FINAL PROJECT REPORT

Project Title: Marker-assisted breeding strategies for large fruit and self-fertility

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Cooperators: Fred Bliss (Davis, California), Dorrie Main (WSU-Pullman), Jim McFerson (WTFRC), Jim Olmstead (Univ. Florida)

Other funding sources

Agency Name: USDA-CSREES NRI Plant Genome
Amount awarded: $400K, Aug 2009 – Aug2011
Notes: "The development of COS markers for comparative mapping in the Rosaceae and their application for understanding variation in fruit size". PI: Iezzoni. Leveraged with WTFRC/OSCC funding. Developed and validates fruit size genetic markers for sweet cherry, providing the marker tools for this project.

Agency Name: WTFRC Apple Review
Amount awarded: $77,616, Feb 2009 – Dec 2010
Notes: "Developing an online toolbox for tree fruit breeding". PI: Main. Co-PIs include Peace and Oraguzie. Databasing support for WSU cherry and apple breeding programs.

Agency Name: USDA-CSREES Specialty Crop Research Initiative
Amount awarded: $2.0 M plus equal matching, Sep 2009 – Aug 2013
Notes: "Tree Fruit GDR: Translating genomics to fruit tree agriculture". PI: Main. Co-PIs include Peace and Oraguzie. Leveraged with WTFRC funding. For practical application of bioinformatics to tree fruit crops.

Agency Name: USDA-CSREES Specialty Crop Research Initiative
Amount awarded: $3.8 M plus equal matching, Sep 2009 – Aug 2013
Notes: "A total systems approach to developing a sustainable, stem-free sweet cherry production, processing and marketing system". PI: Whiting. Co-PIs include Oraguzie. Leveraged with WTFRC/OSCC funding. In addition to developing genomics knowledge on cherry abscission for

amenability to mechanical harvesting, includes cell number and size measurements of local cultivars that integrated with this project.

Agency Name: USDA-CSREES Specialty Crop Research Initiative
Amount awarded: $7.2 M plus equal matching, Sep 2009 – Aug 2013
Notes: "RosBREED: Enabling marker-assisted breeding in Rosaceae". PI: Iezzoni. Co-PIs include Peace and Oraguzie. Broad umbrella project on genetic marker development and application. Leveraged with WTFRC/OSCC funding. Our MAB here for fruit size in Dr. Oraguzie's breeding program is enhanced with higher-resolution genetic markers, and used in RosBREED as a MAB success story.

Agency Name: WTFRC NW Cherry Review
Amount awarded: $95K in 2010, $100K in 2011
Notes: "Breeding and genetics program for PNW sweet cherries". PI: Oraguzie. Beneficiary of MAB for large fruit and self-fertility; phenotypic performance data of seedlings mostly provided by this program.

Agency Name: WTFRC NW Cherry Review
Amount awarded: $13K in 2010
Notes: "Consulting for the Pacific Northwest Sweet Cherry Breeding Program". PI: Iezzoni. Consultancy to enhance success of the PNWSCBP.

Total Project Funding: $88,600

Budget History:

Item	2010	2011	
Salaries			
Benefits			
Wages[1]	26,484	27,032	
Benefits	2,516	2,568	
Equipment			
Supplies[2]	11,000	11,000	
Travel[3]	4,000	4,000	
Miscellaneous			
Total	44,000	44,600	

Footnotes:
[1] Technical assistance - molecular work (Pullman): $15,000 (wages and 9.5% benefits) in year 1 and 4% increase in year 2; Technical assistance - phenotyping work (Prosser): $14,000 (wages and 9.5% benefits) each in year 1 and year 2.
[2] Reagents and consumables - molecular work (Pullman): $1500 for DNA extraction, $2000 for S-genotyping, $14,500 for fruit size marker genotyping, spread evenly over the two years; Consumables for phenotyping (Prosser): $2000 per year
[3] Within-state: Pullman-Prosser for coordination of experimental work: $2000 per year; Interstate: WSU-MSU for coordination among PIs: $2000 per year

RECAP ORIGINAL OBJECTIVES

The primary goal of this project was to finally apply DNA markers for improved efficiency of Pacific Northwest sweet cherry breeding (PNWSCBP), putting to use many years and dollars invested in developing the tools and infrastructure.

