MARKER-ASSISTED SEEDLING SELECTIONS IN SOUR CHERRY FOR CHERRY LEAF SPOT RESISTANCE AND FRUIT FLESH COLOR

By

Fransiska Renita Anon Basundari

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Breeding, Genetics and Biotechnology - Horticulture - Master of Science

2015

ABSTRACT

MARKER-ASSISTED SEEDLING SELECTIONS IN SOUR CHERRY FOR CHERRY LEAF SPOT RESISTANCE AND FRUIT FLESH COLOR

By

Fransiska Renita Anon Basundari

Michigan is the leading producer of sour cherry (*Prunus cerasus* L.) in the United States (U.S.), and 'Montmorency' is the major sour cherry variety grown. This cultivar has high fruit production and bright red skin color that is the basis of the brilliant red color characteristic of cherry pie. Despite those superior qualities, 'Montmorency' is highly susceptible to the cherry leaf spot (CLS) fungus. The goal of the Michigan State University sour cherry breeding program is to develop new cultivars that have fruit with the characteristic 'Montmorency' color and are also disease resistant. Breeding new sour cherry cultivars is expensive due to the long generation time and the high expense of planting and evaluating seedlings in the field. The objective of this study was to implement and evaluate the impact of marker-assisted seedling selection (MASS) for fruit flesh color and CLS resistance in seedlings generated from crosses in 2013 using available DNA diagnostic tests. Implementation of a diagnostic DNA test for CLS resistance resulted in the elimination of the majority of seedlings predicted to be CLS susceptible prior to field planting. Implementation of a diagnostic DNA test for fruit flesh color resulted in the elimination of approximately half of the seedlings prior to field planting. The phenotypes of the original progeny individuals and the remaining progeny were predicted to demonstrate the expected gain from selection with the use of these two DNA tests.

ACKNOWLEDGEMENTS

I would like to thank you to my major advisor Dr. Amy Iezzoni for all of her guidance and huge patience during these two years. Her encouragement, positive thought, and her "tough love" has shaped me to understand the meaning of determination and hard work. It is such an incredible experience working with one of the best professors in Michigan State University and the first sour cherry plant geneticist.

I would like to show my gratitude to my thesis committee members. Dr. James D. Kelly and Dr. Cholani Weebadde for all of the guidance and advice they have given to me. I am so grateful to have them as my thesis committee members.

I would like thank you to Audrey M. Sebolt for all of her technical assistance, in the laboratory, field and computer works. I also want to say thank you for Travis Stegmeir, who introduced me the laboratory works. Thank you for sharing knowledge and experience to me.

I also thank you to all of the graduate students in Department of Horticulture and Crop and Soil Science who have helped me within two years while I am being here. Thank you for the great discussions and togetherness in our busy and rush time as the graduate student.

Finally, my gratitude goes to my beloved mother, my late father, and all of my sisters and brothers, for all of their love, prayers, sacrifice, and everything they have given to me. It really strengthens me during these challenging years.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

LITERATURE REVIEW OF MARKER ASSISTED BREEDING

Marker-assisted breeding (MAB) is defined as the application of molecular biotechnologies, specifically molecular markers, in combination with linkage maps and genomics, to alter and improve plant or animal traits on the basis of genetic assays. This term is used to describe several modern breeding strategies, including marker-assisted selection (MAS) and marker-assisted seedlings selection (MASS). The use of markers for selection in breeding, both of parents and seedlings can be referred to as MAS (Peace et al., 2014); while, the use of DNA markers to provide an early DNA-based evaluation of genetic performance potential of seedlings, with the aim of improving cost or genetics efficiency of seedling selection, is called MASS (Ru et al., 2015). Other MAB strategies include marker-assisted backcrossing (MABC), marker-assisted recurrent selection (MARS), and genome-wide selection (GWS) or genomic selection (GS) (Ribaut et al., 2010; Jiang, 2013).

The concept of MAB was first suggested by Smith and Simpson (1986) and by Soller and Beckmann (1983). These authors put forth the idea that selection using markers genetically linked to the causal gene(s) for the trait of interest would be more efficient than selection based on phenotype alone. The practice of MAB relies upon linkage disequilibrium (LD) existing between a DNA marker and a specific gene (quantitative trait locus; QTL). LD can be exploited by selection, as if the effects are caused by the marker (Ben-Ari and Lavi, 2012). The advantages of MAB result from the fact that many of the traits of interest to breeders are not easily assessed based on phenotype. Thus, selection, which is based on a linked DNA marker, is much more

efficient. Since selection based on markers can be carried out at an early age, it has potential to significantly reduce the number of individuals that must be evaluated in the field by the breeder, thus reducing cost.

MAB is especially advantageous for gene pyramiding (Ben-Ari and Lavi, 2012). Pyramiding is the process of combining several genes together into a single genotype (Collard and Mackill, 2008). Gene pyramiding or combining desirable traits from multiple parental lines is frequently required by plant breeders to develop elite breeding lines and varieties, particularly in the case of disease resistance (Huang et al., 1997; Singh et al., 2001; Luo et al., 2012). The advantage of using markers in this case allows selecting for QTL-allele-linked markers, which have the same phenotypic effect (Jiang, 2013). With linked DNA markers, the number of resistance genes in any plant can be easily determined. The incorporation of quantitative resistance controlled by QTLs offers another promising strategy to develop durable disease resistance (Collard and Mackill, 2008). Pyramiding of multiple genes or QTLs is recommended as a potential strategy to enhance or improve a quantitatively inherited trait in plant breeding (Richardson et al., 2006). It may be achieved through different approaches: multiple-parent crossing or complex crossing, backcrossing, and recurrent selection. A suitable breeding scheme for marker-assisted gene pyramiding (MAGP) depends on the number of genes/QTLs required for improvement of traits, the number of parents that contain the required genes/QTLs, the heritability of traits of interest, and other factors (e.g. marker-gene association, expected duration to complete the plan and relative cost) (Jiang et al., 2013). The cumulative effects of multiple-QTL pyramiding have been proven in crop species like wheat, barley and soybean (Richardson et al., 2006; Jiang et al., 2007a, 2007b; Li et al., 2010; Wang et al., 2012).

Pyramiding genes was also reported by Suh et al. (2013) in developing resistant cultivars from bacterial leaf blight disease of rice caused by *Xanthomonas oryzae pv oryzae* (Xoo). Molecular markers have made it possible to identify and pyramid valuable genes of agronomic importance for resistance breeding in rice. In this study, there were several resistant genes transferred from the indica donor (IRBB57), using a MABC breeding strategy, into a bacterial blight-susceptible, elite japonica rice cultivar, which is high yielding with good grain quality. Several bacterial blight resistance genes identified to date are either race specific or express susceptibility to the emerging races of the pathogen. The study provided some clues to the successful pyramiding of three bacterial blight resistance genes into an elite japonica cultivar to control bacterial blight disease caused by a new race, K3a (Suh et al., 2013).

During the past two or three decades, resistance genes or QTLs and associated markers have been identified for many fungal disease of tomato, including *Alternaria* stem early blight and many *Fusarium* diseases (Foolad and Panthee, 2012). *Fusarium* wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici* (Fol), is a common and devastating disease of tomato worldwide (Agrios, 2004). To date, three races of the pathogen have been reported and four resistance loci conferring vertical resistance to the disease have been identified. PCR-based markers closely linked to this gene are currently available (Foolad and Panthee, 2012).

MASS uses molecular markers to identify and keep plants that contain the desired allele combination and discard those that do not (Francis et al., 2012). Several MASS applications have been reported in apple for determining the scab resistance and good postharvest storability by Tartarini et al. (2000) and Edge-Garza et al. (2010), respectively. Kellerhals et al. (2011) performed MASS to pyramid apple scab resistance alleles and combined resistance for fire blight, scab, and powdery mildew in two seedling populations. DNA tests were used to

determine seedlings with pyramided apple scab resistance alleles at the Rvi6 and Rvi4 loci, fire blight resistance at the FB -F7QTL, and mildew resistance alleles at the Pl2 locus. In those two populations, 3 and 5 % of seedlings were identified with all favorable alleles. Those favorable individuals were selected for further evaluation on fruit and tree characters. MASS in this example showed great potential in improving the efficiency of pyramiding disease resistance alleles (Kellerhals et al., 2011).

Molecular markers are also valuable for confirming parentage. Simple sequence repeats (SSRs) which are codominant and particularly polymorphic, are applicable for these purpose, as has been reported in bur oak, a wind pollinated tree (Dow and Ashley, 1998) and in potato (Buetler et al., 2002). Four SSRs were used to check the paternity of 11 olive progenies thought to come from selfing or controlled crosses involving non-emasculated flowers. The result obtained in this study showed that SSR markers were able to confirm the pollen parent in routine crossing in olive (de la Rosa et al., 2004). Paternity testing in MAS is also done using several SSRs in the perennial forage species, red clover (Riday, 2011). Finally, in sweet cherry, Haldar et al. (2010) used genotyping for the multi-allelic self-incompatibility locus (*S*-locus) to verify the parentage of seedling population and also to determine which seedlings would be self-compatible compared to the less desirable self-incompatible seedlings.

