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A DNA test for fruit flesh color in tetraploid sour cherry (*Prunus cerasus* L.)

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Abstract Fruit flesh color in tetraploid sour cherry (*Prunus cerasus*) is an important market-driven trait in the USA where the fruit from the dominant cultivar has brilliant red skin but clear/yellow flesh. This brilliant red color in the processed products differentiates products from sour cherries grown in the USA compared to those in Europe where the cultivars predominantly have dark purple-red flesh. In sweet cherry (*P. avium*), red skin and flesh colors were shown to be controlled by a major *MYB10*-associated locus. Sour cherry, which is derived from sweet cherry and ground cherry (*P. fruticosa*), also exhibits a range of flesh colors, but the genetic control of flesh color is not known. Our objectives were to test the hypothesis that the *MYB10* locus controls flesh color in sour cherry and develop a predictive DNA test for dark purple-red flesh color. Pedigree-linked sour cherry plant materials were phenotyped for flesh color. Thirteen haplotypes for the sour cherry *MYB10* region were distinguished based on markers scored from the use of the cherry 6K Infinium® II SNP array. Six haplotypes were significantly associated with variation in flesh color, supporting a role for *MYB10* in controlling flesh color variation in sour cherry. A simple sequence repeat primer pair, designed from the peach genome sequence near *MYB10*, amplified a fragment that uniquely identified the haplotype that was associated with the darkest purple-red flesh color. This marker can be used for marker-assisted breeding to identify individuals that are predicted to have dark purple-red flesh.

Keywords Anthocyanin · Marker-assisted seedling selection · Rosaceae

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Introduction

The US sour cherry industry is based on the cultivar 'Montmorency' that has yellow flesh and a brilliant red skin color. This is in contrast to the sour cherry cultivars grown in other major sour cherry-producing countries that typically have dark red, almost purple, flesh color. The brilliant red color of the processed products from 'Montmorency' provides a unique market class for US producers and is the recognizable color of cherry pie. Therefore, one of the major goals of the Michigan State University (MSU) sour cherry breeding program is to develop new improved 'Montmorency' types that retain the characteristic 'Montmorency' red color. Breeding efficiency would be significantly increased if progeny populations

could be enriched for individuals that have the desired flesh color through the use of a DNA test that would allow the elimination prior to field planting of those individuals predicted to have dark purple-red flesh.

The genetic control of the red anthocyanin pigment in sour cherry is not known; however, in apple (*Malus* spp.) an anthocyanin MYB transcription factor, *MdMYB10*, was shown to control red fruit flesh and foliage (Espley et al. 2007; Chagné et al. 2007). In sweet cherry, a quantitative trait locus (QTL) associated with the locus that has the highest homology with *MdMYB10* (*PavMYB10*) was identified as the major determinant of red coloration in sweet cherry skin and flesh (Sooriyapathirana et al. 2010). This major locus, located on *Prunus* linkage group 3, controlled as much as 94 % of the variation for sweet cherry flesh color with red flesh dominant to yellow flesh. Since diploid sweet cherry is one of the two progenitor species of tetraploid sour cherry ($2n = 4x = 32$), the ground cherry being the other progenitor species (Olden and Nybom 1968), we hypothesized that this gene, *MYB10*, may also be the major determinant of flesh color in sour cherry.

To determine the genetic control of flesh color in sour cherry, we took advantage of existing sour cherry breeding populations segregating for fruit flesh color and the ability to obtain genetic data for the parents and progeny individuals using the cherry 6K Infinium® II SNP array as part of the RosBREED project (www.rosbreed.org; Peace et al. 2012). As sour cherry is a segmental allotetraploid that exhibits irregular pairing at meiosis, the generation of a linkage map and a subsequent QTL analysis were not possible (Beaver and Iezzoni 1993; Iezzoni et al. 2005). Therefore, a targeted approach based on the hypothesis that *MYB10* is the major determinant of flesh color was used. The objective was to test this hypothesis and develop a DNA test that would be predictive of flesh color for use in marker-assisted breeding.

Materials and methods

Plant populations and phenotyping

A total of 273 cultivars and seedlings from five bi-parental populations including parents were used in this study (Fig. 1). Populations were as follows: 'Újfehértói Fürtös' ('UF') × 'Surefire' ($n = 69$), M172 × 25-02-29 ($n = 78$), 25-14-20 × 25-02-29 ($n = 58$), 'Montmorency' × 25-02-29 ($n = 44$), and 'Rheinische Schattenmorelle' ('RS') × 'Englaise Timpurii' ('ET') ($n = 24$). These individuals were planted at the MSU Clarksville Research Station, Clarksville, Michigan.

