CONTINUING PROJECT (FINAL REPORT)
WTRFC Project # CH-04-400

Project title: Environmental and Genetic Influences on cherry fruit size
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OBJECTIVES

Maximizing fruit size is critical for profitable sweet cherry production. For any given variety, (e.g., Bing), the grower's goal is to achieve the fruit's genetic potential for size by using "proper" management practices. New varieties with the genetic capacity to produce larger fruit, such as Selah, provide an additional means to achieve large fruit size.

Both environmental and genetic methods of fruit size increase have been studied in the past. Currently, great strides are being made in the understanding of the physiological "carrying-capacity" of cherry trees (i.e., optimal leaf to fruit ratios for desired fruit size). Cultural manipulations such as blossom thinning, pruning, irrigation, and fertilizer management are also important methods of achieving large cherry size. However, fruit size continues to be a concern, particularly with the adoption of dwarfing rootstocks and the potential for over-cropping. New varieties released in the past decade, in most cases, have been selected for large fruit size (among other selection criteria) and their fruits are considerably larger than those from standard varieties. Despite these advances, the basic genetic and environmental mechanisms that result in large vs. small fruit are not well understood, thus limiting our ability to maximize the number of consistently large fruit.

Our overall goal was to understand the bases for achieving large fruit size in sweet cherry. Our experiments are designed to provide knowledge that will be used to design future management and genetic improvement strategies that would ultimately result in maximized fruit size in grower orchards. Our objectives were based upon the premise that fruit size is maximized using both optimal cultural practices (environment) and large-fruited varieties (genotype).

The specific objectives of this research were to:

1. (a) Determine the effect of cultural practices, such as crop load manipulation, on cell size and cell number in Bing and Regina cherry. (b) Determine the developmental timing of these differences.
2. (a) Determine the differences in cell size and cell number associated with genetic differences in fruit size using three varieties that differ dramatically in fruit size. (b) Determine the developmental timing of these differences.
3. Determine the quantitative trait loci (QTL) that contribute to large fruit size. This is the first step towards the identification of the major genes controlling fruit size in sweet cherry.

SCHEDULE OF ACCOMPLISHMENTS

End of YEAR 1
(1) Completion of the comparison of cell size and number from Bing and Regina fruit from crop load treatments.
(2) Completion of a precise determination of the developmental stage(s) in which differences in cell size and cell number occur in Selah, Emperor Francis (EF), and New York 54 (NY54).
(3) Initiated the construction of the sweet cherry linkage map.

During the first project year (2004), Bing and Regina fruit from trees adjusted for crop load were sampled from Prosser. Crop load adjustment resulted in significant fruit size differences (see Results and Discussion). Within each variety, the fruit size increase apparent with thinning was due to increases in cell size, not cell number. In contrast, a comparison of fruit from different varieties exhibiting a wide range of fruit sizes confirmed our previous observation that cell number differences are the primary genetic determinant of fruit size. The period between bloom and pit hardening was identified as the developmental period when cell number differences were first apparent (see Results and Discussion). Construction of a sweet cherry linkage map was initiated.

End of YEAR 2
(1) Completion of a precise determination of the developmental stage(s) in which differences in cell size and cell number occur in Bing and Regina fruit of different sizes subjected to different crop loads.
(2) Completion of the initial QTL analysis to identify regions containing gene(s) contributing to fruit size in sweet cherry.

During the final project year (2005), Bing, Regina, and Selah fruit with significant fruit size differences were harvested at maturity to confirm the previous year's finding that fruit size increase within genetically identical fruit was due to increases in cell size, not cell number. Additionally, fruit from those varieties were sampled during the developmental period identified in the previous year as most important for cell number increase, thus giving a comparison of both the rate and duration of cell division for varieties with very different final fruit sizes (see Results and Discussion). An initial sweet cherry linkage map was developed as a resource for quantitative trait loci (QTL) mapping of genomic regions important for fruit size in cherry.

SIGNIFICANT FINDINGS AND ACCOMPLISHMENTS

- When comparing different sweet cherry varieties, the most important determinant of final fruit size is the number of cells in the flesh.
- Cell number accumulation within a single variety is remarkably stable over both years and different environments.
- Within the same variety, larger fruit have the same number of cells as smaller fruit, but the cells are larger than those of smaller fruit.
- Differences in flesh cell number among different varieties are not apparent until after bloom.
- When comparing different sweet cherry varieties, both the duration and rate of flesh cell division differs.
- Cell division in the flesh does not continue past pit hardening.
- Fruit size QTL were successfully identified for overall fruit size increase but have yet to be found for cell number increase.