Specific objectives were to:

1. Deliver marker-assisted breeding (MAB) strategies for both large fruit and self-fertility to the PNWSCBP.
2. Exploit additional opportunities for key trait improvement in the PNWSCBP using MAB.
3. Coordinate MAB strategy development, implementation, and outreach in the PNWSCBP with Dr. Iezzoni's NRI project and RosBREED, and Dr. Main's Tree Fruit GDR project.
4. Deliver knowledge on cultivar genetic potential for fruit size to PNW cherry growers.

SIGNIFICANT FINDINGS

Objective 1: **Deliver MAB strategies for both large fruit and self-fertility to the PNWSCBP**.

- Strategies for DNA-informed breeding were delivered to the young, modern PNW sweet cherry breeding program, and enacted in the program since spring 2010 to provide more efficient and precise breeding.
- A net projected savings of more than $80K was estimated from the culling of more than 1500 seedlings predicted to be self-infertile or genetically inferior for fruit size ('09 and '10 crosses).
- Segregating populations for the next wave of seedlings ('10 and '11 crosses) were efficiently enriched for self-fertility and large fruit genetic potential by incorporating DNA information into crossing decisions of the last two years.
- The list of advanced selections destined for Phase 2 replicated trials was reduced by consideration of DNA information on fruit size and self-fertility. The potential savings here have not yet been calculated.
- Other aspects of breeding are now conducted more easily, precisely, and cost-effectively through MAB applications such as parentage verification and deduction.
- A full-time Genetic Screening Technician was hired at WSU for a critical role in sustained operations of the PNW Tree Fruit Genotyping Laboratory.
- Successful protocols for high-throughput DNA extraction and high-throughput genotyping were established for sweet cherry, meeting the logistical needs of routine MAB.
- The PNWSCBP is the first stone fruit breeding program in world to routinely conduct high-throughput marker-assisted seedling selection.
- Refinement of the fruit size genetic tests continues. Large datasets were obtained over the last two years for several thousand seedlings ('04, '05, and '06 crosses). Numerous fruit quality traits were evaluated. Genotypes were obtained at three genomic associated with fruit size, and *S*-genotypes. Statistical analyses in late 2011 are expected to provide a comprehensive understanding of the potential for fruit size MAB particularly in the context of other important traits.

Objective 2: **Exploit additional opportunities for key trait improvement**.

- Several further potential genetic tests have arisen and are being translated for use in the breeding program, including for the traits of firmness, sweetness, acidity, fruit color, and a non-'Stella' source of self-fertility.

Objective 3: **Coordinate MAB strategies with large federal projects**.

- Coordination with several large federal projects continued, with information on genetic markers shared among our projects.
- Advances were reported to industry and scientific audiences.
- The PNWSCBP is strategically positioned to utilize large federal investments in Rosaceae genomics, genetics, and breeding.

Objective 4: **Deliver knowledge on cultivar fruit size genetic potential to PNW cherry growers**.

- Components of fruit size are being dissected in detail for representative current industry cultivars, and combined with fruit size genotypes at three genomic regions. Statistical analyses will continue in late 2011, followed by delivery of knowledge to PNW cherry growers.

RESULTS & DISCUSSION

1. Deliver MAB strategies for both large fruit and self-fertility to the PNWSCBP.
Strategies were delivered, positively impacting the efficiency of breeding operations. Further refinement of the strategies will arise from large-scale data analyses in late 2011.

MAB strategies in action:

Crossing decisions in spring 2010 and spring 2010 were supported by DNA information. Examples include:

- Using knowledge on fruit size (and firmness) genetic potential of parents to produce families enriched with genetics for large (and firm) fruit.
- As above to discontinue use of parents with poor genetic potential.
- Using *S*-genotype knowledge of parents to avoid incompatible crosses.
- Using *S*-genotype knowledge of parents to produce families with 25%, 50%, 75%, or 100% of seedlings predicted to be self-fertile, as desired.
- Where selfing is not desired (e.g. to make a specific cross between two different parents), avoiding the use of self-fertile mothers in crosses because they tend to produce a large proportion of selfed seedlings
- Selfing certain self-fertile parents, as resulting seedlings will always be self-fertile and other useful genetics from that parent can be concentrated.
- Using certain recent selections (from ’04 crosses) as new parents – those with predicted superior breeding value based on all available phenotypic, pedigree, and DNA information.