A fruit flesh color rating for each individual was taken in 2011 according to the Sweet Cherry Flesh Color Index from Washington State University (WSU) (Supplementary Fig. S1). A visual rating was given after observing five fruits. The 1–5 score represented clear/yellow flesh color (score of 1), through shades of red (scores 2–4) to dark purple-red color (score of 5). Results from the previous analysis conducted in sweet cherry supported this phenotyping strategy (Sooriyapathirana et al. 2010) as the color index values were highly significantly correlated with quantitative color measurements obtained using a spectrophotometer, and the color index values were highly significantly correlated across 3 years of evaluation.

Genotyping and haplotype construction

Four hundred and two sour cherry individuals, including founders, seedlings, and all 273 individuals in the five bi-parental populations, were genotyped using the 6K Infinium® II SNP array developed as part of the RosBREED project (www.rosbreed.org; Peace et al. 2012). The Illumina® Genome Studio software was used to determine the SNP genotype. Available SNP data from a subset of sweet cherry selections and hybrid seedlings (105 individuals) were included to aid in the determination of dosage by showing the two homozygous (AAAA and BBBB for sour cherry corresponding to AA and BB in sweet cherry) and balanced heterozygous (AABB for sour cherry corresponding to AB in sweet cherry) classes.

A total of 47 SNP markers that spanned the location of the *MYB10* were used to build the sour cherry haplotypes (Supplementary Table S1). These SNPs spanned the physical map location of the three *MYB10* homologs identified on the Peach v1.0 linkage group 3 assembly (scaffold 3, 12.84–12.91 Mb, Verde et al. 2013). For each parent and progeny individual, four haplotypes (to represent the four chromosomes in a tetraploid) were built for the target regions of the genome in an Excel spreadsheet by hand based on

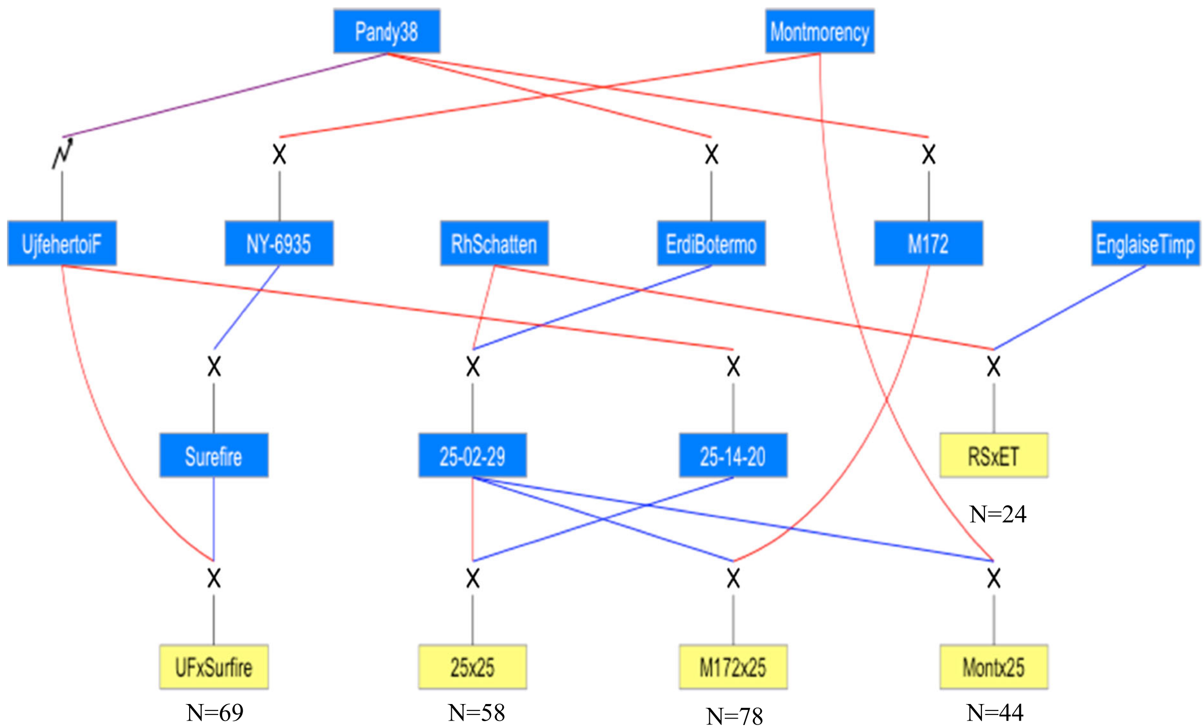

Fig. 1 Pedigrees of plant materials that were used for validating the *MYB10*-associated flesh color QTL in sour cherry visualized using Pedimap Software (Voorrips et al. 2012). The five full sib families used in this study represent a large portion of the diversity found in the breeding germplasm at Michigan State University. N is the number of progeny individuals in each population