Although markers can be used at any stage during a typical plant breeding programs, MASS offers a great advantage in early generations, because plants with undesirable gene combinations can be eliminated. It allows breeders to focus their attention on a lesser number of high-priority lines in subsequent generations (Collard and Mackill, 2008).

REFERENCES

REFERENCES

Agrios, G. N. (2004). Plant Pathology. 5th ed. Elsevier, New York.

Ben-Ari, G., Zenvirth, D., Sherman, A., David, L., Klutstein, M., and Lavi, U., et al. (2006). Four linked genes participate in controlling sporulation efficiency in building yeast. PloS Genetics. 2. E195.

Buetler, M. I. and Labonte, D. R. (2002). Microsatellite-based paternity analysis in polyploidy sweet potato. J. Amer. Soc. Hort. Sci. 127:392-396.

Collard, B. C. Y., and Mackill, D. J. (2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Phil. Trans. R. Soc. B. 363: 557–572

de la Rosa, R., James, C. M., and Tobutt, K. M. 2004. Using microsatellites for paternity testing in olive progenies. HortScience. 39(2):351-354.

Dow, B. D. and Ashley, M. V. (1998). High levels of gene flow in bur oak revealed by paternity analysis using microsatellites. J. Hered. 89:62-70.

Edge-Garza, D. A. and Peace, C. P. (2010). Enabling marker-assisted seedling selection in the Washington apple breeding program. In Bassil, N.V. and Martin, R. (eds). Proc. International Society for Horticultural Science. Acta Hort. 859:369-375.

Francis, D. M. Merk, D. L. Namuth-Covert, D. (2012). Gene Pyramiding Using Molecular Markers. Plant Breeding and Genomics. http://www.extension.org/pages/32465/gene-pyramiding-using-molecular-markers#.VSoB8WbfhNI.

Foolad, M. R. & Panthee, D. R. (2012). Marker-Assisted Selection in Tomato Breeding, Critical Reviews in Plant Sciences. 31(2): 93-123. DOI: 10.1080/07352689.2011.616057.

Gjertson, D.W., Brenner, C.H., Baur, M.P., Carracedo, A., Guidet, F., Luque, J.A., Lessig, R., Mayr, W.R., Pascali, V.L., Prinz, M., Schneider, P.M., and Morling, N. (2007). ISFG: Recommendations on biostatistics in paternity testing. Forensic Sci. Int. Genet. 1: 223-

231.

Huang, N., Angeles, E.R., Domingo, J., Magpantay, G., Singh, S., Zhang, G., Kumaravadivel, N., Bennet, J., and Khush, G.S. (1997). Pyramiding of bacterial blight resistance genes in rice: marker-assisted selection using RFLP and PCR. Theor. Appl. Genet. 95: 313-320.

Jiang, G.-L., Shi, J., and Ward, R. W. (2007a). QTL analysis of resistance to *Fusarium* head blight in the novel wheat germplasm CJ 9306. I. Resistance to fungal spread. Theor. Appl. Genet. 116:3-13.

Jiang, G.-L., Dong, Y. Shi, J., and Ward. R. W. (2007b). QTL. QTL analysis of resistance to *Fusarium* head blight in the novel wheat germplasm CJ 9306. II. Resistance to deoxynivalenol accumulation and grain yield loss. Theor. Appl. Genet. 115:1043-1052.

Jiang, G.-L. (2013). Molecular Markers and Marker-Assisted Breeding in Plants, Plant Breeding from Laboratories to Fields, Prof. Sven Bode Andersen (Ed.), ISBN: 978-953-51-1090-3, InTech, DOI: 10.5772/52583. Available from: http://www.intechopen.com/books/plant-breeding-from-laboratories-to-fields/molecular-markers-and-marker-assisted-breeding-in-plants.

Kellerhals M, Franck L., Baumgartner I. O., Patocchi A., Frey J. E. (2011). Breeding for fire blight resistance in apple. Acta Hort. 896: 385–389.

Li, X., Han, Y., Teng, W., Zhang, S., Yu, K., Poysa, V., Anderson, T., Ding, J., and Li, W. (2010). Pyramided QTL underlying tolerance to Phytophthora root in mega-environment from soybean cultivar 'Conrad' and 'Hefeng 25'. Theor. Appl. Genet. 121: 651-658.

Luo, Y., Sangha, J.S., Wang, S., Li, Z., Yang, J., and Yin, Z. (2012). Marker-assisted breeding of *Xa4*, *Xa21* and *Xa27* in the restorer lines of hybrid rice for broad-spectrum and enhanced disease resistance to bacterial blight. Mol. Breeding. DOI 10.1007/s11032-012-9742-7.

Peace, C. P., Luby, J. J. &. van de Weg, W. E, Bink, M. C. A. M., and Iezzoni, A. F. (2014). A strategy for developing representative germplasm sets for systematic QTL validation, demonstrated for apple, peach, and sweet cherry. Tree Genetics & Genomes. 10:1679–1694.

Ribaut, J.M., de Vicente, M.C., and Delannay, X. (2010). Molecular breeding in developing countries: challenges and perspectives. Current Opinion in Plant Biology 13: 1-6.

Riday, H. 2011. Paternity testing: a non-linkage based marker-assisted selection scheme for outbred forage species. Crop Science. 51:631-641.

Richardson, K.L., M.I. Vales, J.G. Kling, C.C. Mundt, and P.M. Hayes. (2006). Pyramiding and dissecting disease resistance QTL to barley stripe rust. Theor. Appl. Genet. 113: 485-495.

Ru, S. and Main, D., Evans K., and Peace, C. (2015). Current applications, challenges, and perspectives of marker-assisted seedling selection in Rosaceae tree fruit breeding. Tree Genetics & Genomes. 11: 8. DOI 10.1007/s11295-015-0834-5

Singh, S., Sidhu, J.S., Huang, N., Vikal, Y., Li, Z., Brar, D.S., Dhaliwal, H.S., and Khush, G.S. (2001). Pyramiding three bacterial blight resistance genes (*xa5*, *xa13* and *Xa21*) using marker-assisted selection into indica rice cultivar PR106. Theor. Appl. Genet. 102: 1011-1015.

Smith, C. and Simpson, S. P. (1986). The use of genetic polymorphisms in livestock improvement. Journal of Animal Breeding and Genetics. 103:205-217.

Soller, M. and Beckmann, J. S. (1983). Genetic-polymorphism in varietal identification and genetic-improvement. Theoretical and Applied Genetics. 67:25-33.

Suh**,** J.-P., Jeung**,** J.-U., Noh**,** T.-H., Cho**,** Y.-H., Park, S.-H., Park, H.-Y., Mun-Sik Shin, M.-S., Chung-Kon Kim**,** C.-K., and Jena**.** K.-K. (2013). Development of breeding lines with three pyramided resistance genes that confer broad-spectrum bacterial blight resistance and their molecular analysis in rice. *Rice*. 6:5. doi:10.1186/1939-8433-6-5.

Tartarini S, Sansavini S, Vinatzer B, Gennari F, Domizi C (2000). Efficiency of marker assisted selection (MAS) for the Vf scab resistance gene. Acta Hort. 538:549–552

Tsukamoto T, Hauck N. R., Tao, R, Jiang, N, Iezzoni A. F. (2006). Molecular characterization of three non-functional *S*-haplotypes in sour cherry (*Prunus cerasus*). Plant Mol Biol. 62:371–383.

Tsukamoto, T., Tao, R., and Iezzoni, A. F. (2008). PCR markers for mutated *S*-haplotypes enable discrimination between self-incompatible and self-compatible sour cherry selections. Mol. Breeding. 21:67-80.

Wang, X., Jiang, G.-L., Green, M., Scott., R. A., Hyten, D. L., and Cregan, P. B. (2012). Quantitative trait locus analysis of saturated fatty acids in a population of recombinant inbred lines soybean. Mol. Breeding. 30(2): 1163-1179.

