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Development and utilization of a DNA diagnostic test to predict flesh color in tetraploid sour cherry

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SUMMARY: Fruit color in sour cherry (*Prunus cerasus*) is an important market-driven trait in the U.S. where the dominant cultivar 'Montmorency' has a brilliant red color. This unique brilliant red color allows for a differentiation in products from sour cherries grown in the U.S. compared to those in Europe which have predominantly dark-purple flesh. The anthocyanin transcription factor, *MYB10*, has previously been shown to control flesh color in sweet cherry (**Fig. 1**) and other rosaceous species. Our objectives were to test the presence of this *MYB10*-associated flesh color QTL in sour cherry and develop a DNA test that would be predictive of flesh color. Pedigree linked sour cherry plant materials (**Fig. 2**, n=338) were phenotyped for flesh color (**Fig. 3**). Thirteen allelic variants for the sour cherry *MYB10* region were distinguished based on the linkage phase of 47 polymorphic SNPs determined using the 6K Infinium® II SNP array developed as part of the RosBREED project (www.rosbreed.org, **Figs. 4 & 5**). Of these 13 haplotypes (**Table 1**), four haplotypes behaved as dominant alleles conferring dark-flesh color (**Fig. 6**)**.** No SNPs were found in this region which would distinguish haplotypes linked to dark-flesh. Therefore, due to the high synteny in Prunus, the peach genome sequence was used to locate SSRs in this same region of interest (mostly within 1Mb of the three predicted MYB10 genes). Forty SSR markers were then developed which could be screened for their ability to uniquely identify dark-fleshed haplotypes (**Fig. 7**). One SSR primer pair, about 200,000 Kb from the nearest *MYB10* homolog, was found to amplify fragments that successfully differentiated the two darkest-flesh haplotypes (**Fig. 8**)**.** This marker can now be used for marker-assisted seedling selection in any cross where either of these two haplotypes are segregating to cull those individuals that which are predicted to have dark-purple flesh (**Table 2**).

Fig. 1. A flesh color QTL was previously found on LG3 in diploid sweet cherry that co-located with *MYB10*. This allowed a targeted approach in sour cherry with the assumption that the flesh color QTL would be conserved in tetraploid sour cherry. (Figure from Sooripathirana et al. 2010[1])

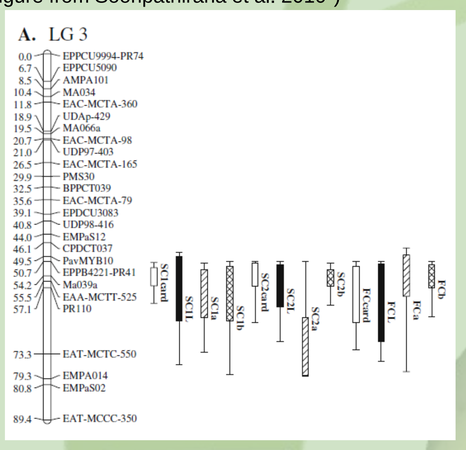

Fig. 2. Plant materials (visualized using Pedimap[2]) that were used for validating the *MYB10*-associated flesh color QTL in sour cherry. The 5 bi-parental sour cherry families used in this study represent a large portion of the diversity found in the breeding germplasm at MSU

Fig. 3. WSU flesh color rating scale used to phenotype individuals, from 1 (clear flesh) to 5 (dark purple flesh).

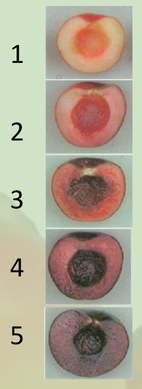

Fig. 4. The cherry 6K Infinium® II SNP array[3] was used to genotype all plant materials. SNP dosage calls were done for each marker, where sweet cherry individuals (yellow) were included to help define the two homozygous (AAAA and BBBB) classes and the balanced heterozygous class (AABB). Determining dosage was necessary to build haplotypes.