progeny inheritance and segregation in each of the bi-parental populations. The haplotypes were initially arbitrarily named with alphabetical letters; however, they were subsequently renamed based upon the presence or absence of a significant phenotypic effect, whereby 'D' and 'd' were used to indicate a significant increase or decrease, respectively, in dark red color associated with the presence of the haplotype, and an '*x*' was used to indicate that no significant effect on color was associated with this haplotype.

Statistical analysis

ANOVA calculations were used to determine whether the *MYB10* haplotypes identified in sour cherry were significantly associated with flesh color. A linear additive model test was implemented with a user-defined design matrix to consider the presence or absence of each haplotype as well as the number of times each haplotype was present (to account for dosage), using a modified R-script 2.15.1 (R Development Core Team 2011). The Proc Mixed least squares means statement in SAS 9.2® (SAS Institute Inc., Cary, NC, USA) was used to determine whether the means of the different haplotypes were significantly different. To confirm the ANOVA calculations and determine whether the *MYB10* haplotypes had a positive or negative effect on the trait, Student's t tests were performed comparing progeny individuals within each family with and without each of the haplotypes that were significant in the linear additive model.

DNA test for flesh color

SSR markers for flesh color that tagged the *MYB10* were designed based on the peach genome v1.0 (Verde et al. 2013) available at www.rosaceae.org. Microsatellite repeats were found using Microsatellite Repeats Finder (www.biophp.org/minitools/microsatellite_repeats_finder/demo.php). The criteria for SSR selection were the same as in Stegmeir et al. (2014): 2-bp minimum

repeat length and 5-bp maximum repeat length with a minimum number of 10 repeats. If multiple SSRs were found in the region of interest, those with the largest number of tandem repeats were selected. Primer pairs were designed using Primer3web (bionifo.ut.ee/primer3/), with product size limited to 150–250 bp, and primers selected on either side of the repeat region with a CG clamp. Primer sequences were compared to the Peach genome v1.0 scaffolds (Verde et al. 2013) using NCBI's BLAST. Primers that co-located to multiple scaffolds with at least 75 % sequence similarity were not used.

To test the ability of the SSR to distinguish between the *MYB10* haplotypes, DNA was extracted from a subset of the parents and hybrid seedlings known to segregate for the dark red-purple flesh haplotype using the procedure described by Edge-Garza et al. (2014). The PCR mixture was as in Olmstead et al. (2008). A touchdown PCR was used for the flesh color diagnostic SSR primer pair called LG3_13.146, which has a forward primer sequence of 5′ ATG TGG CCA AAG GTC AGC 3′ and reverse primer sequence 5′ TGA TCC CAA TCA CGT TTT CC 3′. The conditions were as follows: 94 °C for 5 min followed by nine cycles of 94 °C for 30 s, 60 °C for 45 s (−1 °C per cycle), 72 °C for 1 min, and then 24 cycles of 94 °C for 30 s, 55 °C for 45 s, 72 °C for 1 min with an elongation step of 72 °C for 5 min. PCR fragments were separated on a 6 % polyacrylamide gel and visualized with silver staining as in Olmstead et al. (2008).

MASS was implemented using this SSR for five seedling populations generated from crosses made in 2013 to identify those individuals predicted to have dark red-purple flesh color, and the numbers and percentages of progeny individuals culled were recorded. All crosses had the dark purple-red-fleshed MSU selection 25-14-20 as the seed parent with five different red- and clear/yellow-fleshed MSU selections as pollen parents (27-03-08, 27e-04-54, 27e-05-33, 27e-15-38, and 27e-16-47 (Supplementary Table S2).