CHAPTER 2

MARKER-ASSISTED SEEDLING SELECTIONS IN SOUR CHERRY FOR CHERRY LEAF SPOT RESISTANCE

Introduction

Michigan is the leading sour cherry (*Prunus cerasus* L.) producing state in the U.S., with a production that often exceeds 75% of total U.S. production (Cherry Marketing Institute, 2009; United States Department of Agriculture, 2013). The U.S. sour cherry industry is based on one cultivar 'Montmorency' due to its suitability for processing and its high productivity. However, one of the major limitations of 'Montmorency' is its susceptibility to cherry leaf spot (CLS) disease caused by the fungus *Blumeriella jaapi* (Rehm) Arx (anamorph *Pholeosporella padi* (Lib.) Arx). This is the most important disease of sour cherry in Michigan and throughout the humid growing regions worldwide (Keitt et al., 1937; Wharton et al., 2003). CLS infection results in severe leaf chlorosis and premature defoliation. The fruit will be poorly colored, contain low amounts of soluble solids, and be softer than fruit on healthy trees (Keitt et al., 1937). Early defoliation can also result in reduced winter hardiness, potentially leading to flower bud loss and tree death (Howell and Stackhouse, 1973). Therefore, controlling CLS with frequent fungicide applications is a major production cost for sour cherry producers. As a result, breeding for resistance to CLS has become an industry priority in the United States.

Breeding a new tree crop cultivar is relatively slow compared to annual crops (Folta and Gardiner, 2009). Kappel et al. (2012) reported that the time from seed to flowering of a cherry tree is at least three years, but might be longer in practice. The long period of time from seed to

flowering and the large plant size of cherry trees limits the genetic gain that can be made from classical breeding (Folta and Gardiner, 2009). Cherry breeding programs are cost intensive because of the need to maintain seedlings in the field, which requires fertilizers, pesticides, labor, and equipment. One strategy to reduce the cost of tree breeding is to the use DNA tests that can identify those seedlings predicted to be desirable prior to planting in the field (Edge-Garza and Peace, 2010). Prior knowledge of linkage relationships between marker loci and desired fruit characteristics will increase the efficiency of identifying superior individuals. Consequently, the integration of molecular markers into breeding programs would be a powerful tool for increasing the efficiency of cultivar development in tree crops (Folta and Gardiner, 2009).

A source of CLS resistance had previously been identified from the wild species *P. canescens* (Wharton et al. 2003). A major QTL controlling this *P. canescens*-derived CLS resistance, named *CLSR_G4,* was identified on linkage group 4 (LG4) in sweet cherry, and then validated in sour cherry (Stegmeir et al., 2014). For both sweet and sour cherry, all resistant individuals had the *P. canescens*-derived CLS resistance allele for *CLSR_G4*; however, a small percentage of the seedlings that had the resistance allele were susceptible. For those individuals containing the resistant allele for *CLSR_G4,* approximately one fourth were susceptible (Stegmeir et al., 2014). These results suggested that dominant alleles at two genes are necessary to confer CLS resistance in sour cherry, with the *P. canescens* resistance allele at *CLSR_G4*, being one of these two alleles.

Because the *CLSR_G4* resistance allele is required for a sour cherry individual to be CLS resistant, a DNA test for this resistance allele was developed (Stegmeir et al., 2014). Four SSR markers were designed within the QTL region between SNP markers ss490552323 (4.0 cM, 1.0 Mb) and ss490552500 (13.8 cM, 3.46 Mb) that identified the presence or absence of the *P.*

canescens CLSR_G4 resistance allele. All markers had a unique band representing the *P. canescens* chromosome (Stegmeir et al., 2014). These markers will assist the breeder in discarding more undesirable seedlings at the earliest possible stage during the selection process.

A cross was made in 2013 between the *P. canescens*-derived resistant individual 24-32-37 that had the *CLSR-G4* resistance allele, and susceptible elite sour cherry breeding individual, 27e-05-33. A total of 43 seedlings were obtained and their parentage confirmed using a DNA-test for the self-incompatibility locus (24-32-37, $S_4S_{26}S_{36b}$; 27e 05-05-33, $S_6S_{13'}S_{36a}S_{36b}$, T. Stegmeir, pers. comm.) (see Chapter 3 for a discussion of this paternity test). The objective of this project was to implement MASS for *P. canescens*-derived cherry leaf spot resistance using this new DNA test to increase the efficiency of sour cherry breeding for CLS resistance.

Materials and Methods

Plant materials and DNA extraction

Leaf tissue was collected from the 43 progeny individuals confirmed to be derived from the cross between the CLS resistant maternal parent 24-32-37 and the susceptible paternal parent 27e-05-33 (T. Stegmeir, pers. comm). The leaf samples were dried for two days in tubes containing silica, before grinding with a Mixer Mill (Retsch, Newton, PA, USA). The frequency on Mixer Mill was set to a 27.0 Hz/s for 3 min Once the machine had stopped, the tubes should be taken off and turned around. It was started for another 3 min to ensure all the samples are disrupted equally. On the next day, DNA was extracted from the leaf tissues using the Silica Bead Method (SBM) as described in Edge-Garza et al. (2014).

PCR for MASS

Of four markers, CLS004, CLS005, CLS026, and CLS028 developed by Stegmeir et al (2014), only one marker, CLS028 was used for this study since it has the clearest bands compared to the others. A touchdown PCR was used for the CLS028 primer pair, which has a forward primer of 5'- GAA TGC AGT TGG GGA GTT ACC -3' and a reverse primer of 5'- CTT CTT GCA CCA AAA ACA ACC -3' (Stegmeir et al., 2014). The PCR conditions were as follows: 94 ºC for 5 min followed by 9 cycles of 94 ºC for 30 s, 60 ºC for 45 s, 72 ºC for 1 min, and then 24 cycles of 94 ºC for 30 s, 55 ºC for 45 s, 72 ºC for 1 min with an elongation step of 72 ºC for 5 min (Stegmeir et al., 2014). The reaction mixture contained 10x PCR buffer, 10x dNTPs, 50mM $MgCl_2$, 10 µM of each primer, H_2O, 50 ng/µl of genomic DNA, and Taq polymerase in a 12.5-µl reaction. 2 µl DNA sample and 12 µl master mix of was added into each well of the plate. When the PCR was done, 3 µl DNA buffer were added in each well of the plates, spun for 15 seconds, and kept in the refrigerator. On the next day, the PCR fragments were separated in a 6% polyacrylamide gel and visualized with silver staining.

Results and Discussion

Of the 43 seedlings screened with the *CLSR_G4* marker, 31 progeny individuals (72%) had the 168 bp fragment associated with the resistance allele and were therefore kept for future field planting (Table 2.1; Figure 2.1). The 12 progeny individuals (28%) that did not have the 168 bp fragment were discarded. If it is assumed that 24-32-37 has just one copy of the *CLSR-G4*

Table 2.1. **DNA testing result using CLS028 marker and the prediction ratio of CLS resistance allele segregation based on a simple gene.**

Trait	Expected ratio	Observed (O)	Expected (E)	Deviation (O-E)	Deviation^2 $(d)^2$	d/e
With CLS resistant allele	1/2	31	21.5	20.5	420.25	19.5
Without CLS resistant allele	1/2	12	21.5	-9.5	90.25	4.19
	1	43	43			$X^2 = 23.69$
						$p < 0.001$

Figure 2.1. **A two-gene model for predicting the CLS resistance in sour cherry derived from *P. canescens*.** Individuals are resistant when dominant alleles are present at two unlinked loci, the *P.canescens*-derived R haplotype for CLSR_G4 is represented as locus 'A,' and a proposed second locus, 'B. Disease resistant parent (24-32-37) is shown to be heterozygous in both loci ($A_1a_1a_2a_2B_1b_1b_2b_2$), while the susceptible parent is shown to be homozygous for the 'A' locus and heterozygous for the proposed second locus needed to confer resistance ($a_1a_1a_2a_2B_1b_1b_2b_2$) (Stegmeir et al, 2014). Three-eight (the highlighted columns) of the progeny were predicted to have the CLS resistant. Progeny population predicted to be CLS resistant using CLS028 marker were identified due to the presence of one copy the *CLSR-G4* resistance allele (A_1) in the progeny. This figure is a modification from Stegmeir et al. (2014).

		24-32-37 ($A_1a_1a_2a_2B_1b_1b_2b_2$)			
		$A_1a_2B_1b_2$	$A_1a_2b_1b_2$	$a_1a_2B_1b_2$	$a_1a_2b_1b_2$
27-05-33 ($a_1a_1a_2a_2B_1b_1b_2b_2$)	a_1a_2 B_1b_2	$A_1a_1a_2a_2B_1B_1b_2b_2$	$A_1a_1a_2a_2B_1b_1b_2b_2$	$a_1a_1a_2a_2B_1B_1b_2b_2$	$a_1a_1a_2a_2B_1b_1b_2b_2$
		31		12	
	a_1a_2 b_2b_2	$A_1a_1a_2a_2B_1B_2b_2b_2$	$A_1a_1a_2a_2b_1b_2b_2b_2$	$a_1a_1a_2a_2B_1b_2b_2b_2$	$a_1a_1a_2a_2b_1b_2b_2b_2$

resistance allele, 50% (21-22) of the 43 progeny would be expected to have the resistance allele (Figure 2.2).