Interpretation of seedling performance was supported by DNA information. *S*-genotypes and fruit size genotypes of ’04-‘06 seedlings are known. Parentage of seedlings can now be verified by genetic markers. Examples of using this information include:

- Better estimates of parental breeding values for various traits.
- Verifying germplasm sources of certain valuable and rare traits: very firm fruit, freestone.
- Understanding cases of seedlings that don’t perform as predicted from their supposed parents – because their deduced true parentage is different to that originally recorded.

Advancement to Phase 2 selection decisions were supported by DNA information. Examples include:

- Elevation to selection status of several seedlings with large and firm fruit that also possessed the genotypes predicting such genetic potential.

- When all else is equal, selecting seedlings that are self-fertile (carrying the S_4' allele) rather than self-incompatible.
- Excluding from further consideration an early-season selection that was predicted by genetic markers to have small fruit size genetic potential.

Planting decisions in fall 2010 and twice in spring 2011 were supported by DNA information:

- In fall 2010, 800 seedlings growing in the lath house and destined for fall planting were genotyped with two available predictive markers of genetic potential, and most of those with predicted small to medium fruit size and self-incompatibility were culled. Only 340 seedlings were subsequently planted. This effort was calculated to provide a net projected savings (in efficiency, via reallocation of resources) of around $25,000 by avoiding costs involved in planting and future maintenance and evaluation of inferior seedlings. The cost of DNA analysis of these seedlings was less than 10% of those projected savings!
- In May 2011, 1500 seedlings growing in the lath house and destined for spring planting were genotyped with the above-mentioned genetic tests. Less than half the seedlings were subsequently planted, providing an estimated net savings in future breeding expenditure of about $45,000!
- In June 2011, another 400 seedlings were genetically screened. Less than half of those seedlings were subsequently planted, providing an estimated net projected savings of more than $10,000!

Progress was made toward further refinement of our knowledge of genetic control of fruit size, the effects of selfing, and utility of this knowledge for the PNWSCBP. Our current understanding of the utility of the fruit size genetic tests comes from analyses of 2009 season data for more than 200 fruiting seedlings of ’04 crosses. Many hundreds more seedlings have since fruited, and thousands of seedlings are available. We are using the thousands of fruiting ’04, ’05, and ’06 seedlings of the PNWSCBP as the much-expanded “training population” on which to calculate the explanatory power of DNA tests for fruit size.

Performance evaluation of the training population:

- In 2010, fruit size performance data was obtained for 355 fruiting seedlings (189 of seedlings created from ’04 crosses, and 166 seedlings of ’05 crosses), using phenotyping protocols standardized with MSU. Traits evaluated were ripening date, fruit firmness, fruit weight, fruit length and width, pit weight, pit length and width, stem length, stem pull force, fruit shape, fruit skin color and fruit flesh color (based on both spectrophotometer reading and visual rating), freestone/clingstone, titratable acidity (TA), pH, and total soluble solids (TSS).
- For the ’04 seedlings which were also phenotyped in both the 2009 and 2010 seasons, fruit size was fairly consistent between the two seasons (R=0.64 based on each seedling’s maximum observed fruit weight).
- In 2011, many more seedling trees were fruiting for the first time, the most to date in the history of this young breeding program. Seedling numbers are summarized in Table 1. Evaluated fruit quality traits were the same as in 2010, although given the overwhelming number of fruiting trees this year, only seedlings with field-assessed fruit averaging 10 g or larger were harvested and evaluated.
- Fruit quality data from the 2011 season is still being error-checked at the time of writing. Observed distributions of traits and correlations among them cannot be reported at this time. Such summarization of performance data will be conducted in late 2011.

Table 1. Numbers of seedlings in the breeding program undergoing performance evaluation and genetic screening in recent seasons.