- The results from this two year project were used to successfully obtain a USDA-CSREES-NRI Award of $400,000 over three years [P.I. Amy Iezzoni; Title: Genomic resources to improve fruit size and quality in cherry].

METHODS

Objective 1 (within genotype): (a) Determine the effect of cultural practices on cell size and cell number in Bing and Regina cherry. (b) Determine the developmental timing of these differences.

Plant material: Samples were collected from mature Bing and Regina trees at WSU-Prosser that were subjected to crop load adjustments and/or exhibited significant within-tree variation for fruit size.

Measurements: Prior to the preparation of tissue sections for microscopy, the quality of each individual fruit was evaluated (i.e., weight, diameter, firmness). Cell number and size was visualized by laser confocal microscopy, taking advantage of resources and equipment available at the Center for Advanced Microscopy at Michigan State University. Images created on the confocal microscope were analyzed using digital image processing software. Fruit sections were created according to protocols described previously (Fig. 1).

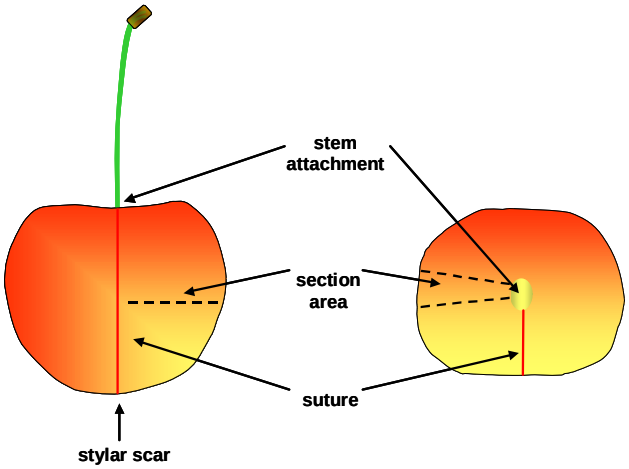

Figure 1. Slide sections were uniformly prepared from the thickest part of the fruit flesh. Radial sections were cut halfway between the point of stem attachment and the stylar scar. Sections consisted of all the flesh from the pit cavity to the epidermal layer.

Objective 2: (a) Determine the differences in cell size and cell number associated with genetic differences in fruit size using three varieties that differ dramatically in fruit size. (b) Determine the developmental timing of these differences.

Plant material: Fruit from three varieties, Selah (~ 12 g), EF (~ 6 g), and NY54 (~ 2 g), were evaluated for differences in fruit cell size and cell number over multiple years. EF and NY54 were used as parents to develop a population designed to identify genes contributing to fruit size by identifying genetic changes associated with domestication.

Measurements: Cell number and cell size measurements were conducted at MSU using the procedures described in Objective 1.

Objective 3: Determine the quantitative trait loci (QTL) that contribute to large fruit size.

Plant material: 200 progeny from reciprocal crosses made in 2001 between EF and NY54 were evaluated and used for development of a molecular marker linkage map. Over 700 additional

progeny from this cross are also planted in Michigan. The larger population will be necessary for future fine mapping and map-based gene cloning.

QTL Analysis: The 200 progeny were scored using "high-thoughput" markers (AFLP and SRAP markers) and markers suitable for comparative mapping with other *Prunus* species (SSR markers). In 2005, the first fruit were available for QTL analysis from the fruit size population developed at Michigan State University. Both overall fruit size and cell number measurements were taken from all progeny fruiting in 2005. QTL analyses were performed using QTL Cartographer.

RESULTS AND DISCUSSION

In our comparison of fruit size between a large size cherry (Selah), a medium size cherry (EF) and a small-fruited mazzard cherry (NY54), the difference in final fruit size was primarily due to a difference in cell number and not cell size (Table 1). The nearly 11.5 gram difference in size between NY54 and Selah was due to a 74% increase in the number of flesh cells and only a 24% increase in the size of those cells. Clearly, cell number increase was the most important factor in the increased fruit size of Selah. The characteristic number of cells in the flesh of each variety also proved to be remarkably stable during the course of this experiment, indicating this trait is under strong genetic control (Table 2).