A chi square (X^2) test was conducted to assess the goodness of fit between observed values and those expected theoretically. As mentioned above the 50% of the progeny would be expected to have *CLSR_G4* resistance allele, or it can be said that the predicted ratio would be 1:1 in the progeny that have the resistance allele and those which do not. The X^2 test presented that the *p* value was less than 0.001 (Table 2.1). It means that only 0.1 percent of this study would have the chance the same as the prediction ratio of 1:1 for the presence and the absence of *A1* allele. Therefore, there is a significant difference between the expected to the observed value. The hypothesis of 1:1 predicted ratio was rejected.

The finding that more than 72% of the progeny (as opposed to 50% of the progeny) had the resistance allele raises the possibility that the resistance allele may be transferred to the next generation at a higher frequency compared to the susceptible alleles. Since the *CLSR-G4* marker only identifies the resistance allele at one of the two predicted QTLs, one-fourth of the 31 individuals with the *CLSR-G4* resistance allele would be predicted to be susceptible (Figure 2.3). However, based on the two-gene model, the remaining ~8 individuals were predicted not to be CLS resistant, since they only presented one copy *CLSR_G4* resistance allele (A1) instead of two alleles, A_1 and B_1, which indicated CLS resistant, in both loci. It would still be maintained in the breeding program and only identified as susceptible upon field planting. The screening of parental genotypes is required to increase the accuracy of the result analysis.

Figure 2.2. Prediction of 1:1 ratio for the individuals expected to have CLS resistance allele and those predicted to be CLS susceptible, screened with CLS028 marker. 50% of the progeny population would be expected to have CLS resistant genotypes (have A_1).

		24-32-37 ($A_1a_1a_2a_2B_1b_1b_2b_2$)			
		$A_1a_2B_1b_2$	$A_1a_2b_1b_2$	$a_1a_2B_1b_2$	$a_1a_2b_1b_2$
27-05-33 ($a_1a_1a_2a_2B_1b_1b_2b_2$)	a_1a_2 B_1b_2	$A_1a_1a_2a_2B_1B_1b_2b_2$ (1/8 – 5.375)	$A_1a_1a_2a_2B_1b_1b_2b_2$ (1/8 – 5.375)	$a_1a_1a_2a_2B_1B_1b_2b_2$ (1/8 – 5.375)	$a_1a_1a_2a_2B_1b_1b_2b_2$ (1/8 – 5.375)
	a_1a_2 b_2b_2	$A_1a_1a_2a_2B_1b_2b_2b_2$ (1/8 – 5.375)	$A_1a_1a_2a_2b_1b_2b_2b_2$ (1/8 – 5.375)	$a_1a_1a_2a_2B_1b_2b_2b_2$ (1/8 – 5.375)	$a_1a_1a_2a_2b_1b_2b_2b_2$ (1/8 – 5.375)

Figure 2.3. **Progeny population predicted to be CLS resistant and CLS susceptible based on two-gene model**. The progeny predicted to be CLS resistant were in the highlighted grey background and diagonal patterned column; while progeny population predicted to be CLS susceptible were in grey highlighted column. The progeny predicted to be CLS resistant should have the R haplotype for *CLSR_G4* in both loci, represent as A_1 and B_1 (Stegmeir et al., 2014). The progeny that do not have those dominant alleles were considered as CLS susceptible. Twenty-three progeny with both dominant alleles (with bold letters in grey highlighted and patterned column) were identified to be CLS resistant. Eight out of 31 numbers of progeny were predicted to be CLS susceptible.

		24-32-37 ($A_1a_1a_2a_2B_1b_1b_2b_2$)			
		$A_1a_2B_1b_2$	A_1a_2 b_1b_2	$a_1a_2B_1b_2$	$a_1a_2b_1b_2$
27-05-33 ($a_1a_1a_2a_2B_1b_1b_2b_2$)	a_1a_2 B_1b_2	**$A_1a_1a_2a_2B_1b_1b_2b_2$** **(23)**	**$A_1a_1a_2a_2B_1B_1b_2b_2$**	$a_1a_1a_2a_2B_1B_1b_2b_2$ (12)	$a_1a_1a_2a_2B_1b_1b_2b_2$
	a_1a_2 b_2b_2	**$A_1a_1a_2a_2B_1b_2b_2b_2$**	$A_1a_1a_2a_2b_1b_2b_2b_2$ (8)	$a_1a_1a_2a_2B_1b_2b_2b_2$	$a_1a_1a_2a_2b_1b_2b_2b_2$

As a result of MASS for CLS resistance, 31 plants were field planted as opposed to 43 plants with a significant cost savings to the breeding program. Additionally because of the use of this DNA marker 74% (23/31) of the plants field planted are predicted to be CLS resistant as opposed to only 37% (16/43) without any genetic testing. These results illustrate the increase in breeding efficiency with the use of MASS for CLS resistance. It allows the plant breeder to discard seedlings predicted to have undesired traits in the possible earliest stage, thus the breeding purpose can be focus on those seedlings that have desired traits.

REFERENCES

REFERENCES

Cherry Marketing Institute. (2009). Tart Cherry Production and Utilization. Retrieved from http://www.cherrymkt.org/.

Folta, K.M. and Gardiner, S. E. (2009). Plant Genetics/Genomics: Genetics and Genomics of Rosaceae. Volume 6. Springer.

Edge-Garza, D. A. and Peace, C. P. (2010). Enabling marker-assisted seedling selection in the Washington apple breeding program. In Bassil, N.V. and Martin, R. (eds). Proc. International Society for Horticultural Science. Acta Hort. 859:369-375.

Howell, D. S and Stackhouse, S. S. (1973). The effect of defoliation time on acclimation and dehardening of tart cherry (*Prunus cerasus* L.). J. Am. Soc. Hortic. Sci. 98: 132-136.

Kappel, K., Granger, A., Hrotkó, K., and Schuster, M. (2012). Cherry. In M.L. Badenes and D.H. Byrne (eds). Handbook of Plant Breeding: Fruit Breeding. Springer Science and Business Media, LLC.

Keitt G. S., Blodgett, E. C., Wilson, E. E., and Magie, R.O. (1937). The epidemiology and control of cherry leaf spot. Univ Wisc Agric Exp Stn Res Bull. 132.

Stegmeir, T., Schuster, M., Sebolt, A., Rosyara, U., Sundin, G. W. and Iezzoni, A. (2014). Cherry leaf spot resistance in cherry (*Prunus*) is associated with a quantitative trait locus on linkage group 4 inherited from *P. canescens*. Mol. Breeding. 34:927-935.

United States Department of Agriculture (USDA). (2013). Non-citrus fruits and nuts: Preliminary Summary. National Agricultural Statistics Service (NASS).

Wharton, P. S., Iezzoni A., and Jones, A. L. (2003). Screening cherry germplasm for resistance leaf spot. The American Phytopathological Society. 87:471-477.

CHAPTER 3

MARKER-ASSISTED SEEDLING SELECTIONS IN SOUR CHERRY FOR FRUIT FLESH COLOR

Introduction

Sour cherry is a *Prunus* specialty crop in the United States that is used for processing (Hummer and Janick, 2009; Iezzoni, 2013). The major sour cherry cultivar variety grown in the U.S. is the red skinned, clear-fleshed cultivar 'Montmorency' (Iezzoni, 1988), while most of the sour cherry cultivars grown in Europe have dark red/purple flesh color (Iezzoni, 2005). This different preference in sour cherry color (brilliant red versus dark red/purple) also results in different fruit color goals for sour cherry breeding programs in Europe compared to the U.S. To fulfill one of the major breeding priorities for a brilliant red fruit color, and to increase breeding efficiency, DNA information is used to predict flesh color at the early seedling stage, which is a major goal of the Michigan State University sour cherry breeding program.

The red class of anthocyanin pigments control flower or fruit pigmentation in many plants including apple, sweet cherry, and sour cherry (Chagné et al., 2007; Chandra et al., 1992; Wang et al., 1997). In sweet cherry, the genetic control of skin and flesh color was investigated using a quantitative trait locus (QTL) approach with progeny derived from a cross between cherry parents representing the two extreme colors (Sooriyapathirana et al., 2010). A major QTL controlling the red skin and flesh color was identified on linkage group (LG) 3. The significance and magnitude of the QTL identified in LG 3 suggested the presence of a major regulatory gene

associated with cherry skin and flesh color *(PavMYB10*). This gene corresponded to the findings of other genetic color studies in apple where *MYB1*/*MYBA* controls skin color (Takos et al., 2006; Ban et al., 2007); and *MdMYB10* controls flesh and foliage color (Chagné et al., 2007; Espley et al., 2007).

