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Phenotyping Protocol for Sour Cherry (*Prunus cerasus* L.) to Enable a Better Understanding of Trait Inheritance

Travis Stegmeir[1], Audrey Sebolt[1] and Amy Iezzoni[1,2]

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Abstract

A phenotyping protocol for sour cherry (*Prunus cerasus* L.) was developed to include evaluations of phenology, productivity and fruit quality. Traits of particular importance for mechanical pitting for processed sour cherries were included such as estimates of fruit and pit shape and pit freestone/clingstone. To evaluate the correlations among the phenotypic traits in the protocol, the traits were evaluated on sour cherry germplasm included in the RosBREED project (www.rosbreed.org). Correlations among the values of these phenotypic traits supported the need to evaluate linear measurements of the fruit and pits as fruit and pit weights were weakly and not significantly correlated with fruit and pit shape, respectively. Highly significant correlations between some traits suggested a genetic correlation as opposed to a correlation based on shared tree/fruit growth and development. These include highly significant correlations between fruit firmness and bloom date and between yield and fruit flavor (measured as soluble solids concentration and acidity). This phenotyping protocol will enable the collection of trait data that can be used in future studies to determine the genetic control of traits important for new sour cherry cultivars.

Sour cherry (*Prunus cerasus* L.) is an important rosaceous fruit crop with worldwide production over 1.2 million tons (FAO, 2011). In the United States, Michigan is the major producer of sour cherries, averaging ~ 70% of the nation's total crop each year (NASS, 2012). Unlike the closely-related sweet cherry (*P. avium* L.), which is usually sold fresh, sour cherry is used in a wide variety of processed products such as juice, jams, pies and dried cherries. Because of the processed nature of sour cherry, considerations for mechanical harvesting and pitting also need to be addressed in breeding programs (Iezzoni, 1996).

Breeding progress in sour cherry has been slow due to the long juvenility of seedlings, where it can take from 3-5 years for a new seedling to bear fruit, and the limited number of seedlings that can be grown due to the large space requirement. As a consequence, most of the cultivars of sour cherry grown today are no more than one generation removed from landrace selections, or the landraces themselves are still in cultivation (Iezzoni, 2008). With this long juvenility period and the high cost of growing seedlings in the field, there is interest in using DNA information to increase breeding efficiency. Marker-assisted parent selection (MAPS) and marker-assisted seedling selection (MASS) have great potential to aid breeders in making crosses that will have a higher percentage of desired seedlings, as well as to cull inferior seedlings before they are planted in the field. This strategy would allow breeders to advance more elite individuals per year, therefore increasing the likelihood of success. For this strategy to work, however, DNA tests are needed that tag chromosome regions that contain genes that control variation for the phenotypic traits of interest.

The analyses that identify these chromosome regions require genetic and phenotypic data on plant materials that exhibit variation for the traits of interest.

[1] Michigan State University, Department of Horticulture, East Lansing, MI 48824
[2] To whom reprint request should be addressed. Email address: iezzoni@msu.edu

Now that genotypic data has become cheaper to obtain in cherry, with markers available that cover the entire cherry genome (Peace et al., 2012), high-quality phenotypic data is needed. In order to obtain high-quality phenotypic data from year-to-year, and between different programs, a standard phenotyping protocol was developed. This paper describes the phenotyping protocol currently developed for sour cherry and its use to phenotype the RosBREED sour cherry germplasm. This project was undertaken as part of the USDA Specialty Crop Research Initiative-funded RosBREED project, which focuses on enabling marker-assisted breeding in the Rosaceae. Standardized phenotyping protocols have also been developed and implemented for other rosaceous crops targeted in RosBREED [specifically apple (Evans et al., 2012), peach (Frett et al., 2012), and strawberry (Mathey et al., 2013)].

Materials and Methods

Phenotyping protocol

Phenology traits and yield (Table 1): Bloom date (BD) was recorded as the morning when 50% of the flowers were open on a tree and converted to growing degree days with a base temperature 4.4°C (40°F). The growing degree days were calculated using hourly weather data from January 1 until 10 a.m. on the bloom date recorded as in Wang et al. (2000). Fruit were harvested on two separate days and the optimum maturity date (MD) was chosen based on an evaluation of the fruit quality data obtained in the laboratory (see Fruit traits). The chosen optimum maturity date for each selection was recorded as was done for bloom date and converted to growing degree days. At the first harvest, yield (YLD) was recorded based on a scale of 0-10, where each number represents a 10% increase in crop, from 0 to 10 (100% crop load). A value of 10 represents the desired crop load based on leaf area to ripen the crop (Iezzoni and Mulinix, 1992).