Table 1. Comparison of fruit anatomical and morphological characteristics among Selah, EF, and NY54.

Variety	Fruit[z] wt. (g)	Fruit dia. (mm)	Cell no. (pit to skin)	Avg. cell length (mm)
Selah	12.8 a[y]	26 a	83 a	0.148 b
EF	6.1 b	21 b	47 b	0.168 a
NY54	1.4 c	12 c	27 c	0.136 b

[z]Avg. of 25 fruit for weight and diameter, 5 for cell size and number.
[y]Mean separation in columns by LSD at $P < 0.05$.

Table 2. Yearly comparison of cell number between Selah, EF, and NY54.

Year	NY54	EF	Selah
2003	27 a[y]	47 a	83 a
2004	29 a	41 b	79 a
2005	28 a	38 b	79 a

[y]Mean separation in columns by LSD at $P < 0.05$.

For the same three varieties, samples taken in 2004 at different fruit developmental stages indicated that differences in cell number approximating the final cell number count were evident by the onset of pit hardening. More importantly, all three varieties sampled at bloom had similar numbers of cells in the ovary wall, tissue destined to become flesh as fruit development proceeds (Table 3). Therefore, the large differences in final cell numbers exhibited in the three varieties happened exclusively during the Stage I fruit development period from bloom to pit hardening. Using this information, an additional set of samples for EF were collected in Michigan, starting at bloom and continuing every 20 growing degree days (base temperature 40 F) until pit hardening occurred. This set of samples further narrowed the time during which fruit cell number increased in EF to a period of 6-10 days after full bloom (Fig. 2). The relatively short duration of this cell division period was surprising, suggesting the basis for final fruit size was determined very early in the fruit developmental period. In 2005, more varieties were evaluated. Samples were collected daily from Selah, Bing, Regina, and NY54 trees at Prosser to determine whether the rate and/or duration of cell division during this time period differed between varieties that had very different final fruit sizes.

Table 3. Comparison of cell number between Selah, EF, and NY54 at different fruit developmental stages.

Variety	Bloom	Pit harden	Harvest
Selah	24 b[y]	70 a	83 a
EF	17 a	40 b	47 b
NY54	25 b	26 c	27 c

[y]Mean separation in columns by LSD at $P < 0.05$.

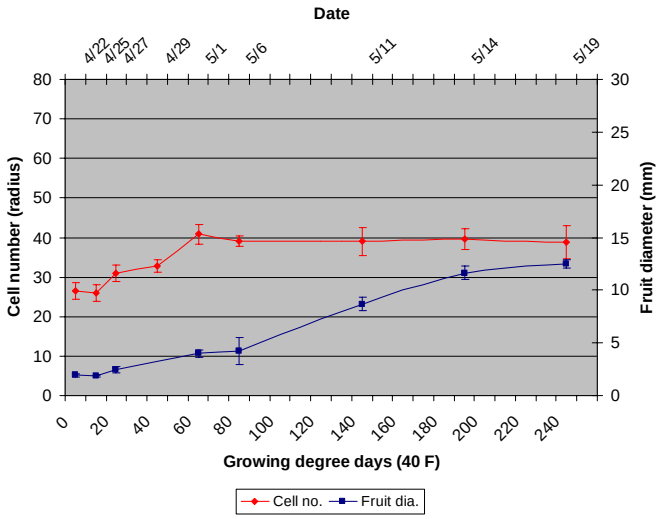

Figure 2. Cell number and fruit diameter increase in EF during Stage I fruit development (Michigan 2004).