Results

Phenotypic variation

The phenotypic distributions for flesh color in all populations ranged from 1 (no red color in the flesh) to 5 (very dark purple-red flesh), but did not show a normal distribution in any of the populations (Supplementary Fig. S2). The three populations that had a very dark purple-red-fleshed parent (underlined), 'UF' × 'Surefire,' 25-14-20 × 25-02-29, and 'RS' × 'ET,' tended to skew toward the darker red flesh colors. In contrast, those populations that did not have a very dark purple-red-fleshed parent, M172 × 25-02-29, and 'Montmorency' × 25-02-29, were slightly skewed to lighter flesh color. The use of 25-02-29 as a parent in three of the five populations allowed a comparison of the combining ability of this selection with parents with color index scores of 5 (25-14-20), 4 (M172), and 2 ('Montmorency'). When 25-02-29 was crossed with 25-14-20, the progeny were skewed toward the dark purple-red color class, possibly due to a dominant allele for red color contributed from 25-14-20. In contrast, when 25-02-29 was crossed with the lighter-colored M172 and then the clear/yellow-fleshed 'Montmorency,' there were increased percentages of lighter-fleshed progeny individuals.

Haplotypes for the *MYB10* region

A total of 47 SNPs that spanned the *MYB10* region (9.73–15. 46 Mb) were used to build 13 haplotypes that represented the alleles identified in the eight sour cherry parents (Supplementary Table S1). These haplotypes could be distinguished using a subset of the constructed SNP region that ranged from 10.57 to 14.02 Mb and represented approximately 10 cM (Table 1). Progeny segregation data for the 13 haplotypes were used to determine the haplotype genotypes of the parents, including whether the haplotype was in single or double dose, such as the presence of two *x2* haplotypes in 25-02-29 (Table 2). The genotypes deduced were consistent with the known parent–offspring relationships. 'UF' and 25-14-20 share the *x1*, *x2,* and *D1* haplotypes which is expected as 'UF' is the maternal parent of 25-14-20 (Fig. 1). Likewise 'RS' and 25-02-29 share the *x2* and *D2* haplotypes as 'RS' is the maternal parent of 25-02-29 (Fig. 1).

Haplotypes associated with fruit color

ANOVA indicated that seven of the thirteen MYB10 haplotypes contribute significantly to flesh color in sour cherry (Supplementary Table S2). Within the five bi-parental populations, t tests confirmed that six of the thirteen *MYB10* haplotypes were significantly

Table 1 Six SNP haplotypes identified in sour cherry for the linkage group 3 region spanning *MYB10* that were significantly associated with flesh color. The SNPs and cM locations are as described in Peace et al. (2012) and Klagges et al. (2013), respectively. Additional SNP haplotypes that were not significantly associated with flesh color are included in Supplementary Table S1. The 31 SNPs included below are required for the differentiation of all 13 haplotypes identified. Additional flanking SNPs are also included in Supplementary Table S1. The four SNPs that uniquely differentiate the *D1* haplotype from the other 12 haplotypes are highlighted in bold