The *MYB10* gene found to control fruit color in sweet cherry was hypothesized to control fruit color in sour cherry because sweet cherry is a progenitor species of sour cherry (Iezzoni, 2013; Beaver and Iezzoni, 1993; Olden and Nybom, 1968). An analysis of the association between the *MYB10* region in sour cherry and flesh color confirmed this hypothesis (Stegmeir et al., submitted). Six out of 13 *MYB10* haplotypes identified in sour cherry were found to be significantly associated with flesh color. Four of the six haplotypes (*D1, D2, D3*, and *D4*) were found to be associated with dark flesh color. The *D1* haplotype had the largest effect on dark flesh color, followed by the remaining three D haplotypes. Two of the six haplotypes, named *d1* and *d2*, were significantly associated with light/clear flesh color. Seven out of the 13 haplotypes, named *x1, x2, x3, x4, x5, x6*, and *x7* were not significantly associated with flesh color (Stegmeir et al., submitted). A DNA test was developed using a SSR marker to identify and select against individuals that have the *D1* haplotype and were therefore predicted to have dark red/purple. The goal was to use this DNA tests at the early seedling stage so only those seedlings predicted to have favorable flesh color would be planted in the breeding field nurseries. The objective of this research was to implement marker-assisted seedling selection (MASS) for flesh color with seedlings derived from crosses in 2013 where one of the parents carried the *D1* haplotype. To permit an accurate analysis of the genetic results, paternity testing of the progeny using the self-incompatibility locus (*S*-locus) was initiated to confirm that the seedlings used for MASS were from the intended cross.

Background

Flesh color phenotypic scale used in sour cherry

The fruit flesh color phenotypic scale was the sweet cherry index color from Washington State University (WSU). Scores ranged from one to five, with clear or yellow flesh color represented as score of 1, pale pink (score of 2), red (score of 3), dark red (score of 4) and purple red (score of 5) (Appendix Figure A1). In sweet cherry study, the fruit flesh color was also quantitatively measured using a spectrophotometer (Sooriyapathirana et al., 2010; Stegmeir et al., submitted).

DNA test for fruit flesh color

A DNA test using a simple sequence repeat (SSR) marker was developed that can uniquely identify the *D1* and *D2* dark red/purple *MYB10* haplotypes (Stegmeir et al., submitted). To develop this DNA test, SSR markers flanking the candidate *MYB10* homolog were screened for possible association with the dark flesh color haplotypes. SSR markers were found using the peach genome sequence (Peace et al., 2012; International Peach Genome Initiative, 2012; Verde et al., 2013). Forty SSR markers were then developed and screened by Stegmeir et al. (submitted), to identify dark-fleshed haplotypes. One SSR marker (named LG3_13.146), about 200,000 Kb from the nearest *MYB10* homolog, was polymorphic and able to distinguish two of haplotypes, *D1* and *D2*, at 218 bp and 220 bp, respectively. This marker, LG3_13.146, was used in this project to select against the individuals predicted to have dark/red purple fruit flesh color.

The use of the S-locus RNase to test paternity

Paternity testing is used to identify an individual's father at some probability when paternal identity is uncertain (Gjertson et al., 2007). Subsequently, tree breeding programs have explored selection based on molecular marker-identified parentage (Kumar et al., 2007; Wang et al., 2010). Paternity testing in this study needs to be done to identify the true parental cross. In sour cherry, the highly polymorphic *S*-locus is currently the locus of choice for paternity testing as it is highly polymorphic, all the alleles are well characterized, the inheritance is known and genotyping is relatively inexpensive (Yamane et al., 2003; Yamane and Tao, 2009).

S-RNase-based self-incompatibility occurs in the Solanaceae, Rosaceae, and Plantaginaceae. In all three families, compatibility is controlled by a polymorphic *S*-locus encoding at least two genes. S-RNases determine the specificity of pollen rejection in the pistil, and *S*-locus F-box proteins fulfill this function in pollen. S-RNases are thought to function as S-specific cytotoxins as well as recognition proteins. Thus, incompatibility results from the cytotoxic activity of S-RNase, while compatible pollen tubes evade S-RNase cytotoxicity (McClure et al, 2011).

In sweet cherry, as in other diploid Gametophytic Self-Incompatibility (GSI) systems, matching *S*-haplotypes in the pollen and style will result in an incompatible reaction, and the growth of this "self"-pollen tube will be inhibited (de Nettancourt, 2001). A basic theory of the *S*-haplotypes found in sour cherry was needed to see the incompatibility and compatibility in the progeny. To date 14 haplotypes have been identified in sour cherry (Hauck et al., 2002; Yamane et al., 2003a; Tobutt et al., 2004; Hauck et al., 2006). Five of 14 sour cherry *S*-haplotypes (S_1, S_4, S_6, S_9, S_{26}) were shown to be functional, and seven *S*-haplotypes ($S_{1'}$, S_{6m}, S_{6m2}, $S_{13'}$, S_a, S_d *and*

S$_{null}$) were shown to be non-functional (Hauck et al., 2002, 2006b; Yamane et al., 2003; Tobutt et al, 2004). Two *S*-haplotypes, *S*$_{12}$ and *S*$_{13}$, have been identified in the self incompatible (SI) sour cherry selection 'Erdi Nagygymolcsu' and 'Tschernokorka' (Yamane et al., 2001) but their functionality has not been tested.

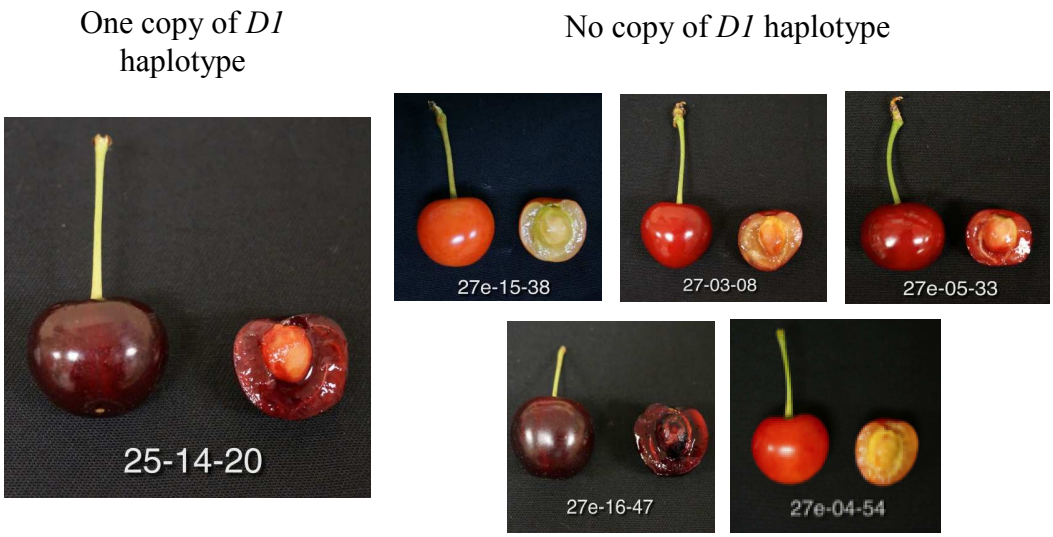
In tetraploid sour cherry, the genetic control of self-pollen recognition is more complicated than sweet cherry because a pollen grain contains two *S*-haplotypes. Sour cherry pollen is incompatible if one or two *S*-haplotypes in the pollen matches an *S*-haplotype in the style (Hauck et al., 2006). In contrast, self-compatible (SC) sour cherry pollen must contain two *S*-haplotypes that can enlist pollen-S and/or pistil-S function, termed nonfunctional *S*-haplotypes. Therefore, the genotype-dependent loss of SI in sour cherry is due to the accumulation of at least two nonfunctional *S*-haplotypes (Hauck et al., 2006b). A sour cherry cultivar must be SC to be commercially successful as it avoids the inefficiencies and costs associated with growing SI types. A study of the utilization of the *S*-locus as genetic marker to distinguish pollen donor for several cultivars in sour cherry was done by Sebolt and Iezzoni (2009). In this study, the use of *S*-locus as a genetic marker to differentiate the pollen donor, required knowledge of the inheritance of compatibility/incompatibility of *S*-haplotype from the pistil and pollen. In the breeding program, early selection using DNA tests for SC types and the elimination of SI types dramatically increases the efficiency and cost-effectiveness of sour cherry breeding (Tsukamoto et al., 2008). In this study the *S*-locus was used as a genetic marker in order to determine the paternal parent of the progeny.