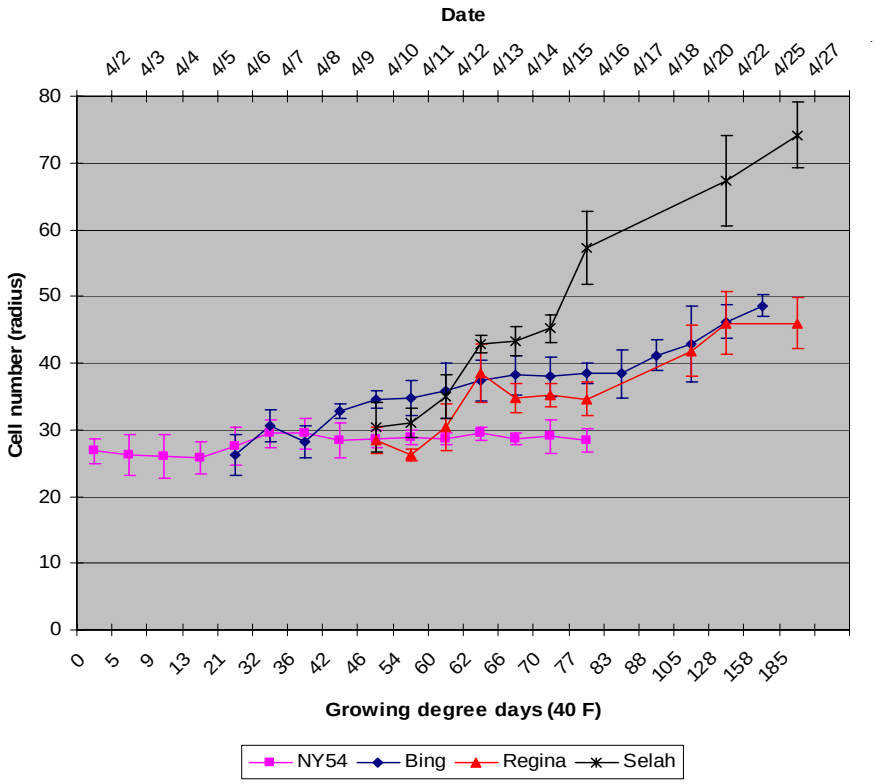

Figure 3. Comparison of cell number increase between NY54, Bing, Regina, and Selah during Stage I fruit development (Washington 2005). Sampling was discontinued when fruit reached cell numbers equivalent to harvest samples.

For these samples, both the duration and rate of cell division differed among varieties (Fig 3). An increase in the number of cells measured in the variety corresponded to an increase in the duration of cell division (Table 4). Selah, the variety with the largest overall fruit size also had the highest rate of cell division during this period.

Table 4. Duration and rate of cell division in NY54, Regina, Bing, and Selah during Stage I fruit development.

Variety	End cell no. pit to skin	GDD accum. when final cell no. reached	Cell division rate (no./GDD)
NY54	28	30	0.06
Regina	45	80	0.22
Bing	47	115	0.19
Selah	79	140	0.34

To better understand the environmental influences on fruit size, fruit of significantly different sizes from the same variety were sampled. Although the samples had a large variation in fruit size, they were genetically identical having been harvested from the same variety. These samples addressed Objective 1, and any cell number or size differences were due to environmental factors and not under genetic control. In 2004, Bing and Regina fruit were harvested from trees that had been thinned to 1 flower bud/spur prior to bloom. Fruit sizes on the thinned Bing trees were nearly two grams larger than the unthinned control at harvest. Thus, the use of crop load adjustment provided a method to generate differences in fruit size within a single variety. However, low crop load on both thinned and unthinned Regina trees resulted in no significant overall fruit size difference between the two treatments. Likewise, spring frost in 2005 prevented random sampling of thinned vs. unthinned treatments for Bing, Regina and Selah from generating significant fruit size differences. In both these cases, large lots of fruit were harvested, and individual fruit were weighed. Two pools of large and small fruit from each variety were created and analyzed. Although no specific treatments were applied to generate fruit size differences, selection of large and small fruit still fit the objective to test environmental differences between fruit from the same variety.

The pertinent question in this experiment was whether fruit size increase in the same variety was due to cell number increase (as indicated in the comparison of Selah, EF, and NY54), or cell size increase. Our results indicate that the large fruit size within a single variety was due to an increase in cell size (Table 5). Both large and small fruit had near identical flesh cell numbers, further illustrating the fact that cell number is under strong genetic control.

The final size of cherry fruit results from both an increase in the number of cells in the fruit and the expansion of those cells. Although expansion of the cell volume contributes greatly to overall fruit size, it is the total number of cells in the fruit flesh that sets the stage for eventual fruit expansion. Our results strongly suggest that the average cell number in the flesh is a genetically controlled trait. Variation in cell number was the most significant and consistent difference between a very small size (NY54) mazzard fruit and the very large sized Selah fruit. There are simply fewer cells available for expansion in NY54 than in Selah. Consistent with our hypothesis that cell number is a strongly genetically controlled trait, analysis of fruit from the same variety revealed similar cell numbers in the flesh regardless of final fruit size. Because all trees of the same variety are genetically

identical, the lack of variation for this trait indicates that variation in fruit size within a single tree is due to environmental influences on cell size.