Fruit traits (Table 1): Fruit were harvested from the field and immediately stored, no longer than 48 hours, in a 4°C cooler. For each fruit sample, fruit firmness (Fir) ($g \cdot mm^{-2}$) was measured from 25 fruit that were at room temperature using the compression test of BioWorks' FirmTech 2 (Wamego, KS). Compression was done from cheek to cheek (perpendicular to the suture) when the stems were still on the fruit. The first five fruit measured for firmness were put in wells of an ice-cube tray in preparation for individual fruit measurements. The stems were removed from the last 20 fruit after the firmness data was taken. These 20 fruit were weighed and the bulk fruit weight (FWB) (g) was determined by dividing the weight by the number of fruit weighed.

The next measurements were taken separately for the five individual fruit, which enabled future calculations using multiple measurements that traced back to the same individual fruits (see Calculations with fruit measurements). However, for the traits measured directly, the phenotypic data point used to represent each selection was the mean of these five fruit. Pull force (PuF) (g) was measured on each of the five fruit using a mechanical force gauge (Hunter Spring Co., Lansdale, PA). Stems were pulled firmly, but smoothly through the pull force meter until the stems detached from the fruit. Fruit weight (FWt) (g) was then taken on each stemless fruit followed by linear fruit measurements (mm) using a digital caliper. Fruit length (FL) was measured from the stem scar to blossom scar, fruit width 1 (FW1) was measured from the suture to the opposite face of the cherry, and fruit width 2 (FW2) was measured from cheek to cheek of the fruit.

The five fruit were then cut in half and the pits were removed. At that time a freestone to clingstone (F/C) (1-5 rating) rating was recorded to describe how much the pit clung to the surrounding flesh as follows: 1 = no cling, 2 = slight cling, 3 = semi cling, 4 = cling, 5 = total cling. Soluble solids concentration (SSC) (Brix %) was measured on each of the five fruit. Juice was squeezed out of each cut fruit and % Brix was measured

Table 1. Phenotypic traits, their descriptions and units of measurement used to characterize sour cherry slections.

Trait	Trait code[a]	Description and Unit of Measurement
		Productivity traits
50% bloom date	BD	Growing degree days accumulated from January 1 to date of 50% bloom calculated with a base of 4.4°C
Maturity date	MD	Growing degree days accumulated from January 1 to date of optimum maturity calculated with a base of 4.4°C
Yield	YLD	0 = no crop; 1 = 10% crop; 2 = 20% crop; 3 = 30% crop; 4 = 40% crop; 5 = 50% crop; 6 = 60% crop; 7 = 70% crop; 8 = 80% crop; 9 = 90%; 10 = 100%
		Fruit quality traits
Fruit firmness	Fir	Measured by compression (cheek to cheek) of 25 fruit at room temperature ($g \cdot mm^{-2}$)
Bulk fruit weight	FWB	Average weight of 20 fruit (g)
Pull force	PuF	Force required to remove the stem from the fruit, avg of 5 largest fruit (g)
Fruit weight	FWt	Average weight of 5 largest fruit (g)
Fruit length	FL	Measured from stem to blossom scar, avg of 5 largest fruit (mm)
Fruit width 1	FW1	Measured from the suture to opposite cheek, avg of 5 largest fruit (mm)
Fruit width 2	FW2	Measured from cheek to cheek, avg of 5 largest fruit (mm)
Freestone	F/C	Pit cling: 1 = no cling, 2 = slight cling, 3 = semi cling, 4 = cling, 5 = total cling (avg of 5 largest fruit)
Soluble solids concentration	SSC	Brix %
Pit weight	PWt	Average pit weight of 5 largest fruit (g)
Pit length	PL	Pit length from point to point, avg of 5 largest fruit (mm)
Pit width 1	PW1	Pit width from suture to back, avg of 5 largest fruit (mm)
Pit width 2	PW2	Pit width perpendicular to width 1, avg of 5 largest fruit (mm)
Skin color	CSk	Visual FCH[b] color chip rating 1-8: 1 = 2c (light yellow), 2 = 26c (cream orange), 3 = 42b (light red), 4 = 178d (red-orange), 5 = 53b (red), 6 = 183b (deep red), 7 = 187b (red-purple), 8 = 187a (mahogany)
Flesh color	CFl	Visual FCH color chip rating 1-5: 1 = 24d (cream), 2 = 50b (pink), 3 = 53b (red), 4 = 185a (red-purple), 5 = 187a (mahogany)
Acidity	AC	10ml strained juice from a thawed frozen sample is titrated to a
Fruit shape	ShF	Ratio of Fruit length / Fruit width 2
Pit shape	ShP	Ratio of Pit length/Width 1
Mesocarp weight	MWt	Pit weight - Fruit weight