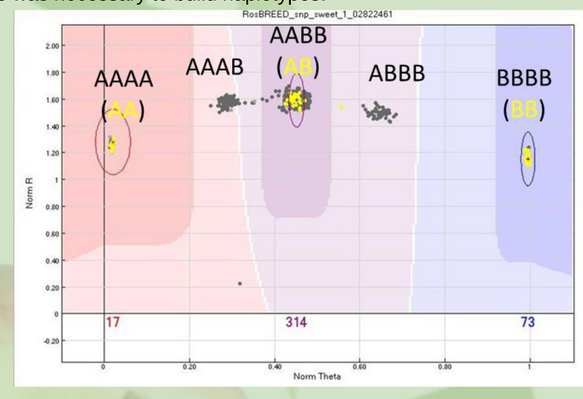

Fig. 5. SNP haplotypes spanning the QTL region for flesh color of the parents of each of the 5 populations used in this study. The red arrow on the left shows the location of *MYB10* homologs. Each haplotype was built manually based on progeny segregation in each of the populations.

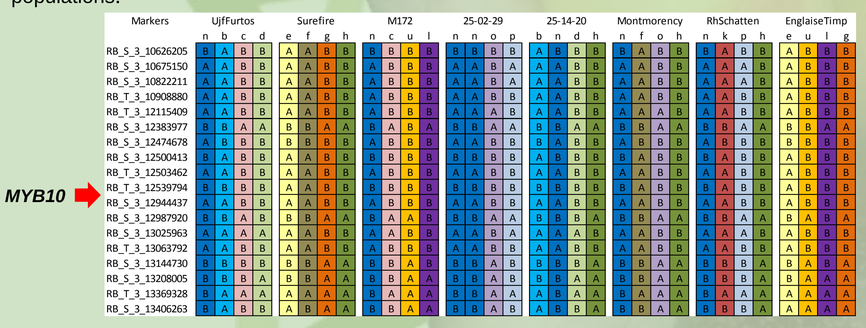

Table 1. A linear additive model test was used to determine which haplotypes contribute significantly to color or lack of color in sour cherry. Significance codes for P-values are 0 '***', 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' '.

Haplotype	Df	Sum Sq	Mean Sq	F value	Pr(>F)	Signif.
b	1	9.9	9.9	9.4	0.002	**
c	1	18.2	18.2	17.4	4.07E-05	***
d	1	115.3	115.3	110.0	2.20E-16	***
e	1	23.6	23.6	22.5	3.34E-06	***
f	1	1.2	1.2	1.1	0.29	
g	1	1.3	1.3	1.2	0.27	
h	1	2.1	2.1	2.0	0.16	
k	1	2.3	2.3	2.2	0.14	
l	1	50.5	50.5	48.2	2.72E-11	***
n	1	0.7	0.7	0.6	0.43	
o	1	98.4	98.4	93.8	2.20E-16	***
p	1	55.1	55.1	52.6	4.06E-12	***
u	1	0.1	0.1	0.1	0.78	

Fig. 6. Haplotypes "d", "p", "l", and "e" were found to contribute to dark flesh color. Mean comparisons were done of these dark flesh alleles in 4 of the populations. Parents of the populations are presented along with the dark flesh haplotype they carry. Progeny mean flesh color scores are sorted by presence or absence of each dark flesh allele. Significantly different (P < 0.05) mean scores are represented by different letters.

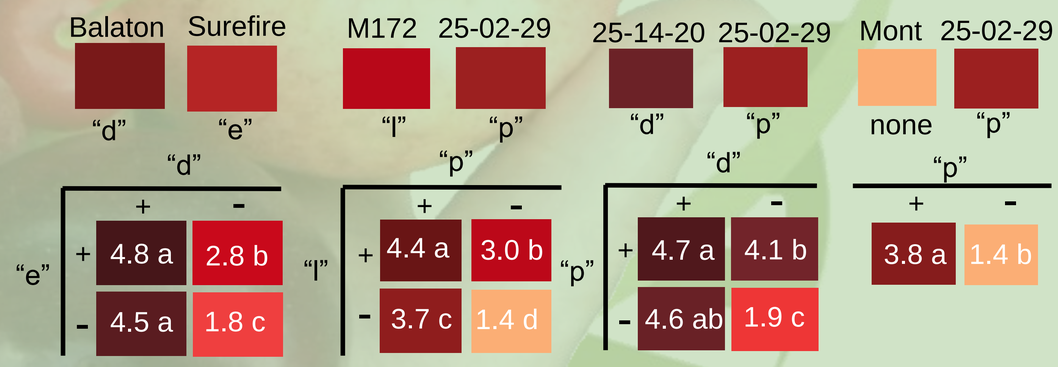

Fig. 7. Locations of SSR markers surrounding the candidate *MYB10* homologs that were screened for possible association with dark-flesh color haplotypes. Markers were found using the peach genome sequence. One marker (LG3_13.146, green star) was found to be polymorphic and able to distinguish two of the dark flesh haplotypes.