Table 5. Comparison of fruit size characteristics between small and large Bing, Regina, and Selah fruit.

		2004			2005		
Variety	Treatment	Avg.[z] fruit wt. (g)	Cell no. (pit to skin)	Avg. cell length (mm)	Avg.[z] fruit wt. (g)	Cell no. (pit to skin)	Avg. cell length (mm)
Bing	High wt.	9.4***	49 ns	0.196*	11.3***	49 ns	0.208*
Bing	Low wt.	7.6***	48 ns	0.181*	7.5***	48 ns	0.185*
Regina	High wt.	10.3***	46 ns	0.214*	12.4***	47 ns	0.219*
Regina	Low wt.	7.7***	44 ns	0.195*	8.3***	47 ns	0.176*
Selah	High wt.				13.7***	79 ns	0.137 ns
Selah	Low wt.				8.8***	78 ns	0.125 ns

[z]Mean separation in columns by LSD; n.s. = non significant, *, **, *** = significance at $P<0.05$, 0.01, 0.001 respectively

Due to low heterozygosity in the NY54 parent, a complete genetic linkage map is not yet available. Currently, 7 and 5 of the 8 total linkage groups for sweet cherry are available for QTL analysis for EF and NY54, respectively. The EF parental map comprises a total distance of 494.4 cM, while the NY54 map only measures 214 cM (Fig. 4). Linkage groups EF1, EF4, EF5, EF6, EF7, and NY5 can currently be aligned with the consensus *Prunus* linkage map available through the Genome Database for Rosaceae (http://mainlab.clemson.edu/gdr/). Although there are still several large gaps in both parental maps, these are anticipated to be filled with continued high-throughput AFLP marker development. Map development is ongoing, as the construction of a high density sweet cherry linkage map is on of the goals of the USDA-CSREES-NRI Award.

Fruit were available for 67 of the 200 progeny from the NY54 x EF mapping population. Because of the apparent stability and importance in overall fruit size, analysis efforts were concentrated on the cell number differences among progeny in the population. The population mean for this trait was 31 cells, significantly skewed toward the NY54 parent (Fig. 5). Although the stability of the cell number trait should significantly enhance our ability to locate corresponding QTL, none have yet been identified. This may be due to incomplete coverage of the current linkage map or low numbers of fruiting progeny. Although a QTL for the cell number trait was not found, one significant QTL explaining 23% of the phenotypic variation for fruit diameter was identified on linkage group 6 of the EF parent (Fig. 6). This QTL spans the region occupied by a marker from the AFLP primer combination EcoRI+AA/MseI+CCC.