[a] Trait code is used for correlation matrix in Table 2

[b] Flower Council of Holland, Leiden (The Royal Horticultural Society, London)

using a digital refractometer (PAL-1, Atago, Tokyo, Japan).

Pit weight (PWt) (g) was determined from each pit following removal of the flesh. Paper towels were used to rub off any remaining fruit/juice. Pit measurements (mm) were taken on each of the pits using a digital caliper. Pit length (PL) was measured from the top to bottom of the pit along the suture, pit width 1 (PW1) was measured from the suture to the back of the pit, and pit width 2 (PW2) was measured as the distance perpendicular to the suture, corresponding to the cheek to cheek measurement of the cherry fruit.

Visual color scores were recorded for the sour cherry skin and flesh. Because sour cherry skin does not have a "blush" characteristic as with some sweet cherries, only one skin color was recorded. Skin color (CSk) ratings ranged from 1-8 and the numbers correspond to color chips from the Flower Council of Holland (FCH), Leiden (The Royal Horticultural Society, London). Skin color ratings are as follows: 1 = 2c (light yellow), 2 = 26c (cream orange), 3 = 42b (light red), 4 = 178d (red-orange), 5 = 53b (red), 6 = 183b (deep red), 7 = 187b (red-purple), and 8 = 187a (mahogany). The skin colors 5-8 were equivalent to the color card numbers on the Sweet Cherry Maturity Index manufactured by Colorcurve Systems, Inc. (East Lansing, MI). Flesh color (CFl) was taken by cutting the fruit along the suture and rated 1-5 using the Washington State University Sweet Cherry Flesh Color Index card, which can be seen in the tart cherry phenotyping protocol available at www.rosbreed.org/resources/fruit-evaluation. As with skin color, the flesh color ratings 1-5 corresponded to color chips from the Flower Council of Holland as follows: 1 = 24d (cream), 2 = 50b (pink), 3 = 53b (red), 4 = 185a (red-purple), and 5 = 187a (mahogany).

Fruit acidity (AC) (% acidity) was the only trait taken from frozen fruit. Twenty frozen fruit, with their stems removed, were thawed and their juice was pressed through a Kimwipe (low-lint paper wipes) using a potato/rice masher. Juice (10 mL) was then placed in 100 mL of water and titrated to a pH of 8.2 using 0.1 N NaOH. Percent acidity (% acidity = [mLs NaOH used] x [0.1N NaOH] x [0.067 (meq factor)] x [100] / mLs of sample) was calculated using an automatic titrator, coupled to an auto-sampler and control unit (Titroline 96, Schott, Germany).

Calculations with fruit measurements: Sour cherries are primarily processed where pits are removed with a punching action that pushes the pit out of the fruit. Fruit to be pitted are placed on a conveyer belt where they fall into individual round cups that hold the fruit during the punching/pitting step. Round fruits and pits are desired so that the fruits are always oriented with the pits in the center of the cup and pit breakage, associated with oblong pits, does not occur. To record whether the fruit is round, fruit shape (ShF) was calculated as the ratio of fruit length and fruit width 2. This value was calculated for each fruit individually, and then averaged between the five largest fruit. Pit shape (ShP) was calculated as the ratio of pit length and pit width 1. This value was calculated for the pit from each individual fruit, and then averaged between the five largest fruit. Fruit width 2, and pit width 1 were chosen for these measurements as these tend to be the widest parts of the fruit and pit, respectively. Mesocarp weight (MWt) (g) was calculated for each fruit individually by subtracting pit weight from fruit weight and then averaging the mesocarp weight for the five largest fruit.

A slide presentation of the sour cherry phenotyping protocol (www.rosbreed.org/resources/fruit-evaluation) and videos of fruit phenotyping (www.rosbreed.org/resources/fruit-evaluation/phenotyping-videos/tartcherry) are available.