Materials and Methods

Plant materials and DNA extractions

Five of many progeny populations from crosses made by A. Iezzoni in 2013 were used for MASS, since these populations were shown to be segregating for fruit flesh color (A. Iezzoni, pers. comm). All five populations had the same maternal parent, 25-14-20, previously shown to carry one copy of the dark red/purple flesh color haplotype *D1* (Stegmeir et al., submitted), while the different paternal parents do not have *D1* haplotype (Figure 3.1). All pollen parents had light red/clear juice color. The five progeny populations were derived from these crosses: 1) 25-14-20 × 27-03-08; 2) 25-14-20 × 27e-04-54; 3) 25-14-20 × 27e-05-33; 4) 25-14-20 × 27e-15-38; 5) 25-14-20 × 27e-16-47 (Table 3.1). For all parents, their *MYB10* haplotype genotypes were known (Stegmeir, 2013), allowing the prediction of the possibly progeny outcomes (Table 3.2). Leaf tissues from the seedling populations grown in the growth chamber were collected from the seedling progeny populations for DNA extraction. These samples were dried for two days in the tubes contained silica, before it would be ground with a Mixer Mill. The frequency on Mixer Mill was set to a 27.0 Hz/s for 3 min (Retsch, Newton, PA, USA). Once the machine had stopped, the plates should be taken off and turned around and started for another 3 min to ensure all the samples are disrupted equally. On the next day, DNA was extracted from the leaf tissues by Silica Bead Method (SBM) as described in Edge-Garza et al. (2014).

Figure 3.1. Fruit color of plant materials crosses made in 2013 (A. Iezzoni, pers. comm.), used in DNA testing for *D1* haplotype segregation in the progeny



PCR for MASS

A touchdown PCR was used for the flesh color SSR marker LG3_13.146, which has a forward primer sequence of 5'- ATG TGG CCA AAG GTC AGC -3' and reverse primer sequence of 5'- TGA TCC CAA TCA CGT TTT -3'(Stegmeir et al., submitted). The conditions were as follows: 94^{o}C for 5 min followed by 9 cycles of 94 ^{o}C for 30 s, 60 ^{o}C for 45 s, 72 ^{o}C for 1 min, and then 24 cycles of 94 ^{o}C for 30 s, 55 ^{o}C for 45 s, 72 ^{o}C for 1 min with an elongation step of 72 ^{o}C for 5 min. The reaction mixture contained 10x PCR buffer, 10x NTPs, 50mM $MgCl_2$, 10 µM of each primer, H_2O, 50 ng/µl of genomic DNA, and Taq polymerase in a 12.5-µl reaction. 2 µl DNA sample and 12 µl master mix of was added into each well of the plate. When the PCR was done, 3 µl DNA buffer were added in each well of the plates, spun it for 15 seconds, and kept in the refrigerator. On the next day, the PCR fragments were separated in a 6% polyacrylamide gel and visualized with silver staining (Olmstead et al., 2008). The presence or absence of the *D1* allele of the PCR products amplified at 218 bp was recorded.

PCR for confirmation of true cross

Paternity testing was used to confirm the parentage for the seedlings using markers diagnostic for the *S*-locus were known. Paternity testing needs to be done to ensure the true ancestry of the progeny, and to see the inheritance of *S*-allele of paternal parent in the progeny. The individual 25-14-20, found to have $S_1S_6S_{36a}S_{36b}$, was used as the maternal parent in this study. This individual was crossed with five paternal parents, 27-03-08, 27e-04-54, 27e-03-33, 27e-16-47, and 27e-05-33 which were found to have $S_1S_{13}S_{35}S_{36a}$, $S_{13m}S_{13}S_{36a}S_{36a}$, $S_6S_{13}S_{36a}S_{36b}$,

$S_4S_{13'}S_{13'}S_{36a}$, and $S_6S_{13'}S_{36a}S_{36b}$, respectively (Table A3.1.1-A3.1.4). All of the *S*-allele genotypes in those individual parents were known previously (*Iezzoni, unpublished data*). In this study, PCR for confirmation of true cross of the population was done for 56 progeny of the cross 25-14-20 × 27-03-08, with the prediction *S*-alleles inheritance to the progeny showed in Table 3.3.

S-allele genotyping was done using the S-RNase Pru-C2/PCE-R marker (Tao et al, 1999; Yamane et al., 2001) that has a forward primer sequence of 5'-CTA TGG CCA AGT AAT TAT TCA AAC C -3'and a reverse sequence of 5'- TGT TTG TTC CAT TCG CYT TCC C -3'; while the Pc-SFB13 marker (Yamane et al. 2001; Hauck et al. 2006; Tsukamoto et al. 2006) has a forward sequence of 5'- AGT TAA TGA CTG CAA GGC TGT AAG G -3' and a reverse sequence of 5'- CCC GAT TGT ACG ATA ATT GTA ATC C- 3' (Invitrogen). The reaction mixture contained 10x PCR buffer, MgCl2, 10xdNTPs, 50mM $MgCl_2$, 10 µM of each primer, H_2O, 50 ng/µl of genomic DNA, and Taq polymerase in a 12.5-µl reaction. 2 µl DNA sample and 12 µl master mix of was added into each well of the plate. PCR fragments were separated in 2% agarose gel (Tsukamoto et al., 2010), and were visualized with GelRed.

Results and Discussion

MASS for D1

Five progeny populations generated from parental crosses made in 2013 were screened using SSR marker LG3_13.146 to identify the presence or absence of the *D1* haplotype, associated with the allele resulted in the darkest red/purple flesh color (see the example of the *D1* haplotype

Table 3.1. Five seedlings populations segregating for the presence or absence of the *D1* haplotype screened by LG3_13.146 marker.

Maternal Parent	Paternal Parents	Number of DNA tested	Number (%) of plants with *D1* and discarded	Number (%) of plants without *D1* and kept	X^2 value (Prob) for a 1:1 ratio[a]
25-12-20	27-03-08	400	222 (56)	178 (44)	4.84 (0.03)
25-12-20	27e-04-54	91	59 (65)	32 (35)	8.10 (0.004)
25-12-20	27e-05-33	18	10 (56)	8 (44)	0.22 (0.6)
25-12-20	27e-15-38	26	12 (46)	14 (54)	0.14 (0.7)
25-12-20	27e-16-47	84	55 (65)	39 (35)	8.04 (0.04)
Total		619	358 (58)	261 (42)	

[a] See Appendix Table 3.2 for calculations of X^2 values

Table 3.2. Possible progeny genotypes for the MYB10 haplotypes in five progeny population generated from 2013 parental crosses. *D1, D2*, *D3*, and *D4* are the haplotypes with decrease significant effect to dark flesh color, respectively; *d1* and *d2* are the haplotypes with significant effect with the light flesh color; *x1, x2, x3*, and *x5* are the haplotypes that do not have significant effect on flesh color, either dark or light flesh color (Stegmeir et al, submitted); *x1/x2*, *D2/d1*, or *x2/D1*, it means that there is crossover from haplotype *x1* to *x2*, *D2* to *d1*, or *x2* to *D1*, respectively.

Maternal parent (G3 haplotypes)	Paternal parents (G3 haplotypes)					
25-14-20 *x1 x2 D1 x5*	**27-03-08** (*x1*/x2)[a]*d2x3x5*					
	(x1/x2)d2	*(x1/x2)x3*	*(x1/x2)x5*	*d2x3*	*d2x5*	*x3x5*
x1x2	*x1x2(x1/x2)d2*	*x1x2(x1/x2)x3*	*x1x2(x1/x2)x5*	*x1x2d2x3*	*x1x2d2x5*	*x1x2x3x5*
x1D1	*x1D1(x1/x2)d2*	*x1D1(x1/x2)x3*	*x1D1(x1/x2)x5*	*x1D1d2x3*	*x1D1d2x5*	*x1D1x3x5*
x1x5	*x1x5(x1/x2)d2*	*x1x5(x1/x2)x3*	*x1x5(x1/x2)x5*	*x1x5d2x3*	*x1x5d2x5*	*x1x5x3x5*
x2D1	*x2D1(x1/x2)d2*	*x2D1(x1/x2)x3*	*x2D1(x1/x2)x5*	*x2D1d2x3*	*x2D1d2x5*	*x2D1x3x5*
x2x5	*x2x5(x1/x2)d2*	*x2x5(x1/x2)x3*	*x2x5(x1/x2)x5*	*x2x5d2x3*	*x2x5d2x5*	*x2x5x3x5*
D1x5	*D1x5(x1/x2)d2*	*D1x5(x1/x2)x3*	*D1x5(x1/x2)x5*	*D1x5d2x3*	*D1x5d2x5*	*D1x5x3x5*

[a] see Appendix Figure A3.3 for detail crossover.

Table 3.2 (cont'd).

