FINAL PROJECT REPORT

Project Title: After RosBREED: Developing and deploying new sweet cherry DNA tests

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Other funding sources

Agency Name: USDA-NIFA Specialty Crop Research Initiative
Amount awarded: $10.0 M (Sep 2014 – Aug 2019)
Notes: "RosBREED: Combining disease resistance with horticultural quality in new rosaceous cultivars." PI: Iezzoni. Co-PIs include Peace, Oraguzie, and Main.

Agency Name: USDA-NIFA Specialty Crop Research Initiative
Amount awarded: $2.74 M (Sep 2014 – Aug 2019)
Notes: "Genome Database for Rosaceae: Empowering specialty crop research through big-data driven discovery and application in breeding." PI: Main. Co-PIs include Peace.

Agency Name: WTFRC/OSCC
Amount awarded: $52,844 (2014–2015)
Notes: "New genomic regions controlling production and fruit disorder traits." PI: Oraguzie. Co-PIs include Peace.

Agency Name: WTFRC/OSCC
Amount awarded: $10,000 (2014-2015)
Notes: "Sweet cherry breeding toolbox." PI: Main. Co-PIs include Peace and Oraguzie.

Agency Name: WTFRC/OSCC
Amount awarded: $13,000 (2015-2016)
Notes: "Consulting for the sweet cherry breeding program" PI: Iezzoni.

Agency Name: WTFRC/OSCC
Amount awarded: $442,847 (2012–2014)
Notes: "PNW sweet cherry breeding and genetics program." PI: Oraguzie. Co-PI: Peace.

Total Project Funding:

Budget History:

Item	**Year 1:** 2014	**Year 2:** 2015	**Year 3:** 2016
Salaries	17,651	18,440	19,265
Benefits	11,242	11,916	12,632
Wages			
Benefits			
Equipment			
Supplies	9,107	0	9,103
Travel	2,000	2,000	2,000
Plot Fees			
Miscellaneous			
Total	40,000	32,356	43,000

RECAP ORIGINAL OBJECTIVES

Overall goal: Improve prospects for sweet cherry breeding efficiency, accuracy, creativity, and speed by actively devising new predictive DNA tests that strategically target the region's valuable traits.

Specific objectives:

1. Begin with developing new DNA tests for **maturity time**, **fruit color**, and **fruit firmness** – those traits for which the most promising discoveries were made within the RosBREED project

2. Develop new DNA tests for **pitting** and **cracking incidence**, **fruit-pedicel abscission**, **resistance to bacterial canker** and **powdery mildew**, **sweetness**, and **acidity** – those traits for which discoveries are anticipated from other sources during the project period

3. Ensure appropriate use of new DNA tests by devising and trialing strategies for their routine deployment within the context of existing tests and ongoing Pacific Northwest Sweet Cherry Breeding Program (PNWSCBP) operations

SIGNIFICANT FINDINGS

- **Powdery mildew** (PM) resistance DNA test developed (foliar genetic resistance/susceptibility type**; might also target fruit incidence)

- **Maturity time** DNA test developed (early vs. late predisposition*)

- **Fruit color** DNA test developed (mahogany vs. blush fruit type)

- **Fruit-pedicel abscission** DNA test developed (PFRF level predisposition)

- **Fruit cracking** DNA test developed (presence vs. absence of resistance predisposition)

- **Fruit size & firmness** DNA test developed (size and firmness level predisposition)

- Improved maturity time DNA test under development – targeting multiple genomic regions with a single DNA test to maximize predictiveness

- Predictiveness of each DNA test established across PNWSCBP germplasm using new analytical method that provides greater accuracy by accounting for genetic background

- DNA information on **self-fertility**, **foliar PM incidence**, **fruit color**, **maturity time**, **fruit-pedicel abscission**, **fruit cracking**, **fruit size**, and **fruit firmness** obtained for <u>all</u> PNWSCBP selections and seedlings to aid in selection

- Four-stage deployment strategy devised to optimally utilize many DNA tests now available

* "predisposition" means the test is not deterministic but rather determines some degree of genetic propensity for certain trait levels

** "type" means deterministic: the DNA test appears to identify clearly one type vs. another

RESULTS & DISCUSSION

Objectives 1 & 2: DNA test development

Using genomic discoveries made within the RosBREED project, the WTFRC/OSCC project "New genomic regions controlling production and fruit disorder traits", and through international collaborations, DNA tests were developed for the PNW region's valuable market class-defining, essential, and enhancing traits levels. Adding to previously available DNA tests for self-fertility and fruit size, new DNA tests were developed for the traits of **fruit color**, **maturity time, foliar PM** incidence, **fruit size**, **fruit firmness**, **fruit-pedicel abscission**, and **fruit cracking** (Fig. 1).