Cross year	No. of seedlings				
	Available	Genotyped	Phenotyped in 2009	Phenotyped in 2010	Phenotyped in 2011[a]
'04	250	all	219	189	96
'05	1500	all	0	166	220
'06	3600	2120	0	0	330

[a] Only a subset of fruiting seedlings, those with superior field performance, were evaluated for fruit quality in 2011

Genetic screening of the training population:

- A full-time Genetic Screening Technician was hired at WSU: Terry Rowland, who reliably performs routine genetic screening for both the PNWSCBP and the Washington apple breeding program (approx. half-time) as well as working on separately funded research projects (approx. half-time). The establishment of this critical permanent position helps ensure that the skilled labor for genetic screening is now available throughout the year when needed by WSU's tree fruit breeding programs.
- Successful protocols for high-throughput DNA extraction (using the silica bead method) and high-throughput genotyping (using an ABI 3730xl DNA Analyzer purchased through a WTFRC grant in 2009) were developed for sweet cherry in our PNW Tree Fruit Genotyping Laboratory in Pullman (managed by C. Peace's Associate in Research, Daniel Edge-Garza). Such processing ability had eluded us in previous years. The protocols can now be routinely conducted for *S*-genotyping and fruit size genotyping, using leaf material collected in spring through to September. In the meantime, we continue to tweak the protocols for maximum efficiency.
- DNA was extracted from approximately 4000 seedlings of '05 and '06 crosses, with a success rate of >95%.
- Genotyping of these seedlings (success rate >95%) was conducted for the *S* locus controlling self-fertility/cross-compatibility (*S*-universal and S_4' genetic tests) and three fruit size genetic loci (five markers, mostly obtained from Dr. Iezzoni's federal NRI project). Scoring of this data is ongoing.
- Seedlings with unintended parentage remains a significant proportion in '06 populations as in '04 (Figure 1), but at least the genetic screening can identify them.
- Much genetic variation, in the form of multiple alleles, has been observed in the '04, 05, and '06 breeding populations for markers at the fruit size genetic loci and at the *S* locus. An example is shown in Figure 2. This genetic variation in our training population represents opportunities to discover or verify "jewels in the genome". This genetic variation evident in breeding populations also represents opportunities for MAB to fine-tune crosses and planted trees to focus later performance evaluation efforts on the very best genetics.

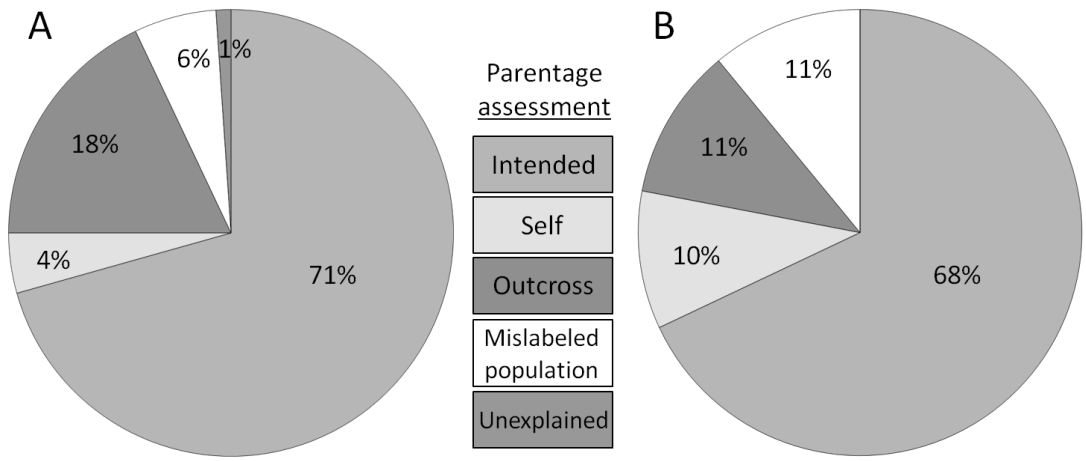

Figure 1. Parentage proportions of some sweet cherry seedlings of the PNW sweet cherry breeding program as determined by genotypes at seven markers. **A**. For a 375 seedling subset of ’06 progenies, resulting from seven crosses among nine parent cultivars. **B**. For all 243 of the ’04 seedlings, resulting from 22 crosses among 17 parents (data from 2009 final report, “Establishing the Marker-Assisted Breeding Pipeline for sweet cherry”). All parentage types other than “intended” are unintended crossing outcomes.