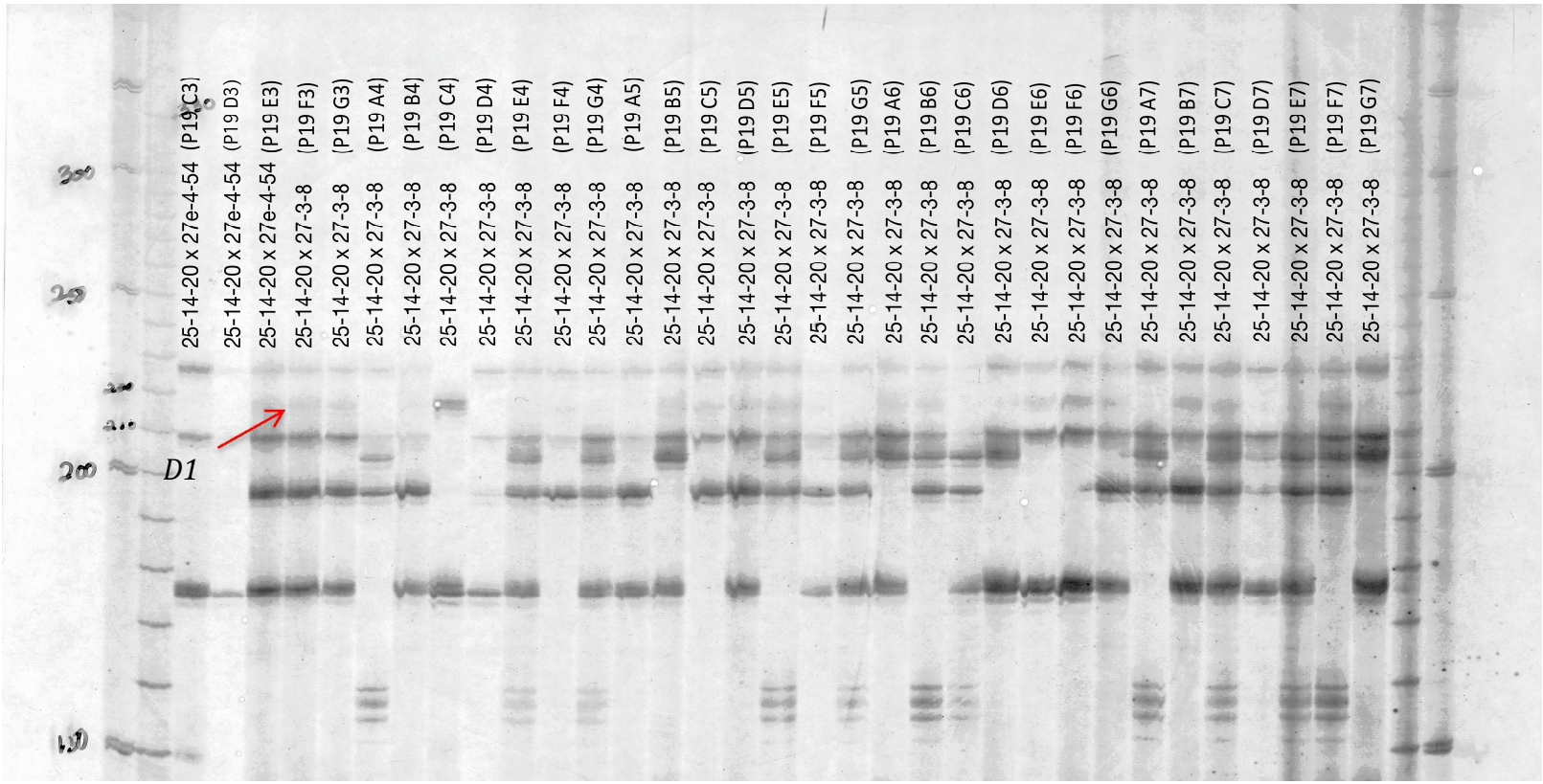
Maternal parent (G3 haplotypes)	**Paternal parents** (G3 haplotypes)					
25-14-20 *x1 x2 D1x5*	**27e-04-54** *x2 (D2/d1)[b]x3 x5*					
	x2(D2/d1)	*x2x3*	*x2x5*	*(D2/d1)x3*	*(D2/d1)x5*	*x3x5*
x1x2	*x1x2(D2/d1)*	*x1x2x2x3*	*x1x2x2x5*	*x1x2(D2/d1)x3*	*x1x2(D2/d1)x5*	*x1x2x3x5*
x1D1	*x1D1(D2/d1)*	*x1D1x2x3*	*x1D1x2x5*	*x1D1(D2/d1)x3*	*x1D1(D2/d1)x5*	*x1D1x3x5*
x1x5	*x1x5(D2/d1)*	*x1x5x2x3*	*x1x5x2x5*	*x1x5(D2/d1)x3*	*x1x5(D2/d1)x5*	*x1x5x3x5*
x2D1	*x2D1(D2/d1)*	*x2D1x2x3*	*x2D1x2x5*	*x2D1(D2/d1)x3*	*x2D1(D2/d1)x5*	*x2D1x3x5*
x2x5	*x2x5(D2/d1)*	*x2x5x2x3*	*x2x5x2x5*	*x2x5(D2/d1)x3*	*x2x5(D2/d1)x5*	*x2x5x3x5*
D1x5	*D1x5(D2/d1)*	*D1x5(D2/d1)x3*	*D1x5(D2/d1)x3*	*D1x5(D2/d1)x3*	*D1x5(D2/d1)x5*	*D1x5x3x5*
25-14-20 *x1 x2 D1 x5*	**27e-05-33** *x2 D4 (x2/D1)[c]x5*					
	x2D4	*x2(x2/D1)*	*x2x5*	*D4(x2/D1)*	*D4x5*	*(x2/D1)x5*
x1x2	*x1x2x2D4*	*x1x2x2(x2/D1)*	*x1x2x2x5*	*x1x2D4(x2/D1)*	*x1x2 D4x5*	*x1x2(x2/D1)x5*
x1D1	*x1D1x2D4*	*x1D1x2(x2/D1)*	*x1D1x2x5*	*x1D1D4(x2/D1)*	*x1D1D4x5*	*x1D1(x2/D1)x5*
x1x5	*x1x5x2D4*	*x1x5(x2/D1)*	*x1x5x2x5*	*x1x5D4(x2/D1)*	*x1x5D4x5*	*x1x5(x2/D1)x5*
x2D1	*x2D1x2D4*	*x2D1(x2/D1)*	*x2D1x2x5*	*x2D1D4(x2/D1)*	*x2D1D4x5*	*x2D1(x2/D1)x5*
x2x5	*x2x5x2D4*	*x2x5(x2/D1)*	*x2x5 x2x5*	*x2x5D4(x2/D1)*	*x2x5D4x5*	*x2x5(x2/D1)x5*
D1x5	*D1x5x2D4*	*D1x5(x2/D1)*	*D1x5x2x5*	*D1x5D4(x2/D1)*	*D1x5D4x5*	*D1x5(x2/D1)x5*

[b] see Appendix Figure A3.4 for detail crossover.
[c] see Appendix Figure A3.5 for detail crossover.

Table 3.2 (cont'd)

Maternal parent (G3 haplotypes)	Paternal parents (G3 haplotypes)					
25-14-20 *x1x2D1x5*	**27e-15-38** *x2x6x2d1*					
	x2x6	*x2x2*	*x2d1*	*x6x2*	*x6d1*	*x2d1*
x1x2	*x1x2x2x6*	*x1x2x2x2*	*x1x2x2d1*	*x1x2x6x2*	*x1x2x6d1*	*x1x2x2d1*
x1D1	*x1D1x2x6*	*x1D1x2x2*	*x1D1x2d1*	*x1D1x6x2*	*x1D1x6d1*	*x1D1x2d1*
x1x5	*x1x5x2x6*	*x1x5x2x2*	*x1x5x2d1*	*x1x5x6x2*	*x1x5x6d1*	*x1x5x2d1*
x2D1	*x2D1x2x6*	*x2D1x2x2*	*x2D1x2d1*	*x2D1x6x2*	*x2D1x6d1*	*x2D1x2d1*
x2x5	*x2x5x2x6*	*x2x5x2x2*	*x2x5x2d1*	*x2x5x6x2*	*x2x5x6d1*	*x2x5x2d1*
D1x5	*D1x5x2x6*	*D1x5x2x2*	*D1x5x2d1*	*D1x5x6x2*	*D1x5x6d1*	*D1x5x2d1*
25-14-20 *x1 x2 D1 x5*	**27e-16-47** *d2D4x2d1*					
	d2D4	*d2x2*	*d2d1*	*D4x2*	*D4d1*	*x2d1*
x1x2	*x1x2d2D4*	*x1x2d2x2*	*x1x2d2d1*	*x1x2D4x2*	*x1x2D4d1*	*x1x2x2d1*
x1D1	*x1D1d2D4*	*x1D1d2x2*	*x1D1d2d1*	*x1D1D4x2*	*x1D1D4d1*	*x1D1x2d1*
x1x5	*x1x5d2D4*	*x1x5d2x2*	*x1x5d2d1*	*x1x5D4x2*	*x1x5D4d1*	*x1x5x2d1*
x2D1	*x2D1d2D4*	*x2D1d2x2*	*x2D1d2d1*	*x2D1D4x2*	*x2D1D4d1*	*x2D1x2d1*
x2x5	*x2x5d2D4*	*x2x5d2x2*	*x2x5d2d1*	*x2x5D4x2*	*x2x5D4d1*	*x2x5x2d1*
D1x5	*D1x5d2D4*	*D1x5d2x2*	*D1x5d2d1*	*D1x5D4x2*	*D1x5D4d1*	*D1x5x2d1*