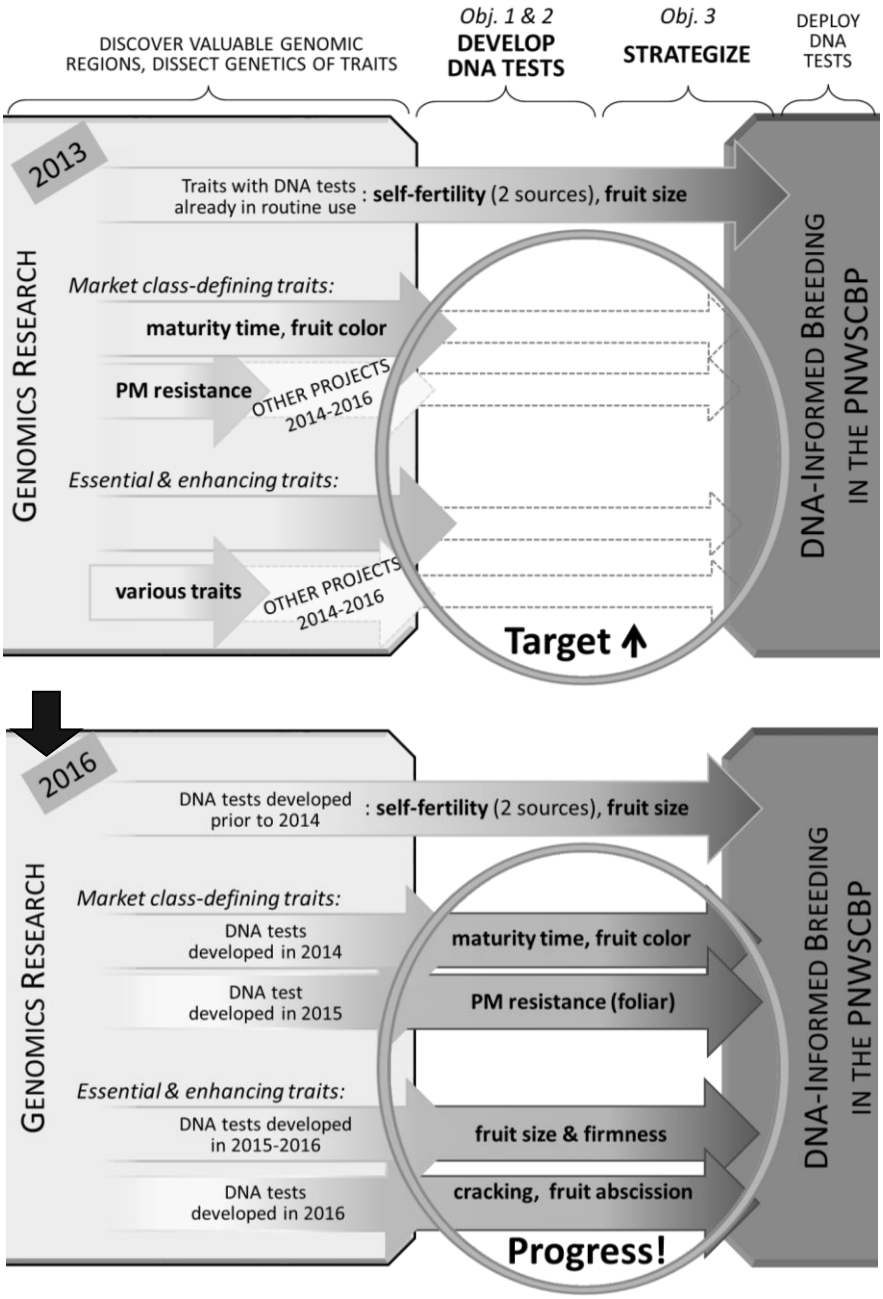

Figure 1: Progress made during project, which has and will continue to improve the efficiency, accuracy, creativity, and pace of the Pacific Northwest Sweet Cherry Breeding Program (PNWSCBP) via DNA-informed breeding.

Foliar PM DNA test

A new DNA test for routine prediction of **foliar PM** resistance/susceptibility was developed. This advance was made possible by the discovery of a genomic region associated with this trait in the WTFRC/OSCC-funded project, "New genomic regions controlling production and fruit disorder traits". The DNA test developed, "Pav-G5PM-SSR", is able to identify individuals with resistance to PM from the three sources, 'PMR-1', 'Moreau', and 'Mildew Immune Mazzard'. When screened across a large germplasm set representative of the breeding program, a significant, breeding-relevant difference was observed between those individuals with one or two resistance alleles compared to those without (Fig. 2). In PNWSCBP germplasm, this DNA test explains approximately 15% of the phenotypic and approximately 25% of the genotypic variation observed for foliar PM incidence. With Pav-G5PM-SSR, the costly and error-prone phenotyping that is traditionally used to evaluate foliar PM incidence can be avoided. Based on correspondence of DNA test genotypes among cultivars resistant vs. susceptible for fruit PM incidence, this test might also be effective to identify individuals with resistance to fruit PM; however, additional research, specifically the collection of new, accurate phenotypic data on fruit PM incidence, is needed to test this hypothesis. Pav-G5PM-SSR is currently being screened across all PNWSCBP seedlings to identify individuals resistant to foliar PM, especially in the late season mahogany market class where a new cultivar must be resistant to this costly disease.