Correlations of phenotypic traits

To determine the correlations between the phenotypic traits in the phenotyping protocol, a total of 370 sour cherry individuals were evaluated that represented the genetic diversity used in the Michigan State Univer-

sity sour cherry breeding program. This included 50 cultivars and breeding selections and 320 progenies resulting from families whose ancestry traces back to the following five founder cultivars: 'Montmorency', 'Schattenmorelle', 'Pandy 38', 'Eugenia' and 'Englaise Timpurii' (Stegmeir, 2013). Correlations were calculated with the proc corr procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC).

Results and Discussion

Bloom date (BD) was significantly and positively associated with maturity date (MD), however the correlation was low (0.19) indicating that individuals that bloom early and ripen late, and individuals that bloom late and ripen early do exist (Table 2). The phenotypic trait most highly associated with bloom date was fruit firmness (Fir) with those individuals blooming late tending to be the most firm. Maturity date (MD) was also the most highly correlated with fruit firmness with later-maturing individuals tending to have firmer fruit.

Yield (YLD) was most highly correlated with fruit soluble solids (-0.29) and acidity (0.28) indicating that those selections with higher yield ratings had fruit that had less sugar and higher acid contents. It is not likely that this correlation is due to insufficient resources to mature the crop as the vast majority of the selections had very low crop loads. The mean crop load was a rating of three, and only 11 (3%) and 15 (4%) of the 370 sour cherry individuals evaluated had crop load ratings of 10 and 9, respectively.

The correlations among all the fruit size measurements (FWB, Fwt, FL, FW1, FW2, MWt) were all positive and highly significant ranging from 0.79 to 0.99 (Table 2). The correlations among all the pit size measurements (PWt, PL, PW1, PW2) were also all positive and highly significant; however, the values (0.53 to 0.85) were less than that for fruit size. The lowest correlation was for pit length and pit width 2. Fruit shape (ShF) was significantly associated with fruit weight (FWt), mesocarp weight (MWt), and the linear measurements of fruit size (FL, FW1, FW2). However, the correlation values for fruit shape and fruit weight and bulk weight were very low (-0.17 and -0.16, respectively). Pit shape was significantly associated with the pit length and width measurements but not pit weight. These results indicate that measurements of fruit weight and pit weight alone were not predictive of fruit and pit shape.

Fruit firmness is an important trait for mechanical harvesting and pit removal is more difficult from soft fruit. This trait was negatively correlated with all of the fruit size traits but not the pit size traits. These negative correlations indicate that larger fruit were generally less firm than smaller fruit. Therefore, care must be taken when selecting for either of these traits as selecting for one trait could result in more individuals that do not meet industry standards for the other trait. The finding that quantitative trait loci (QTLs) for fruit firmness and fruit size co-locate at four positions in the sweet cherry genome [linkage group 2 (C. Peace, pers. comm., Dirlewanger et al., 2012a) and on linkage groups 1, 3, and 6 (Dirlewanger et al., 2012a)] suggests a genetic linkage between genes controlling these two traits. Fruit shape was significantly associated with firmness suggesting that those fruit that are more round tended to be firmer than oblong fruit. Firmness was also significantly negatively correlated with acidity (-0.37) indicating that those fruit that were more firm tended to have lower acidity values.

Stem pull force (PuF) was significantly associated with all the fruit size traits suggesting that those selections with larger fruit tended to have higher values for stem pull force. The correlations between stem pull force and pit size were also significant but at a lower level of significance.

All fruit size measurements were significantly associated with the degree of freestone-clingstone (0.24 - 0.29) (F/C) indicating that larger fruit tended to be

Table 2. Phenotypic correlations among 22 sour cherry (*Prunus cerasus*) fruit traits for 2013 at the Clarksville Research Station, Clarksville, MI[1,2].