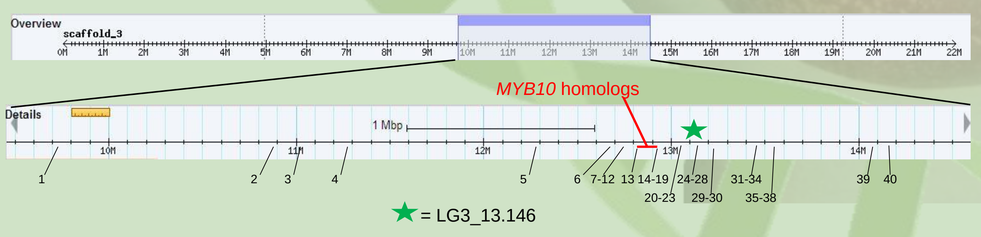

Fig. 8. Acrylamide gel of SSR marker LG3_13.146 which can be used to identify the two dark flesh color haplotypes "d" and "p". This SSR marker results in a unique fragment for haplotype "d" at 218 bp and another for haplotype "p" at 220 bp.

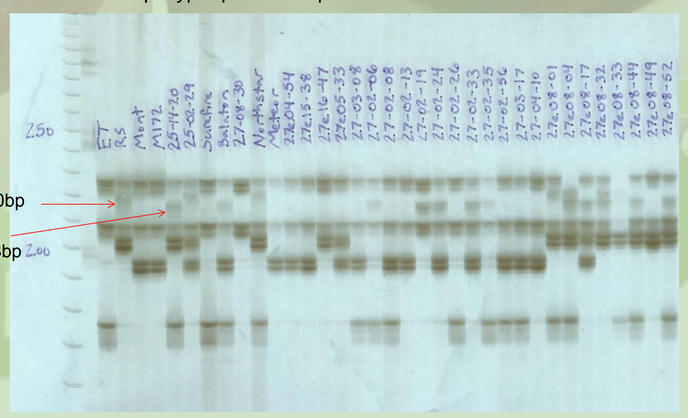

Table 2. Seedling populations segregating for presence or absence of the "d" haplotype were screened using the SSR marker LG3_13.146. Those carrying the "d" fragment were culled.

Maternal Parent	Paternal Parent	# Plants DNA tested	# Plants discarded	% Plants kept
25-14-20	27-03-08	*371*	*206*	44
25-14-20	27e-04-54	*88*	*58*	34
25-14-20	27e-05-33	*18*	*10*	44
25-14-20	27e-15-38	*26*	*12*	54
25-14-20	27e-16-47	*84*	*55*	35

Comment – marker development in polyploids

In sour cherry, the SNPs used to define the flesh color QTL region were not sub-genome specific between the *P. avium* and *P. fruticosa* genomes. Additionally, no combination of SNPs was able to completely distinguish the haplotypes in an unknown genotype as it is SNP phase, not the SNP itself that is unique. By designing and screening SSR markers for this region, we were able to find one SSR (LG3_13.146) which differentiated, based on a unique fragment size, haplotype "d" which carries the allele which confers the darkest flesh color to individuals. We could then select against this allele since our goal is to have a brilliant red cherry, and not one that is dark mahogany. In summary, this DNA diagnostic test allowed us to achieve our marker-assisted breeding goal, as our breeding goal did not require differentiation of all possible haplotypes.

[1]Sooriyapathirana S, Khan A, Sebolt A, Wang D, Bushakra J, Lin-Wang K, Allan A, Gardiner S, Chagné D, Iezzoni A. 2010. QTL analysis and candidate gene mapping for skin and flesh color in sweet cherry fruit (*Prunus avium* L.). Tree Genet Genomes 6:821–832
[2]Voorrips RE, Bink MC, van de Weg WE. 2012. Pedimap: Software for visualization of genetic and phenotypic data in pedigrees. J Hered. 103(6):903-7
[3]Peace C, Bassil N, Main D, Ficklin S, Rosyara UR, Stegmeir T, Sebolt A, Gilmore B, Lawley C, Mockler TC, Bryant DW, Iezzoni A. 2012. Development and evaluation of a genome-wide 6K SNP array for diploid sweet cherry and tetraploid sour cherry. PLoS ONE 7(12): e48305.

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USDA United States Department of Agriculture National Institute of Food and Agriculture NIFA