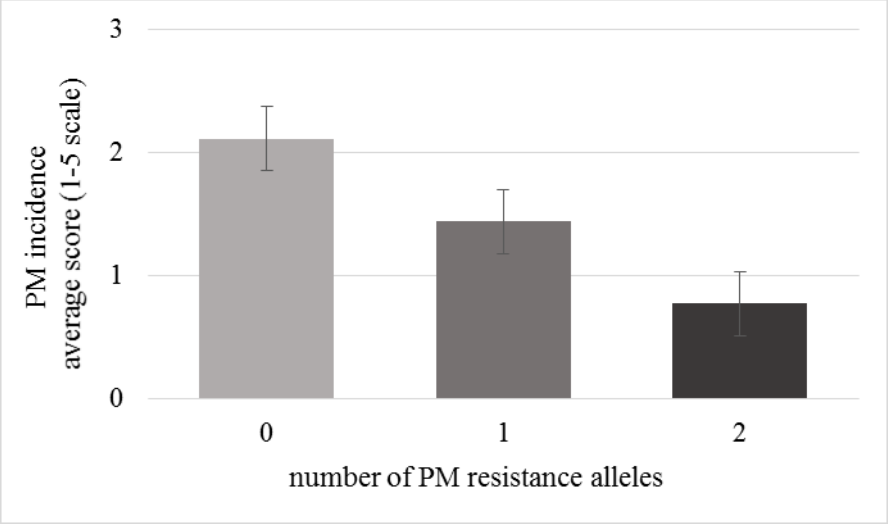

Figure 2: Predicted difference in foliar powdery mildew infection between those individuals carrying no PM resistance alleles, one resistance allele, or two resistance alleles as revealed by the new DNA test, Pav-G5PM-SSR. Predictions based on three years phenotypic data using 1-5 scale (1 = 1–20%, 2 = 21–50%, 3 = 51–80%, 4 = 81–99%, 5 = 100%) and corrected for major influencing factors such as family (genetic background) and year effects.

Fruit maturity time DNA test

A new DNA test for routine prediction of **fruit maturity time** was developed, Pav-Fht-SSR. The test targets the largest-effect genomic region associated with variation in sweet cherry fruit maturity time. In PNWSCBP germplasm, this DNA test explains approximately 27% of the phenotypic and approximately 35% of the genotypic variation observed for maturity time. This test reveals two functional alleles, termed "early" and "late", that in combination can predict differences in harvest

date of an average of approximately one week (Fig. 3); however, this test does not explain the early harvest date of 'Chelan'. Due to the importance of the 'Chelan' maturity time to the PNW industry, an effort was made in Year 3 of the project to identify additional genomic regions. New genomic regions on chromosome 1, 4, and 6 were found to be associated with additional variation in maturity time and preliminary analysis indicates that these regions, in combination with Pav-Fht-SSR, might provide additional genetic contributions to DNA-based predictions of maturity time. Development of a DNA test with improved predictivness for fruit maturity time is major focus of the remaining months of the project.

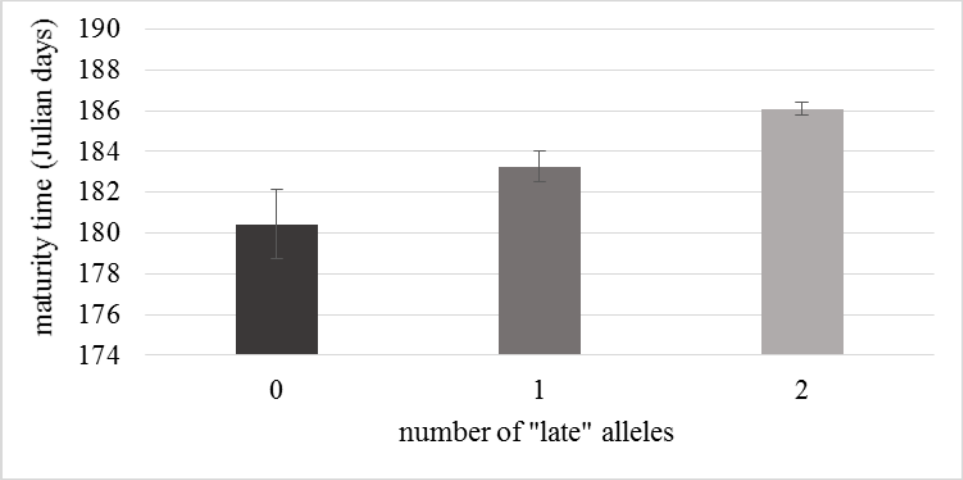

Figure 3: Predicted difference in maturity time between those individuals carrying no late alleles, one late allele, or two late alleles as revealed by the new DNA test, Pav-Fht-SSR. Predictions based on two years phenotypic data measured as number of days to harvest from January 1 and corrected for major influencing factors such as family (genetic background) and year effects.