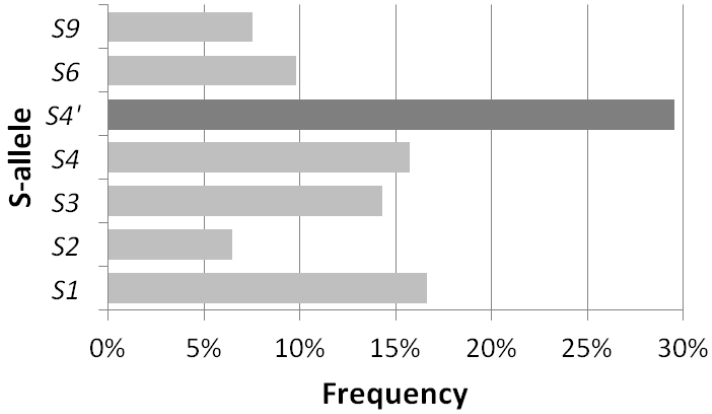

Figure 2. Allelic frequency at the *S* locus for 375 seedlings from seven ’06 crosses among nine parent cultivars, a snapshot of the 4000 seedlings under scrutiny for seven genetic markers. The S_4' allele here provides self-fertility to seedlings possessing it, and was the most common allele observed.

Explanatory power of DNA tests for fruit size

- Analyses to determine the explanatory power of DNA tests for fruit size will be conducted in late 2011. The results will be used to further refine MAB strategies will arise from large-scale data analyses in late 2011.
- In the meantime, the categories of largest and smallest fruit described by the genotype at a single DNA marker from the 2009 season data were again predictive of fruit size in 2010.

Further refinements and delivery of MAB strategies for fruit size, self-fertility, and other traits

- Statistical analyses in late 2011 are expected to provide a comprehensive understanding of the potential for fruit size MAB particularly in the context of other important traits. Specific aims will be to:
    - Determine the impact of marker-assisted seedling selection (MASS) for fruit size on achieving sufficient genetic gain in other essential breeding target traits (i.e., firmness and flavor components).
    - Determine the genetic basis for low occurrence of large-fruited seedlings in sweet cherry crosses.
    - Determine the effect of MASS for self-fertility on obtaining enough seedlings with large fruit size from which to make selections for other traits.

2. Exploit additional opportunities for key trait improvement in the PNWSCBP using MAB.
Several further potential genetic tests have arisen and are being translated for use in the breeding program. A genetic marker for acidity and fruit color (the genomic region happens to be associated with these two otherwise independent traits) was taken further though the MAB Pipeline toward routine use in the PNWSCBP. This marker has been given extra attention in the RosBREED project (together with our main fruit size markers), which is helping its Pipelining. Firmness and sweetness are associated with our main genetic test for fruit size on chromosome 2, and because those traits are routinely evaluated on seedlings in the breeding program, genetic tools for their improvement are also proceeding. A marker for a novel source of self-fertility from the Spanish cultivar Cristobalina was reported by a European lab, and will be used in late 2011 to identify Cristobalina seedlings that can be immediately used as parents in spring 2012 to confer their self-fertility. Efforts in the RosBREED project are furthering the development of markers for all of the PNWSCBP's Primary traits, Secondary traits, and Market-defining traits, and we expect major/many discoveries by early 2012 using RosBREED, resources, approaches, and momentum.

3. Coordinate MAB strategy development, implementation, and outreach in the PNWSCBP with Dr. Iezzoni's NRI project and RosBREED, and Dr. Main's Tree Fruit Genome Database Resources project.
Coordination with other projects continued throughout the last two years, with information on genetic markers shared among our projects. Latest advances were described in the 2010 and 2011 Cherry Field Days at IAREC in Prosser, WA. Our main fruit size genomic region is a target for detailed genomic dissection in both if Dr. Iezzoni's federally funded projects, to find the underlying genes as well as better understand its predictive power. This genetic test was chosen as the major example of impactful MAB in presentations and reports to scientific audiences by Dr. Peace: a RosBREED talk by at the American Society for Horticultural Science annual conference in Aug 2010 (Palm Desert, CA), a talk at the International Rosaceae Genomics Conference in Nov 2010 (Stellenbosch, South Africa), a poster at the American Society for Horticultural Science annual conference in Sep 2011 (Waikoloa, HI), and the Community Breeders' Page article for the Nov 2011 quarterly RosBREED Newsletter. Other genomic regions influencing cherry fruit quality also currently targeted in RosBREED. In Dr. Main's federal project, genotypic data collected on the parent pool of the breeding program is being uploaded to the secure Breeders' Toolbox database for this breeding program, for ready access to DNA information by Dr. Oraguzie. Further functionalities in the Breeders' Toolbox to facilitate ready incorporation of DNA information into crossing decisions are being developed in RosBREED. The PNWSCBP is strategically positioned to utilize large federal investments in Rosaceae genomics, genetics, and breeding.