Traits	MD	YLD	Fir	FWB	PuF	FWt	FL	FW1	FW2	F/C	SSC	PWt	PL	PW1	PW2	CSk	CFl	AC	ShF	ShP	MWt
BD	*0.19*	NS	0.21	NS	NS	-0.15	-0.14	*-0.18*	*-0.17*	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	-0.16
MD		NS	0.35	0.17	*0.16*	*0.20*	NS	NS	NS	NS	NS	0.27	0.18	0.26	*0.18*	*-0.20*	-0.18	NS	NS	NS	0.20
YLD			-0.17	NS	0.15	NS	NS	NS	NS	NS	-0.29	NS	NS	*-0.21*	*-0.18*	NS	NS	0.28	NS	NS	NS
Fir				-0.30	NS	-0.33	*-0.18*	-0.29	-0.35	NS	NS	NS	NS	NS	NS	-0.15	NS	-0.37	0.27	NS	-0.35
FWB					0.23	0.94	0.79	0.87	0.85	0.24	-0.15	0.68	0.66	0.71	0.64	NS	NS	0.16	-0.16	NS	0.94
PuF						0.20	*0.18*	0.25	0.24	*0.18*	NS	0.15	0.14	0.16	NS	NS	NS	NS	-0.14	NS	0.22
FWt							0.82	0.91	0.89	0.24	-0.15	0.71	0.69	0.75	0.68	NS	NS	0.17	*-0.17*	NS	0.99
FL								0.86	0.83	0.29	-0.15	0.62	0.78	0.70	0.61	NS	NS	NS	*0.17*	0.14	0.82
FW1									0.96	0.26	-0.14	0.62	0.63	0.78	0.70	NS	NS	NS	-0.25	NS	0.91
FW2										0.24	NS	0.58	0.60	0.74	0.68	NS	NS	NS	-0.37	NS	0.90
F/C											NS	*0.19*	*0.19*	*0.18*	0.16	NS	NS	NS	NS	NS	0.24
SSC												-0.14	*-0.18*	NS	NS	NS	0.15	NS	-0.14	NS	-0.16
PWt													0.69	0.75	0.72	NS	NS	NS	NS	NS	0.68
PL														0.66	0.53	NS	NS	NS	*0.19*	0.47	0.68
PW1															0.85	NS	NS	NS	-0.15	-0.34	0.74
PW2																NS	NS	NS	-0.20	-0.33	0.67
CSk																	0.77	NS	NS	NS	NS
CFl																		NS	NS	NS	NS
AC																			NS	NS	0.18
ShF																				0.43	*-0.18*
ShP																					NS

[1] Correlation values significantly different from zero at the <0.0001 level are underlined, 0.001 level in italics and all remaining values are at the 0.01 level. Values not significant at any of these levels are identified as non-significant (NS).

[2] BD = bloom date, MD = maturity date, YLD = yield, Fir = Firmness, FWB = fruit weight bulk, PuF = pull force, FWt = fruit weight, FL = fruit length, FW1 = fruit width 1, FW2 = fruit width 2, F/C = freestone/clingstone, SSC = soluble solids concentration, PWt = pit weight, PL = pit length, PW1 = pit width 1, PW2 = pit width 2, CSk = color of skin, CFI = color of flesh, AC = acidity, ShF = fruit shape, ShP = pit shape, and MWt = mesocarp weight

more clingstone than smaller fruit. Pit measurements were also all significantly associated (0.16 to 0.19) with freestone-clingstone, with larger pits clinging to the flesh more than smaller pits.

Skin and flesh color ratings can be done quantitatively with the use of a Chroma Meter (CR-400, Konica Minolta, Tokyo, Japan). Intensity (L*) is measured as -L* = dark and +L* = light; green/red (a*) where -a* is green and +a* is red, and blue/yellow (b*) where -b* is blue and +b* is yellow. However, as these quantitative values are very highly correlated with the visual ratings (Sooriyapathirana et al., 2010), and taking quantitative measurements is very time and labor consuming, only visual ratings were included in this phenotyping protocol.

Visual ratings of skin and flesh color (CSk and CFl) were highly correlated (0.77) and significant correlations were only identified with maturity date and to a lesser extent with firmness. There was a significant trend for late maturing selections to have lighter skin and flesh colors. This suggests that the major gene controlling skin and flesh color on *Prunus* linkage group 3, *PavMYB10* (Sooriyapathirana et al., 2010), may be linked to loci contributing to variation for maturity date. In sweet cherry, a QTL for maturity date has been reported in the region of *PavMYB10* (Dirlewanger et al., 2012b).

Conclusions

The phenotyping protocol for sour cherry includes measurements that are critical to the successful mechanical harvesting and pitting of sour cherry. These include pull force, fruit firmness, fruit and pit shape, and freestone/clingstone. Also included are linear measurements of fruit and pit lengths and widths, as these measurements provide a more accurate determination of fruit and pit shape compared with measuring fruit weight alone. Correlations among some of the traits such as maturity date and fruit firmness, may suggest a genetic linkage of genes controlling these traits. Use of this standardized phenotyping protocol will enable the comparison of data between years and programs, and aid in identifying sour cherry chromosome regions that control trait variation.

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