Fruit color DNA test

A new DNA test for sweet cherry fruit color ("Pav-R_f-SSR") was developed. This DNA test clearly differentiates *blush* types from *mahogany* types, and can be used to differentiate "pure" mahogany types – those that possess two mahogany alleles (R_f^{SSR}) and tend to have a slightly darker color from those that possess only one mahogany allele and have a slightly lighter red hue (Fig. 3). A manuscript describing the development and utility of Pav-R_f-SSR was published in the journal Molecular Breeding [Sandefur P, Oraguzie N, Peace C (2016) A DNA test for routine prediction in breeding of sweet cherry fruit color, Pav-R_f-SSR. Molecular Breeding 36:33.].

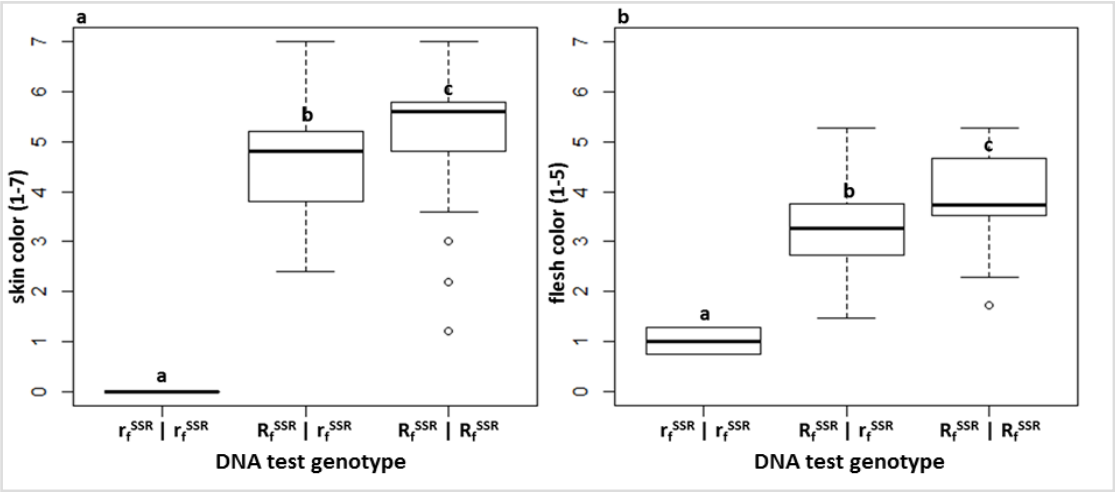

Figure 4. Skin color (a) and flesh color (b) of Pav-R_f-SSR genotypes in PNWSCBP sweet cherry germplasm. R_f^{ssr} is the dominant mahogany allele and r_f^{ssr} is the recessive blush allele. Significant differences among groups are indicated by different letters (Kruskalmc multiple comparisons test; $P < 0.01$). The 2011 and 2012 data was combined after removing significant year effects

Fruit-pedicel abscission

A new DNA test was developed for **fruit-pedicel abscission** [measured as "pedicel-fruit retention force" (PFRF)]. During Year 2, our collaborators in the WTFRC/OSC funded project "New genomic regions controlling production and fruit disorder traits" discovered a genomic region on chromosome 2 associated with PFRF that overlapped the region already targeted by Pav-G2-SSR for fruit size. However, additional analysis in Year 3 found that this region accounted for less of the phenotypic variation for PFRF in PNWSCBP germplasm than expected. It is still likely that in specific germplasm (e.g.in elite germplasm, such as 'Selah' and 'Cowiche') the DNA test detects a significant contrast in readiness of fruit to abscise from the pedicel. Additionally, the Spanish landrace 'Ambrunes' and its offspring in the breeding program carry a unique allele with breeding promise. A major focus of the remaining months of the project is to further investigate the utility of Pav-G2-SSR for PFRF in PNWSCBP germplasm.