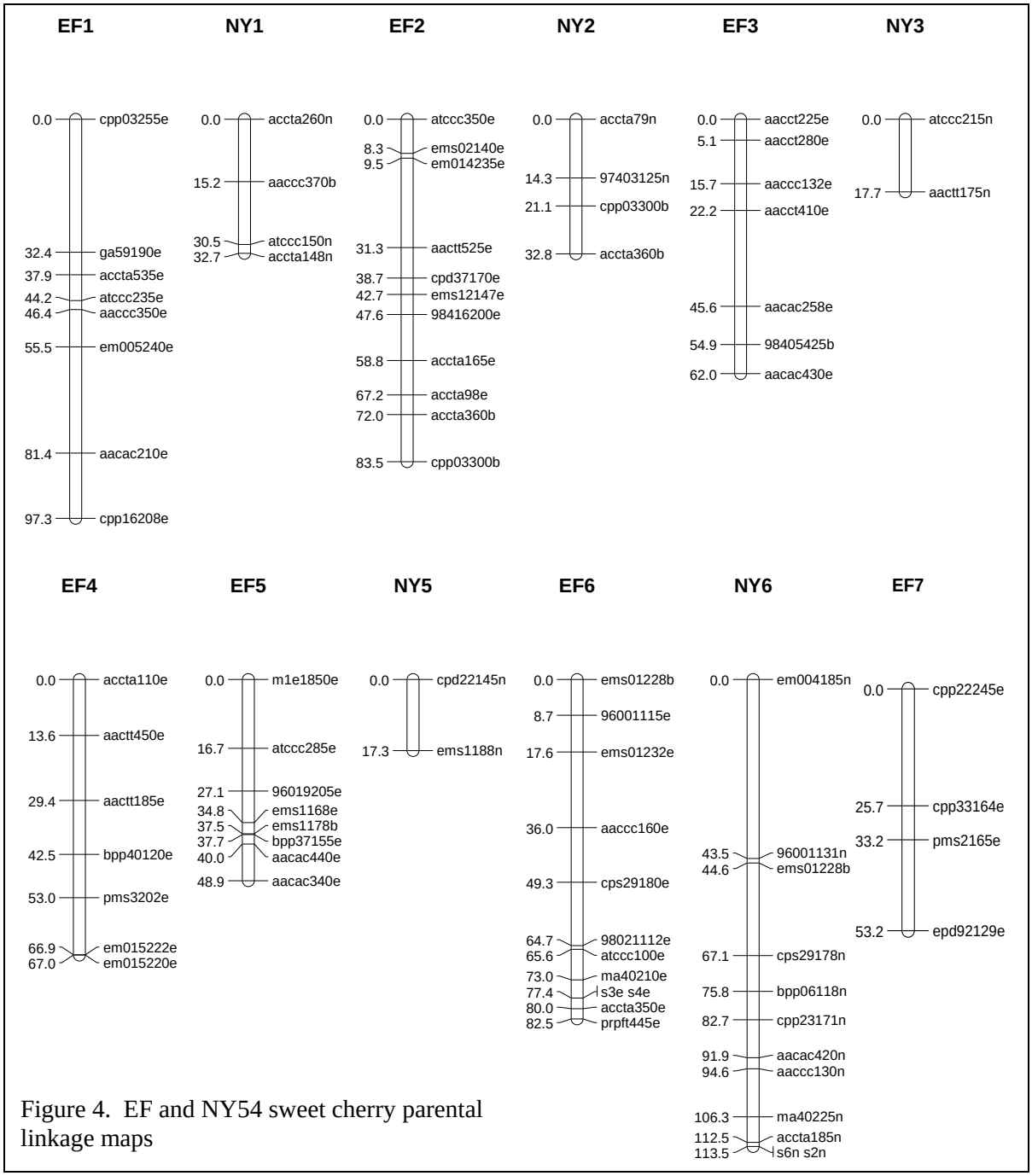

Figure 4. EF and NY54 sweet cherry parental linkage maps

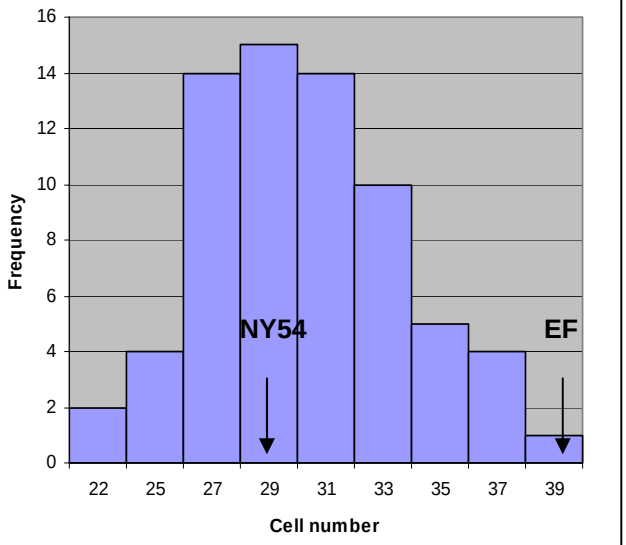

Figure 5. NY54 x EF population distribution for the cell number trait.