NCBI SS#	Linkage map position (cM)	Original full name	Haplotypes					
			D1	D2	D3	D4	d1	d2
ss490551577		RosBREED_snp_sweet_cherry_Pp3_10573974	B	B	A	B	B	B
ss490547944		RosBREED_snp_tart_cherry_a_Pp3_10590166	B	B	A	B	B	B
ss490551581		RosBREED_snp_sweet_cherry_Pp3_10626205	B	B	A	B	B	B
ss490551584	43.8	RosBREED_snp_sweet_cherry_Pp3_10675150	B	A	A	B	B	B
ss490551593	45.1	RosBREED_snp_sweet_cherry_Pp3_10822211	B	A	A	B	B	B
ss490547952		RosBREED_snp_tart_cherry_a_Pp3_10908880	B	B	A	B	B	B
ss490547960		RosBREED_snp_tart_cherry_a_Pp3_12115409	B	B	A	B	A	B
ss490551635		RosBREED_snp_sweet_cherry_Pp3_12383977	A	A	B	A	A	A
ss490551642		RosBREED_snp_sweet_cherry_Pp3_12474678	B	B	B	B	B	B
ss490551648		RosBREED_snp_sweet_cherry_Pp3_12500413	B	B	A	B	B	B
ss490547972		RosBREED_snp_tart_cherry_f_Pp3_12503462	B	B	A	B	B	B
ss490547976	49.7	RosBREED_snp_tart_cherry_f_Pp3_12539794	**B**	B	A	B	B	B
3 MYB10 homologs	47.5	12.84–12.91 Mb						
ss490551672		RosBREED_snp_sweet_cherry_Pp3_12944437	**B**	B	A	B	B	B
ss490551678	50	RosBREED_snp_sweet_cherry_Pp3_12987920	**B**	A	B	B	A	A
ss490551684	51	RosBREED_snp_sweet_cherry_Pp3_13025963	**A**	A	A	B	B	A
ss490547992		RosBREED_snp_tart_cherry_a_Pp3_13063792	B	B	A	B	B	B
ss490551699	51.1	RosBREED_snp_sweet_cherry_Pp3_13144730	B	B	B	B	A	B
ss490551705	51	RosBREED_snp_sweet_cherry_Pp3_13208005	A	B	B	A	A	B
ss490548000		RosBREED_snp_tart_cherry_f_Pp3_13369328	A	A	A	A	A	A
ss490551720	51.3	RosBREED_snp_sweet_cherry_Pp3_13406263	B	B	A	A	A	B
ss490551723		RosBREED_snp_sweet_cherry_Pp3_13433848	B	B	B	A	A	A
ss490551730		RosBREED_snp_sweet_cherry_Pp3_13466702	B	A	B	A	A	B
ss490551739		RosBREED_snp_sweet_cherry_Pp3_13520194	B	B	B	A	B	A
ss490551746		RosBREED_snp_sweet_cherry_Pp3_13563908	B	B	A	B	B	B
ss490551749	52.8	RosBREED_snp_sweet_cherry_Pp3_13567593	B	B	A	B	A	B
ss490551771		RosBREED_snp_sweet_cherry_Pp3_13724726	B	B	A	B	B	B
ss490551778	53.4	RosBREED_snp_sweet_cherry_Pp3_13754793	A	B	B	A	A	A
ss490551784		RosBREED_snp_sweet_cherry_Pp3_13795019	A	A	B	A	A	A
ss490559450	52.9	RosCOS3766-391_snp_sweet_cherry_Pp3_13878008	B	A	A	A	A	A
ss490548016		RosBREED_snp_tart_cherry_a_Pp3_13881088	B	A	A	A	A	A
ss490551803	52.9	RosBREED_snp_sweet_cherry_Pp3_14024780	A	A	A	B	A	B

associated with flesh color (Table 2). Progeny that inherited the *D1* haplotype from the dark purple-red-fleshed 'UF' or its dark purple-red-fleshed offspring 25-14-20 had significantly darker red flesh color than those progeny that did not inherit the *D1* haplotype. Progeny that inherited the *D2* haplotype from 'RS' or its offspring 25-02-29 also had significantly darker red flesh than those that did not inherit the *D2* haplotype. Inheritance of the *D3* haplotype from 'Surefire' or 'ET' also resulted in progeny that had significantly

Table 2 Phenotypic means for flesh color[a] for the presence or absence of the *MYB10* haplotypes[b] for all sour cherry individuals within the five full sib families. Only haplotypes with significant differences in at least one family are presented

Family parental genotypes	Flesh Color			
	Haplotype[c]	Number[d]	Mean[e]	*P* value
UF × Surefire	**D1**/no D1	27/42	$4.63^{a}/2.43^{b}$	<0.0001
D1d2x1x2 × D3x3x4x5	**D3**/no D3	26/43	$3.92^{a}/2.91^{b}$	0.002
	d2/**no d2**	37/32	$2.62^{a}/4.06^{b}$	<0.0001
25-14-20 × 25-02-29	**D1**/no D1	25/33	$4.68^{a}/2.97^{b}$	<0.0001
D1x1x2x5 × D2d1x2x2	**D2**/no D2	29/29	$4.52^{a}/2.90^{b}$	<0.0001
	d1/**no d1**	27/31	$2.96^{a}/4.35^{b}$	0.003
M172 × 25-02-29	**D2**/no D2	37/41	$3.92^{a}/2.20^{b}$	<0.0001
D4d2x2x6 × D2d1x2x2	**D4**/no D4	35/42	$3.71^{a}/2.48^{b}$	<0.0001
	d1/**no d1**	39/39	$2.13^{a}/3.90^{b}$	<0.0001
	d2/no d2	38/40	$2.76^{a}/3.15^{a}$	0.22
Montmorency × 25-02-29	**D2**/no D2	20/22	$3.90^{a}/1.63^{b}$	<0.0001
d1x2x3x5 × D2d1x2x2	d1/**no d1**	33/8	$2.33^{a}/4.13^{b}$	0.002
RS × ET	**D2**/no D2	13/11	$4.62^{a}/3.36^{b}$	0.03
D2x2x5x7 × D3D4x4x6	**D4**/no D4	13/11	$4.62^{a}/3.36^{b}$	0.02
	D3/no D3	10/14	$4.40^{a}/3.79^{a}$	0.21