Fruit cracking

A new DNA test was developed for rain-induced fruit cracking. Collaborating French researchers (led by Dr. Jose Quero Garcia, INRA) discovered a genomic region associated with rain-induced fruit cracking. This exciting discovery was made in a family with 'Regina', a low-incidence cracking cultivar, as a parent. In 2016, we confirmed the association between fruit cracking incidence and the newly discovered genomic region in germplasm of the PNWSCBP. Based on this confirmation, a DNA test was developed for performance prediction of this valuable trait (Fig. 5). The new DNA test, Pav-G5Crack-SSR, can be used to identify individuals highly resistant to rain-induced cracking. Although valuable, this test might only differentiate individuals that are highly resistant from individuals that have some susceptibility. Such a test would only be useful in some breeding applications, such as in parent selection and in seedling selection within families particularly targeted for genetic improvement in cracking incidence. If the test does not differentiate individuals that are highly susceptible (a fatal flaw) from those that are only moderately susceptible (not a fatal flaw), it should not be used on all seedlings. The DNA test might be effective for various cracking incidence levels; however, additional phenotypic data is needed to fully understand the predictiveness and maximize the utility of Pav-G5Crack-SSR.

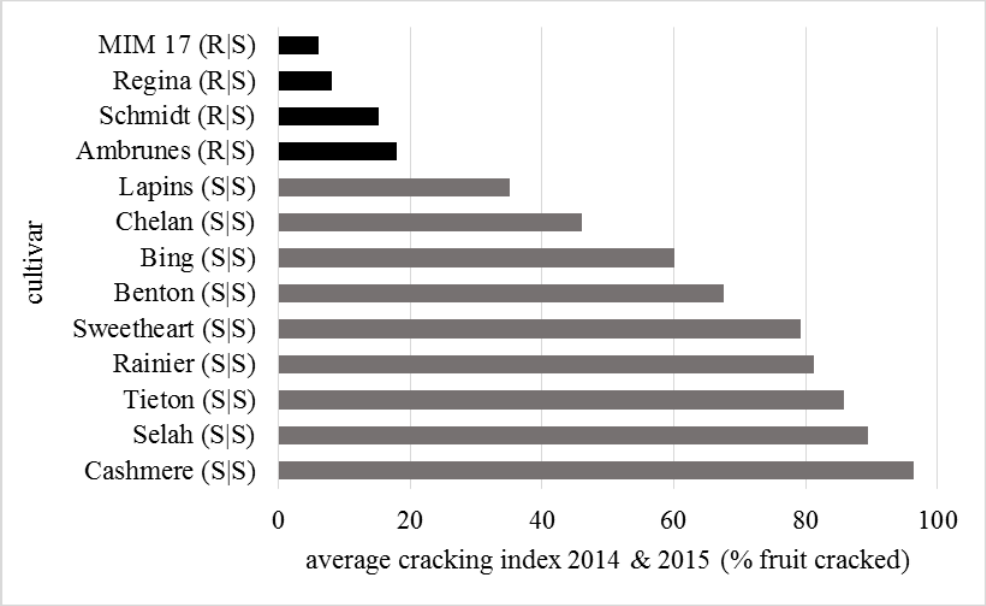

Figure 5. Fruit cracking index (2014-2015 average) of some sweet cherry cultivars that carry a single resistance allele ("R") and a susceptible allele ("S") or two susceptibility alleles as revealed by Pav-G5Crack-SSR.

Fruit size & firmness

DNA tests for fruit size and firmness have been refined by targeting additional genomic regions influencing these traits. Prior to 2014, the CPSCT038-BPPCT034 fruit size DNA test, targeting a region on chromosome 2 originally discovered by Amy Iezzoni's research group, was routinely used in the PNWSCBP. Since 2014, two more genomic regions associated with fruit size and firmness were reported by French (INRA) collaborators. We designed DNA-based assays to target these two new regions. We estimate that a single assay combining these multiple tests will explain approximately 20% of the phenotypic variation and 25% of the genotypic variation for fruit size and firmness. These proportions indicate that when used in seedling selection, only those seedlings with the worst genotypes should be discarded. In the remaining months of the project, we will finish the development of this combined DNA test and confirm its breeding utility.

Further refinement of existing DNA tests

In Year 3 of the project, quantitative statistical analyses were conducted to refine the prediction accuracy of all our DNA tests. The method used software developed by Dr. Craig Hardner within the WTFRC-funded apple project, "After RosBREED: Developing and deploying new apple DNA tests". The new analyses accounted for the genetic background of the PNWSCBP individuals, removing confounding effects associated with individual families, to more accurately estimate the outcomes of selecting for, or against, a specific DNA test allele or allelic combination. In addition, the outcomes were determined for selecting for, or against, a specific allele on other traits other than that targeted by a DNA test.