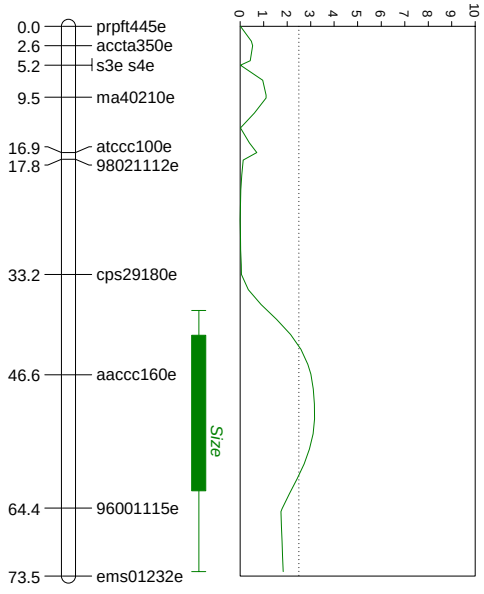

Figure 6. QTL for fruit size located on EF linkage group 6. Green bar indicates significant QTL region.

CONCLUSIONS

The first major outcome of this work is a new understanding of how cell number and cell size contribute to overall fruit size in sweet cherry, and how these parameters are altered given different environmental and genetic influences. In summary, in the set of varieties we have tested, the flowers had approximately the same cell number at bloom. The primary cellular difference between varieties with significant final fruit sizes was an increase in the number of cells in the flesh. However, cell division, and thus final cell numbers was not affected by environmental differences. Surprisingly, cell division in the developing cherry fruit occurred very early in Stage I growth, with larger-fruited varieties undergoing cell division for a longer period of time. This detailed knowledge can be utilized by cherry physiologists to target cultural practices aimed at maximizing fruit size, and geneticists to dissect the genetic control of this important trait.

The second major outcome of this work was the initiation of linkage map construction and QTL analysis for fruit size. This information will be used to design molecular markers for the early identification of selections with large fruit size in the breeding program. Additionally, the data obtained in this project was used to write a proposal that resulted in a $400,000 USDA-CSREES-NRI Award to not only continue this search for fruit size QTL, but broaden this search to include QTL for other fruit quality traits (sugars, acids, etc.). Therefore no continued funding is requested. Below is an abstract of the NRI Award that began June 1, 2005.

Genomic resources to improve fruit size and quality traits in cherry

Amy Iezzoni (Principal Investigator), Wayne Loescher, Esther van der Knaap*, & Dechun Wang
Michigan State University & *Ohio State University

Fruit size and quality (e.g. firmness, color, sugar etc.) are the most important market driven traits in cherry. For profitable cherry production, growers must achieve sufficient fruit size and quality standards. Unfortunately consistently maximizing fruit size and quality in grower orchards and in new varieties thorough breeding has been difficult because the genetic control of these traits is not well understood and the genetic tools to facilitate trait improvement do not exist. This USDA Award will result in the development of the genomics tools necessary to implement more efficient selection for fruit size and quality traits in breeding programs. Our analyses will also lead to a better understanding of the developmental timing and genetic control of these traits. This opens up the possibility of targeting cultural interventions to enhance or delay a desired fruit characteristic.

BUDGET

Title: Environmental and Genetic Influences on the Components of Fruit Size in Sweet Cherry

P.I.: Amy Iezzoni
Project Duration: 2 years (2004-2005)
Current Year: 2005
Project Total (2 years): $50,442

Item	Year 1 (2004)	Year 2 (2005)
Salary[1]	$16,344	16,998
Wages[2]	500	500
User fee – confocal microscope[3]	1,500	1,500
Supplies[4]	4,000	4,000
Travel[5]	550	550
Plot fees at CHES[6]	1,000	1,000
Prosser costs[7]	1,000	1,000
Total	$24,894	$25,548

[1]Salary for Ph.D. student.
[2] Wages for student labor to help with sample preparation.
[3] Fees for the use of the Confocal Laser Scanning Microscope at MSU's Center for Advanced Microscopy. The user fee is $15/hr.[http://www.ceo.msu.edu/Services.htm]
[4] Microscopy supplies are budgeted at $200/yr. Cost of supplies for DNA extraction and marker genotyping is $3,800/yr. This is based upon supply cost to genotype 190 progeny and the two parents using 25 AFLP primer pairs and 32 SSR primer pairs.
[5]Travel to Prosser at sweet cherry harvest to meet with Matt Whiting relative to the cultural experiments.
[6]Starting in 2004, plot fees are charged at all MSU Horticultural Research Stations. These costs are based upon a fee structure that reflects the cost of standard plot maintenance.
[7]These funds are for to Matt Whiting to cover the cost sampling and overnight shipping.