[a] Washington State University color card rating 1 to 5 scale (See Supplementary Fig. S1)

[b] Haplotypes representing the alleles for the *MYB10* region are defined in Table 1

[c] The allelic states significantly associated with increased trait values are identified in bold

[d] Numbers of progeny individuals with or without the individual haplotype

[e] Means with the same letter within a row are not significantly different ($P > 0.05$)

redder flesh than those without the *D3* haplotype. Finally, the inheritance of the *D4* haplotype from M172 or 'ET' also resulted in significantly redder flesh color. Collectively, the effect of these haplotypes on color was greatest for the *D1* haplotype followed by the *D2*, *D3,* and *D4* haplotypes.

Two haplotypes were significantly associated with lighter flesh color in at least one population (Table 2). The presence in the offspring of the *d1* haplotype from 25-02-29 and 'Montmorency' was significantly associated with lighter flesh color. The presence of the *d2* haplotype derived from 'UF' was significantly associated with light flesh color, but the effect of *d2* derived from 'M172' was nonsignificant. The significance of the *d2* haplotype from 'UF' may be due to the absence of inheriting the *D1* haplotype, whereas the lighter red-fleshed 'M172' does not have the very purple-red *D1* haplotype.

Since no parent had more than one significant dark red flesh haplotype (with the exception of 'ET' which had two), the effect of an individual having two dark red-fleshed haplotypes was examined in the three largest progeny populations. For 'UF' × 'Surefire,' progeny individuals that had neither the *D1* nor *D3* haplotypes had light flesh (mean = 1.9; Fig. 2a). The presence of a *D3* haplotype, in the absence of the *D1* haplotype, resulted in a significant increase in flesh color (mean = 3.0). However, any progeny individual that had a *D1* haplotype had significantly darker flesh color (means of 4.8 and 4.5) irrespective of the presence of the *D3* haplotype. The effect of the *D1* haplotype was similar when compared to the *D2* haplotype, suggesting that the *D1* haplotype by itself results in the maximum purple-red flesh color (Fig. 2b). This is consistent with the 'UF' × 'Surefire' and 25-02-29 × 25-24-20 progeny phenotypic data that are highly skewed to the dark purple-red color class when one parent contains the *D1* haplotype (Fig. 3). The vast majority of those progeny that had a color index score of 5 had the *D1* haplotype (Fig. 3). In contrast, the two 'weaker' red-fleshed haplotypes (*D2* and *D4*) exhibited an additive effect on flesh color with the presence of both haplotypes having a significantly increased red flesh color compared to the presence of

A UF × Surefire (D1, D3)

		D1 +	D1 -
D3	+	4.8 a	3.0 b
	-	4.5 a	1.9 c

B 25-14-20 × 25-02-29 (D1, D2)

		D1 +	D1 -
D2	+	4.9 a	4.2 b
	-	4.4 ab	1.6 c

C M172 × 25-02-29 (D4, D2)

		D2 +	D2 -
D4	+	4.2 a	3.2 b
	-	3.6 c	1.3 d

Fig. 2 Within population mean comparisons of dark flesh haplotypes *D1*, *D2*, *D3*, and *D4* for populations, **a** 'UF' × 'Surefire,' **b** 25-14-20 × 25-02-29, and **c** M172 × 25-02-29. Each parent contributes one dark red-fleshed haplotype. Within each population, means that were significantly different ($P < 0.05$) are identified with *different letters*