Traits for which DNA tests were not made or that need additional confirmation

Several traits were evaluated over the three years but effective DNA tests could not be developed. Bacterial canker resistance and pitting phenotypic data obtained in the PNWSCBP was insufficient for detection of robust genomic regions. Additional phenotypic data would be needed that adequately characterizes genetic susceptibility for these diseases. Although a fruit cracking DNA test was

developed, standardized phenotypic data on diverse breeding seedling families is needed to confirm the test's utility in the PNWSCBP. DNA test development for other traits of high priority for the PNW sweet cherry industry, such as incidence of fruit powdery mildew and the storage disorders of stem browning, shrivel, and luster loss, is also hampered by the lack of robust phenotypic data. DNA tests for these traits would be valuable because routine acquisition of performance data for these traits during breeding operations is expensive, logistically difficult to obtain, or both. A new proposal for 2017 has been submitted to the WTFRC/OSCC to fund the necessary phenotypic data collection so that new or refined DNA tests can be developed for resistance to fruit powdery mildew, cracking, pitting, shrivel, luster loss, and stem browning. Use of such DNA tests in the breeding program at the parent and/or young seedling stage, rather than older-plant fruiting stage, would reduce ongoing program operational costs.

Objective 3: DNA test deployment

Deployment strategies for all currently available DNA tests were developed and are ready for application. DNA information on prospective parents in the PNWSCBP for maturity time and fruit color, in addition to fruit size and firmness, self-fertility, and cross-compatibility, was used to guide 2014 crossing decisions. These same DNA tests were deployed for seedling selection in the families resulting from these targeted crosses, and resulted in a double deployment of DNA information thereby maximizing the genetic potential (new cultivar potential) of the offspring produced. Continued use of this double deployment is expected to improve the overall efficiency and impact of the PNWSCBP.

With so many DNA tests of different types now in the toolkit of the PNWSCBP (as described above) sophisticated deployment strategies are needed to best take advantage of the new opportunities. A four-step strategy was developed in this project.

STAGE 1: Effectual parent combining

With availability of the new **maturity time** and **fruit color** DNA tests, the PNWSCBP can more accurately align family sizes with established market class priorities. Creating the best cross combinations with predictable proportions of seedlings producing blush or mahogany and early-, mid-, or late-season fruit, and selecting those seedlings with the greatest potential to meet industry needs within each market class is more tractable than ever before.

A major market class-defining trait is **fruit color**, with 70% of the PNWSBP's resources to be allocated to mahogany-fruited individuals and only 30% to blush. Using the **fruit color** DNA test Pav-R_f-SSR, crosses producing a majority of mahogany offspring can now be targeted. Similarly, the DNA test Pav-G4Mat-SSR can be used to target another major market class-defining trait, **maturity time**, whereby crosses resulting in a majority of late-season and early-season individuals can be targeted. When combined, Pav-R_f-SSR and Pav-G4Mat-SSR provide the breeder with a convenient tool to create market class-targeted cross combinations.

For a new cultivar to be successful it must have the genetic potential to perform above a minimum level for many traits. Such minimums are what we call *essential attributes*. These essential attributes typically include **self-fertility**, **fruit siz**e >10 g, and **firmness** >300 g. The fewer seedlings that need to be culled for essential attributes, the greater the selection pressure that can be placed on all other traits, and greater selection pressure raises the genetic potential of remaining individuals. DNA information on parents can be used to identify parental combinations where little or even no culling for that trait is required on resulting families. For example, a cross using a homozygous (doubled-up) for the S4' self-fertility allele will only produce self-fertile seedlings – eliminating the need to cull any self-infertile seedlings. The parent selection stage is also a useful time to consider those parents possessing alleles for *enhancing* attributes. Enhancing attributes are those trait levels that enhance the value of a new cultivar but are not required for it to be viable in the marketplace, such as extra-large size, extra-firmness, or strong fruit-pedicel abscission. DNA tests now available

for **self-fertility, fruit size & firmness**, **fruit-pedicel abscission**, and **fruit cracking** and **powdery mildew resistance** will help to maximize effectual parental combining.

STAGE 2: Punctilious seedling sorting

After generating seedling families from superior parental combinations, specific proportions of industry driven market class individuals to be field-planted can be established by using DNA information for seedling sorting. After sorting, specific trait thresholds for each market class can be used to guide selection decisions.

STAGE 3: Surgical seedling culling

After careful consideration of parental combinations and targeted seedling sorting, the amount of seedling culling required can be reduced substantially and accurately applied to each market class. DNA tests that target essential attributes are first used to support "hard' selection decisions. For these "hard" selection decisions, the selection thresholds depend on the market class to which each seedling has been sorted. By deploying the current suite of sweet cherry DNA tests to target essential attributes, most seedlings field-planted and evaluated at adulthood for fruit are likely to meet target trait thresholds for each specific market class.

STAGE 4: Versatile family enhancement

Surgical seedling culling is followed by versatile family enhancement, where specific seedling families are screened with DNA tests for desired enhancing attributes to support "soft" selection decisions. Just like surgical seedling culling, the selection thresholds depend on the market class to which each seedling has been sorted, but here the selection is more nuanced with thresholds possibly varying from family to family. By following the Stage 3 "hard" selection decisions with "soft" selection decisions, most seedlings later evaluated for performance at adulthood are likely to meet essential target trait thresholds and also carry numerous enhancing attributes to suit desired targets and field capacity.