4. Deliver knowledge on cultivar genetic potential for fruit size to PNW cherry growers.
To determine what physiological components of fruit size are associated with genotypic categories for fruit size potential, 15 cultivars were evaluated over two seasons. Considerable phenotypic variation was observed, with some consistency between years. However, to be able to place any cultivar into a

genetic category that can predict fruit size and levels of its physiological components, full results from Objective 1 activities are required.

Cultivars and selections were chosen to cover the available range of our current idea of fruit size genotype groups, with one to three representative cultivars per group. To maximize expression of fruit size genetic potential, whole trees or two large branches were bloom-thinned to one bud per spur for 15 cultivars and selections: 14 in 2010 (Benton, Bing, Cashmere, Chelan, Cowiche, Rainier, Selah, Skeena, Summit, Tieton, Van, BB, CC, and DD) and 11 in 2011 (Benton, Bing, Cashmere, Chelan, Cowiche, Kiona, Selah, Skeena, Summit, CC, and DD); underlined cultivars were those not evaluated in the other year. Recorded for these individuals were various components of fruit size: fruit weight, fruit shape, fruit width at cheeks, fruit width at suture, fruit length, fruit volume, pit weight, pit width in two different planes, and pit length (fruit samples were frozen for later determination of cell number and cell size). Cowiche consistently had the largest fruit, but only a modest fruit weight to pit weight ratio due to relatively large pits, whereas Selah had large fruit and an exceptionally high proportion of that being flesh and not pit (Figures 3 and 4).

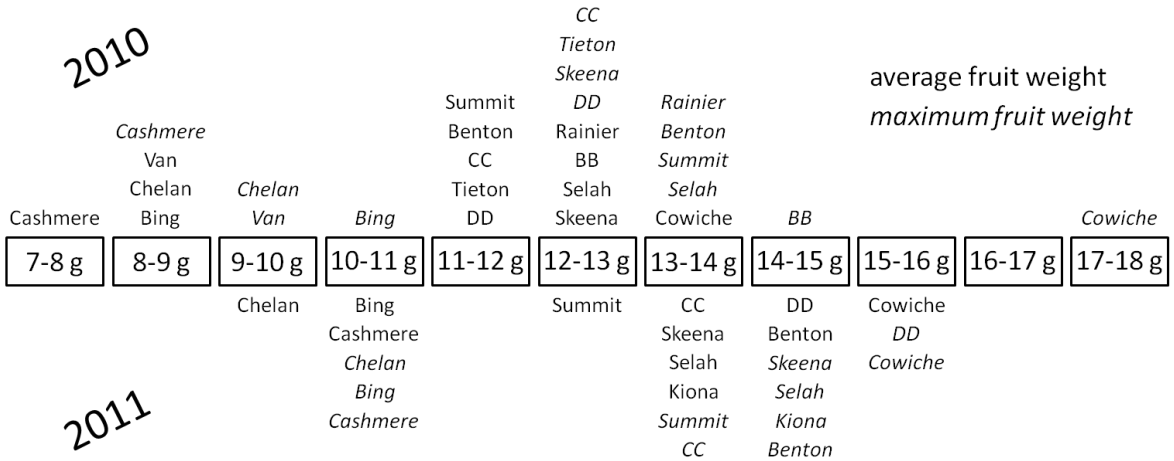

Figure 3. Fruit weight observations for 15 sweet cherry cultivars in two fruiting seasons. Shown are weights for an average of the largest five fruit on a tree ("average fruit weight") and the largest single fruit on a tree ("maximum fruit weight"), following thinning manipulations to maximize fruit size.