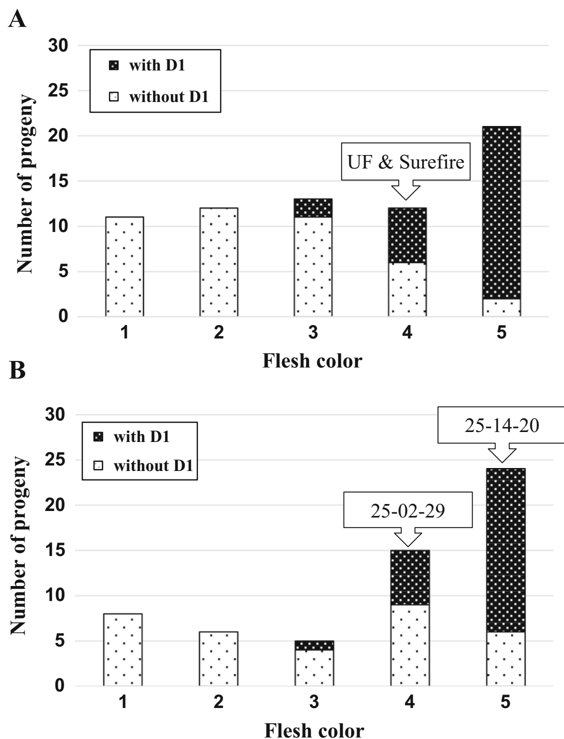

Fig. 3 Flesh color ratings for the 69 progeny from the 'UF' × 'Surefire' population (**a**) and 58 progeny from the 25-14-20 × 25-02-29 population (**b**). Those progeny individuals that have the *D1* haplotype are identified with *white dots* on a *black* background and those that do not have the *D1* haplotype are identified with *black dots* on a *white* background. *Arrows* mark the flesh color ratings of the parents

only one haplotype (Fig. 2c). The interaction of these flesh color haplotypes is consistent with the transgressive segregation for flesh color (scores 1–5) seen in the cross M172 × 25-02-29, where both parents have a color index score of 4 (Supplementary Fig. S2).

Marker-assisted seedling selection (MASS)

The presence of one *D1* haplotype was significantly associated with the darkest flesh color class (purple-red) that is not preferred for the US sour cherry markets. Therefore, the effects of implementing MASS against the *D1* haplotype were examined in the 'UF' × 'Surefire' and the 25-14-20 × 25-02-29 populations (Fig. 3). If MASS had been implemented in these two populations, 41 % (52/127) of the progeny would have been discarded. None of the progeny discarded would have had color index scores of 1 and 2. The progeny that would have been discarded would have overwhelmingly had a color index score of 5 (71 %), followed by a score of 4 (23 %) and a score of 3 (6 %). Of those progeny that did not have the *D1* haplotype and would not have been discarded, 11 % (8/75) had the color index score of 5. Therefore, MASS against the *D1* haplotype collectively in these two populations would have reduced the frequency of progeny individuals with the dark purple-red flesh color from 35 to 11 %. This enrichment of the remaining seedlings was sufficiently compelling to implement in the sour cherry breeding program.

To enable MASS for the presence or absence of the *D1* haplotype in a progeny individual, a DNA marker had to be developed that uniquely identified the *D1* haplotype. The *D1* haplotype differs from all the other *MYB10* haplotypes by the presence of BBBA for four consecutive SNPs (Table 1, see bold). A SNP diagnostic marker, however, could not be developed because none of the SNPs were unique to this haplotype. Instead, it was the phase of the SNP markers (coupling versus repulsion for the A and B alleles) that distinguished this haplotype. Therefore,

the strategy was to design an SSR marker that had a repeat length that uniquely distinguished the *D1* haplotype.

Taking advantage of the synteny between peach and cherry, forty SSRs were identified within 1 Mb of the *MYB10* and tested for their ability to uniquely distinguish the *D1* haplotype (Supplementary Fig. S3). Of the forty SSRs, only one that was located ~200,000 kb from *MYB10* at position 13.146 Mb was found to uniquely differentiate the *D1* haplotype. A 218-bp fragment amplified with this SSR uniquely co-segregated with the presence/absence of the *D1* haplotype. This SSR also amplified a 220-bp fragment that uniquely co-segregated with the presence/absence of the *D2* haplotype (Supplementary Fig. S4). Therefore, the SSR marker, LG3_13.146, fit the criteria for use for MASS for the *D1* haplotype.

MASS against the *D1* haplotype was implemented using the LG3_13.146 marker in progeny populations where the maternal parent was the dark purple-red-fleshed selection 25-14-20 that has one copy of the *D1* haplotype (Supplementary Table S3). As none of the pollen parents have the *D1* haplotype, 50 % of the progeny would be expected to have the *D1* haplotype resulting in the potential of discarding a half of the seedlings. Across all populations, 619 progeny were screened and the 358 progeny (58 %) were found to have the *D1* haplotype. These plants were subsequently discarded; therefore, the number of plants advanced to the breeding orchard totaled 261 as opposed to 619.