DNA-based evaluation of Phase 2 selections

DNA-based performance predictions were obtained for current Phase 2 selections by screening them with all available DNA tests. The resulting data are being used to aid in the selection process, providing confidence to decisions to remove selections from trials or to include them in 2017 phenotypic evaluations. Utilization of this new DNA information is a major focus of the remaining months of the project. This DNA information has made some intriguing revelations (Fig. 6). For example, 62% of P2 selections have mahogany fruit color but of those only ~25% carry two mahogany Pav-R_f-SSR alleles indicating a possible selection bias toward mahogany fruit with a lighter red hue [carrying only 1 mahogany allele (Fig. 4; Fig. 6)]. Only 23% of mahogany selections carry a single early-harvest-time allele (none have two) – thus there appears to be a large opportunity to develop earlier-season selections. For fruit size, only 17% of P2 selections carry the maximum of two of the CPSCT038-BPPCT034 large fruit size alleles, with only 43% carrying the self-fertile allele – thus there is still much opportunity to enrich future cohorts of P2 selections with desirable alleles.

Although sweet cherry fruit color, mahogany vs. blush, might seem like a trait that is easy to evaluate phenotypically, fruit color is directly influenced by fruit maturity (e.g., mahogany fruit can appear to be blush-type when not fully ripe) and environmental conditions (e.g., fruit might appear blush in low light or mahogany in high light environments). As a specific example, the selection R21 was classified phenotypically in 2014 as mahogany, in 2015 as blush, and in 2016 as "possibly blush, but likely mahogany". Using the Pav-R_f-SSR DNA test, we are now able to definitively say that R21 is mahogany, therefore having the genetic potential to produce fruit with a full red overcolor and red flesh when ripe. By using DNA test information on Phase 2 selections in this manner, we can reveal their true genetic potential.

One of the most important attributes to the success of a new cultivar in the PNW is self-fertility (Fig 6). Therefore, the self-fertility DNA test has been used since 2010 to screen all PNWSCBP seedlings to facilitate planting of only those that are self-fertile. However, many of the Phase 2 individuals were derived from seedlings planted prior to use of this DNA test and therefore are not necessarily self-fertile. By screening all Phase 2 selections with the self-fertility DNA test, we have found that only half of the current P2 selections are self-fertile. The genetic identification for each Phase 2 selection as self-fertile or non-self-fertile will help decide retention in vs. removal from field trials.