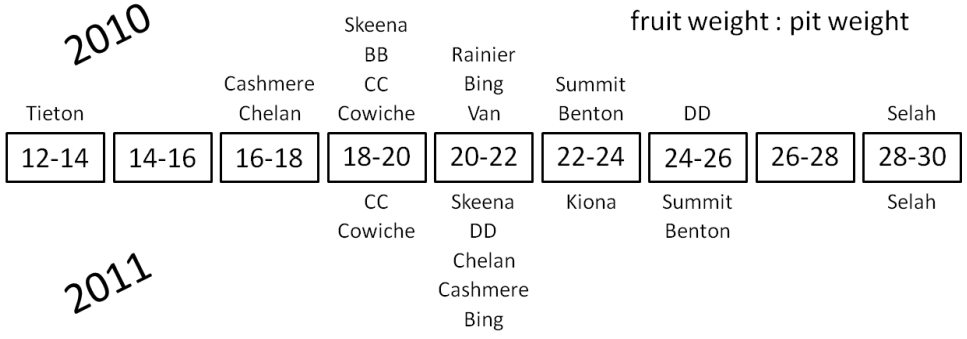

Figure 4. An example of cultivar variation for physiological components of fruit size: ratios of fruit weight to pit weight for 15 sweet cherry cultivars in two fruiting seasons. Measurements are from the average of the largest five fruit on a tree, following thinning manipulations to maximize fruit size.

EXECUTIVE SUMMARY

Our 2010-2011 project on developing and delivering marker-assisted breeding (MAB) strategies for the Pacific Northwest sweet cherry breeding program (PNWSCBP) achieved several important milestones for this young, modern cultivar development program. The primary goal of this project was to finally apply DNA markers for improved sweet cherry breeding efficiency, putting to use many years and dollars invested in developing the tools and infrastructure. This goal was achieved.

The PNWSCBP now applies DNA-based genetic markers to enhance the efficiency and precision of breeding operations. DNA information is incorporated in crossing decisions, seedling field evaluation decisions, and selection advancement decisions. Marker-assisted seedling selection on several thousand seedlings at a time helps eliminate genetically inferior material. *S*-genotyping and fruit size genotyping of new cultivar releases is also available. The PNWSCBP is the first stone fruit breeding program in world to routinely conduct high-throughput marker-assisted seedling selection. Leadership of the PIs and Cooperators in allied projects has strategically positioned the PNWSCBP to utilize large federal investments in Rosaceae genomics, genetics, and breeding.

MAB delivery accomplishments include:

- A net projected savings estimated at more than $80K, by culling more than 1500 seedlings predicted to be self-infertile or genetically inferior for fruit size.
- Efficient enrichment for self-fertility and large fruit genetic potential of the next wave of seedlings, by incorporating DNA information into crossing decisions of the last two years.
- Reduction of the number of advanced selections destined for Phase 2 replicated trials, by consideration of DNA information on fruit size and self-fertility.

Other accomplishments include:

- Filling the full-time Genetic Screening Technician position for a critical role in sustained operations of the PNW Tree Fruit Genotyping Laboratory.
- Establishment of successful protocols for high-throughput DNA extraction and high-throughput genotyping for sweet cherry, meeting the logistical needs of routine MAB.

Projected cost savings are already reflected in the requested budget for the next three years of the breeding program because having fewer planted trees is cheaper (lower land use fees and lower maintenance costs). Savings should also become evident in future years as the trees that survived the genotyping cull begin to fruit, with fruit quality evaluation efforts not wasted on many genetically inferior seedlings. Savings can also manifest as resource reallocation, if a certain limited amount of land, labor, and other resources are used as efficiently as possible for generating superior new cultivars. Such decisions are still in flux as the repercussions of routine MAB sink in for all involved.

Some major aspects of the project are continuing through to the end of 2011. Refinement continues for three fruit size genetic tests. Large phenotypic and genotypic datasets obtained over the last two years, for cultivars, selections, and several thousand seedlings of the earliest crosses and, will be statistically analyzed in late 2011. The major expected outcome is a comprehensive understanding of the genetic potential for fruit size, particularly in the context of other important traits. Such knowledge will be immediately developed into refined MAB strategies in breeding, and delivered to the PNW sweet cherry industry as genetic categories that define cultivar fruit size genetic potential.

Future Directions:
Determine the role for MAB in a cost-efficient PNWSCBP; Optimize replicated trials and support cultivar release and adoption decisions by revealing and communicating genetic potential for commercial performance; Take promising new trait markers through the MAB Pipeline; Enhance and utilize bioinformatics support for maximized access to performance and DNA-based data; Coordinate with the RosBREED and tfGDR projects to maximize benefit for the PNW sweet cherry industry.