Discussion

Thirteen unique SNP haplotypes were identified spanning the *MYB10* locus from analyses of eight sour cherry parents. For seven of these haplotypes, no significant effect on fruit flesh color was identified. However, four and two of the haplotypes were significantly associated with dark purple-red versus light red/yellow flesh colors in sour cherry, respectively, validating the *MYB10*-associated fruit flesh color QTL previously identified in sweet cherry (Sooriyapathirana et al. 2010). This supports the previous finding that the *MYB10* locus on *Prunus* linkage group 3 is a major factor contributing to sweet cherry red flesh color. However, even the presence of the darkest purple-red-fleshed haplotype *D1* for *MYB10* was not 100 % predictive of dark red flesh color. For example, in the cross 'UF' × 'Surefire,' three out of 21 progeny with the darkest color index score of 5 did not have the *D1* haplotype, and two of the 13 with the *D1* haplotype had the color index value of 3. Although *MYB10* is likely the major determinant of flesh color in sour cherry, these results indicate that other loci are contributing to flesh color. This is similar to the findings in sweet cherry and peach. In sweet cherry, alleles at loci on chromosomes 6 and 8 influenced the fruit color predicted based on *Pav-MYB10* alone (Sooriyapathirana et al. 2010). In peach (*P. persica*), a major QTL for red blush color was significantly associated with the linkage group 3 *MYB10* region; however, minor QTLs were identified on linkage groups 4 and 7 (Frett et al. 2014). Identifying these additional loci influencing fruit anthocyanin pigmentation will be critical to increase the predictive ability of marker-assisted breeding for red flesh color in cherry.

This study was unable to determine how multiple copies of the same haplotype would influence flesh color, since no populations studied had more than one copy of any dark red flesh haplotype. For example, there were no individuals that had more than one copy of the *D1* haplotype. However, based on the finding that the moderate flesh color haplotypes *D3*, *D4,* and *D2* tended to have an additive affect when found together in the same individual, it could be inferred that two copies of the same dark flesh haplotype would have a similar result.

The *D1* haplotype was determined to be an excellent candidate for marker-assisted breeding in sour cherry as it was very significantly associated with the dark purple-red fruit flesh color that is not preferred in the US markets and it tended to exhibit dominant gene action. Reducing the frequency of the *D1* haplotype in the MSU breeding seedling populations would likely result in enrichment of progeny for the desired lighter flesh colors, resulting in significant cost savings. For example, those seedlings that were discarded due to the presence of the *D1* haplotype would otherwise have been planted and grown in the breeding orchard for three or four years before the flesh color would have been determined. In Europe, where a very dark purple-red flesh color is preferred for cherry juice and certain other processed products, a DNA test for the *D1* haplotype would also potentially be useful. However, in their case, MASS would likely be used to identify those progeny individuals predicted

to have very dark red-purple flesh color so that they could be maintained.

Our ability to develop a DNA test that provided sufficient confidence for use in the breeding program was due in a large part to the examination of a wide range of germplasm that represented the diversity in the breeding program. This allowed the identification of multiple haplotypes segregating in the genepool and the characterization of the functional haplotypes in different genetic backgrounds. A DNA test that met the specific breeding goal could then be designed. This approach of QTL validation in pedigree-linked germplasm is a central strategy used in the 'RosBREED' project (Peace et al. 2014) to enable marker-assisted breeding in rosaceous crops.

As sweet cherry is one of the two progenitor species of sour cherry, the sour cherry haplotypes were further compared to the sweet cherry haplotypes identified to date in a diverse set of 268 sweet cherry individuals in the 'RosBREED' sweet cherry Crop Reference Set (Peace et al. 2012) using three common SNPs that flanked the *MYB10* region (ss490551678, ss490551684, and ss490551723) (sweet cherry data provided by P. Sandefur and C. Peace, per. comm.). Based on these three SNPs, the sour cherry haplotypes *D1*, *D2*, *D3*, *x1*, *x2*, *x3*, and *x7* were not identified in sweet cherry, suggesting that these haplotypes may be unique to sour cherry, possibly contributed by the ground cherry subgenome. In particular, the dark purple-red flesh color haplotype *D1* was not identified in sweet cherry. For the six other sour cherry haplotypes that had SNP markers in common with those in sweet cherry, it was not possible to confirm identity based on the SNPs alone. Additionally, it is possible that the SNP haplotypes overrepresent the number of *MYB10* alleles. Further characterization of the *MYB10* alleles would require sequence data spanning the *MYB10* region. However, the different effects of the four red color haplotypes in sour cherry suggest that there are multiple functional variants of the *MYB10* locus that result in differences in the presence and intensity of red flesh color.

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