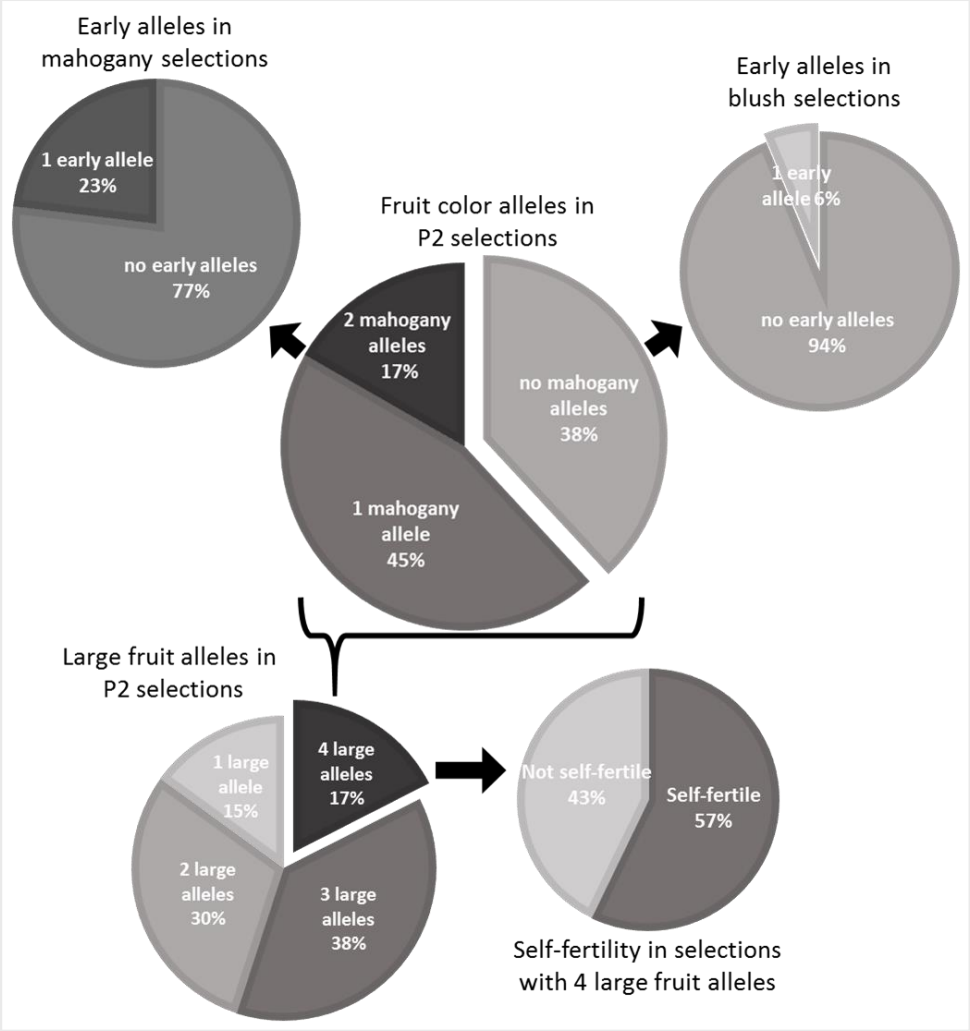

Figure 6: Pav-Rf-SSR fruit color, Pav-Fht-SSR fruit harvest time, CPSCT038-BPPCT034 fruit size, and S4Pav fruit self-fertility DNA test results for PNWSCBP Phase 2 selections.

Evaluation of Phase 1 seedlings

All Phase 1 seedlings are currently being screened with all available DNA tests. The resulting data will be used to aid in the selection process, providing confidence in decisions to remove seedlings from current trials, to advance seedlings to Phase 2, or to identify individuals that should receive additional phenotypic evaluation in 2017.

EXECUTIVE SUMMARY

Genetic improvement underpins the long-term economic sustainability of the Pacific Northwest sweet cherry industry. Focusing on industry priority traits, our goal was to deliver routinely implementable DNA tests to the Pacific Northwest Sweet Cherry Breeding Program (PNWSCBP). The two objectives of this project were to:

1. Develop new DNA tests for traits for which the most promising discoveries were made within the RosBREED project – maturity time, fruit color and fruit firmness and for which discoveries were anticipated from other sources during the project period – pitting and cracking incidence, fruit-pedicel abscission, resistance to bacterial canker and powdery mildew, sweetness, and acidity.
2. Ensure appropriate use of new DNA tests by devising and trialing strategies for their routine deployment within the context of existing tests and ongoing Pacific Northwest Sweet Cherry Breeding Program operations.

By successfully fulfilling these objectives, we expect to have improved the PNWSCBP's prospects for developing superior new cultivars that have fruit quality and disease/disorder resistance that is superior to the market-class standards. We believe that we can increase the PNWSCBP's development of such superior cultivars through the routine application of predictive DNA tests for the regional industry's priority traits. The appropriate deployment of trait-predictive DNA tests can efficiently reveal genetic potential for superior performance across the spectrum of breeding germplasm in each program phase. DNA tests for industry priority traits were developed and deployed on all PNWSCBP germplasm in this project.

- **Foliar powdery mildew** DNA test developed – this test can be used to identify individuals carrying the major-effect resistance allele that imparts resistance to foliar powdery mildew. This DNA test might also cover fruit PM incidence.
- **Maturity time** DNA test developed and improvement continues – this test can be used to differentiate individuals that will have, on average, up to one week difference in harvest date. However, this test does not explain the extra-early harvest of 'Chelan'. Additional genomic regions are being targeted to maximize predictiveness.
- **Fruit color** DNA test developed – this test can be used to differentiate individuals that will produce blush fruit from those that will produce mahogany fruit and can be used to identify individuals that will produce darker or lighter mahogany fruit.
- **Fruit-pedicel abscission** DNA test developed – this test should be useful in specific PNWSCBP families for differentiating fruit-pedicel abscission tendencies.
- **Fruit cracking** DNA test developed – this test appears to differentiate individuals that are highly resistant from those that are moderately to highly susceptible.
- **Fruit size & firmness** DNA test developed and improvement continues – multiple genomic regions are targeted with a single DNA test to maximize predictiveness.
- A new method was used that refines accuracy of DNA test predictions by accounting for genetic background and controlling non-genetic effects.
- DNA information on **self-fertility**, **foliar powdery mildew**, **fruit color**, **maturity time**, **fruit-pedicel abscission**, **fruit cracking**, **fruit size**, and **fruit firmness** was obtained for <u>all</u> PNWSCBP P2 selections and P1 seedlings to aid in the selection process.
- A four-stage deployment strategy (market class-defining in crosses then in seedling sorting, followed by hard selection for essential attributes within each market class, then soft selection for enhancing attributes) was devised to optimally utilize the many DNA tests now available.

In the remaining months of this project, we will conduct final refinements to DNA tests to streamline their future deployment and we will use the results from DNA-testing of all P2 selections and P1 seedlings to improve the accuracy of identifying germplasm with genetic potential for superior performance.