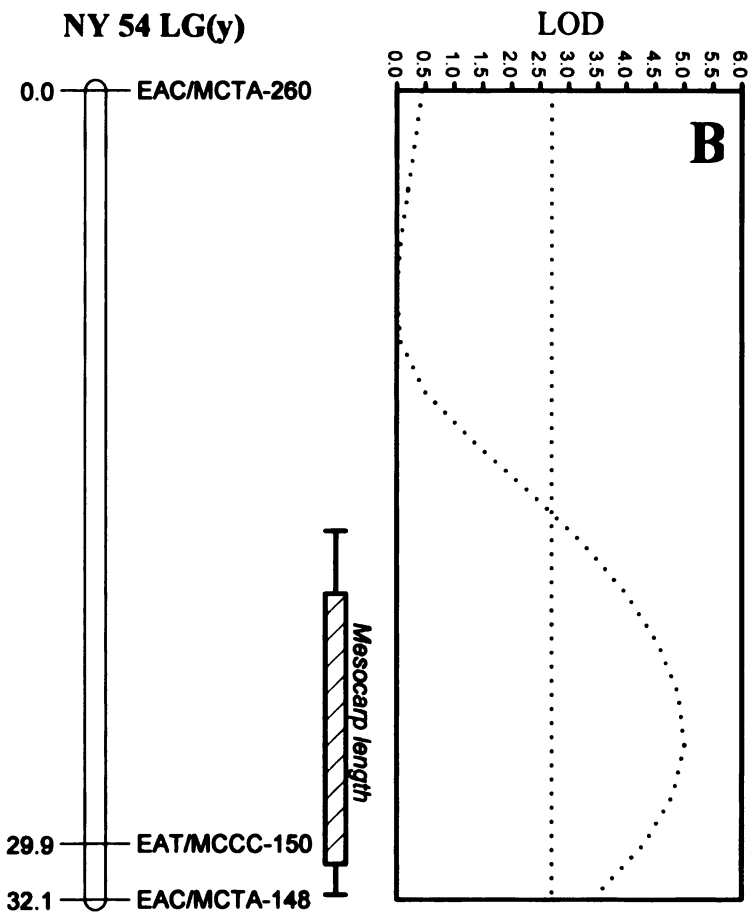


Figure 4. Significant QTL for fruit mesocarp cell length (A) and mesocarp radial length (B) at endocarp hardening on 'NY 54' LG (y). Vertical lines on the graph indicate the LOD significance threshold based on 1000 permutations. Bars between the graph and linkage group indicate 1-LOD (filled) and 2-LOD (bars) interval for the QTL peak on the graph.





CHAPTER SIX

SUMMARY AND CONCLUSIONS

In an increasingly competitive market, production of high-quality sweet (*Prunus avium* L.) and sour (*P. cerasus* L.) cherries has become essential. For fresh markets, large fruit size is critical for profitable production. Increased and consistently large fruit size is an area of continued horticultural and physiological research with existing production cultivars. However, breeding efforts will continue to be an important avenue to increase cherry fruit size. Unfortunately, the long generation time of perennial tree fruit crops such as cherry and the quantitative nature of the fruit size trait make breeding for improved fruit size inefficient. A better understanding of the cellular basis for fruit size potential, both among and within cultivars, could increase selection efficiency in cherry breeding programs. Further increases in efficiency would be realized if initial selection for fruit size was based on genotypic markers rather than phenotypic expression of the trait after the juvenility period has passed.

The research reported herein examined fruit mesocarp cellular differences between cultivars with a wide range of average fruit sizes and within fruit from single cultivars exhibiting significant size differences (Chapter 2). Mesocarp cell number differences between cultivars were correlated with increasing fruit size, while mesocarp cell size was not. However, differences in cell size were observed between cultivars. For example, mesocarp cell sizes in fruit from 'Selah', the cultivar examined with the largest fruit size, were not significantly different than those in 'New York 54' ('NY 54'), the cultivar with the smallest fruit size. 'Bing', 'Regina', and 'Emperor Francis' ('EF'), all with fruit sizes falling between 'Selah' and 'NY 54', had significantly larger cell sizes. Mesocarp cell number was environmentally stable and did not differ when fruit thinning treatments were applied; whereas mesocarp cell size contributed to the increase in fruit

size gained from reduced crop load. The low environmental variance exhibited for mesocarp cell number makes it an obvious selection criterion for improved fruit size in cherry breeding programs.

To further examine the genetic control of fruit size in sweet cherry, a linkage map was constructed for reciprocal crosses between 'NY 54' and 'EF' (Chapter 3). These parents were selected to represent the genetic differences accumulated during the domestication of sweet cherry, by crossing a wild example ('NY 54') with an early domesticate ('EF'). Simple sequence repeat (SSR) markers developed in other *Prunus* species were used extensively to facilitate map comparison within *Prunus*. Although the map is incomplete and only the first year of fruit phenotypic data was available, a preliminary quantitative trait loci (QTL) analysis identified fruit size QTL, predominantly on LG 6 of both parents (Chapter 5). This linkage group represents an important chromosome in *Prunus*, as it also contains the self-incompatibility locus (*S*), and fruit size QTL on LG 6 have been identified previously in peach [*P. persica* (L.) Batsch.]. Fruit size QTL on LG 6 were examined further using a targeted mapping approach, whereby only SSR loci previously mapped to LG 6 in other *Prunus* species were used to develop a linkage map for the 'Újfehértói Fürtös' ('UF') × 'Surefire' sour cherry population (Chapter 4). A QTL three cM from the *S* locus explaining 26.4% of the phenotypic variation was identified in the 'UF' × 'Surefire' population. Additionally, a fruit shape QTL was also located on LG 6, co-segregating with the CPSCT012 marker and explaining up to 22.6% of the phenotypic variation for fruit shape.