Figure 3.2. **PCR amplification for segregation of fruit color alleles of 33 sour cherry individuals - derived from 25-14-20 × 27e-04-54 (P19C3, P19D3, and P19E3) and 25-14-20 × 27-03-08 (P19G3-P19G7).** Genomic DNA was amplified by PCR with LG3_13.146 primer set (Stegmeir et al., 2014). PCR products were separated on 6% polyacrylamide gels and visualized with silver staining. The arrow pointed at one of the bands of PCR products of *D1* allele, with the fragment size 218 bp. The plant ID written after the parental crosses code means that the sample individual of each progeny was placed in plate 19, on specific letter column and specific numeral rows.



scoring in Figure 3.2). In the progeny population from the cross 25-14-20 × 27-03-08, 222 (56%) of 400 progeny individuals in this population were identified to have the *D1* allele. The chi-square (X^2) value of 4.84 (p= 0.030) was just below the 0.05 probability level, and did not fit the expected 1:1 ratio for the transmission of *D1* to the progeny (Table 3.1).

Fifty-nine seedlings (65%) from the cross 25-14-20 × 27e-04-54 were detected to have *D1* allele and could be discarded. In this population, the X^2 value of 8.1 ($p=0.004$) was less than 0.05 (Table 3.1), and it did not fit the expected ratio of 1:1 for the segregation of *D1* to the progeny.

Of the 18 plants screened on the progeny population of 25-14-20 × 27e-05-33, 10 individuals (56%) were discarded due to the presence of the *D1* allele. The X^2 value of 0.22 ($p=0.64$) showed that it was fit to the expected 1:1 ratio.

Of the 26 seedlings DNA tested in the progeny population of the cross 25-14-20 × 27e-15-38, 12 plants (46%) of the population were identified to have the *D1* haplotype and discarded. In this progeny population, the X^2 value of 0.14 with p=0.71 (Table 3.1) which was greater than 0.05 and it fitted the 1:1 predicted ratio.

Of the 84 plants screened in the population of 25-14-20 × 27e-05-33, 55 (65%) of the progeny were identified to have the *D1* haplotype. The X^2 value of 8.04 resulted in p value of 0.04 (Table 3.1). This p value showed that the observed value did not fit the 1:1 predicted ratio for the segregation of *D1* haplotype in the progeny.

Of five progeny populations being tested, three progeny populations did not fit the 1:1 ratio predicted. The other two progeny populations fitted the 1:1 predicted ratio of transmitting the *D1* haplotype in the progeny. Three progeny populations that did not fit the prediction ratio could be due to gametophytic selection. The gametophyte of higher plants is an independent

organism that expresses its own genetic information, is exposed to selection and consequently can influence the genetic constitution of the resulting sporophytic generation (Mulcahy, 1979). In this study, the *D1* haplotype segregation was significantly different from the predicted ratio could be influenced by genes linked to *D1*. In data result, the percentage number of plants that have *D1* haplotype and discarded skewed to the dark allele. This condition indicated that there were some excess of *D1* allele in the seedlings populations, caused by self-pollination in some individuals in the populations. The other possible reasons why the result did not fit the 1:1 prediction ratio was due to poor seedling germination of the progeny populations, or the progeny were not true hybrid from the parents. This evidence showed the importance of paternity testing using the *S*-locus information that may confirm that the seedlings used for MASS were from the intended cross.

Paternity Testing

At this point, paternity testing was done only for 56 progeny from the parental cross 25-14-20 × 27-03-08. The result of the analysis markers presented that 20 of the 56 progeny exhibited the $S_{13'}$ allele from the paternal parent, and were verified to have true parentage (Table 3.3). Since the *S*-locus screening that had been done only using the $PcSFBS_{13}$ to identify the specific allele from the paternal parent, it is assumed that there was a possibility that 30 progeny that could not detected for having the $S_{13'}$ allele, could be having the S_{35} allele from 27-03-08 (Figure 3.3; Table 3.4). To get a final paternity verification, the remaining progeny population that were not verified to have $S_{13'}$ allele, should be screened with another specific marker that can identify the presence of S_{35} allele in the progeny, derived from the paternal parent. Six of the

Table 3.3. **Paternity verification for some of the progeny generated from parental crosses 25-14-20 ($S_1S_6S_{36a}S_{36b}$) × 27-03-08 ($S_1S_{13'}S_{35}S_{36a}$).** "YES" means that the individuals found to be have $S_{13'}$ screened by the PcSFBS$_{13}$ primers. The asterisk (*) symbol means that the individuals did not have the $S_{13'}$ allele showed on the agarose gel. It is assumed that the individuals could be have the S_{35} allele from the paternal parent, but need to be screened using the appropriate *S*-locus marker. Double asterisk (**) means that the DNA samples could not be amplified.

Maternal Parent	Paternal Parent	Seedling ID	Paternity verification
25-14-20	27-03-08	P19 A4	YES
25-14-20	27-03-08	P19 B4	YES
25-14-20	27-03-08	P19 D4	*
25-14-20	27-03-08	P19 E4	YES
25-14-20	27-03-08	P19 G4	*
25-14-20	27-03-08	P19 A5	YES
25-14-20	27-03-08	P19 F5	*
25-14-20	27-03-08	P19 G5	YES
25-14-20	27-03-08	P19 C6	*
25-14-20	27-03-08	P19 D6	*
25-14-20	27-03-08	P19 A7	YES
25-14-20	27-03-08	P19 D7	YES
25-14-20	27-03-08	P19 E7	YES
25-14-20	27-03-08	P19 G7	*
25-14-20	27-03-08	P1 E1	**
25-14-20	27-03-08	P1 B2	*
25-14-20	27-03-08	P1 F2	YES
25-14-20	27-03-08	P1 G2	*
25-14-20	27-03-08	P1 A3	**
25-14-20	27-03-08	P1 C3	*
25-14-20	27-03-08	P1 E3	**
25-14-20	27-03-08	P1 G3	*
25-14-20	27-03-08	P1 C4	*
25-14-20	27-03-08	P1 D4	*
25-14-20	27-03-08	P1 E4	*
25-14-20	27-03-08	P1 F4	*
25-14-20	27-03-08	P1 A5	**
25-14-20	27-03-08	P1 D5	**
25-14-20	27-03-08	P1 A6	*
25-14-20	27-03-08	P1 D6	*

Table 3.3 (cont'd)

Maternal Parent	Paternal Parent	Seedling ID	Paternity verification
25-14-20	27-03-08	P1 E6	*
25-14-20	27-03-08	P1 G6	*
25-14-20	27-03-08	P1 A7	**
25-14-20	27-03-08	P1 C7	*
25-14-20	27-03-08	P1E7	*
25-14-20	27-03-08	P1 F7	YES
25-14-20	27-03-08	P1 G7	YES
25-14-20	27-03-08	P1 B8	**
25-14-20	27-03-08	P1 G8	YES
25-14-20	27-03-08	P1 A9	YES
25-14-20	27-03-08	P1 B9	*
25-14-20	27-03-08	P1 C9	*
25-14-20	27-03-08	P1 D9	*
25-14-20	27-03-08	P1 F9	*
25-14-20	27-03-08	P1 A10	**
25-14-20	27-03-08	P1 B10	YES
25-14-20	27-03-08	P1 D10	*
25-14-20	27-03-08	P1 A11	YES
25-14-20	27-03-08	P1 D11	*
25-14-20	27-03-08	P1 E11	*
25-14-20	27-03-08	P1 G11	YES
25-14-20	27-03-08	P1 A12	*
25-14-20	27-03-08	P1 B12	*
25-14-20	27-03-08	P1 D12	*
25-14-20	27-03-08	P1 E12	YES
25-14-20	27-03-08	P1 F12	YES

Figure 3.3. **PCR amplification for *S*-allele segregation of four individuals of the progeny derived from 25-14-20 × 27-03-08 in agarose gel.** Genomic DNA was amplified by PCR with consensus primer set of PruC2/PREC to identify the non-specific *S*-allele, and PcSFBS$_{13}$ to identify the specific allele of $S_{13'}$ (Tao et al., 2008). PCR products were separated on 2% of agarose gels and visualized with GelRed. The arrows indicate the band of PCR products of $S_{1'}$, S_6, S_{36a}, and S_{36b}-RNase and $S_{13'}$. The individual 27-03-08 was used as the paternal control, and the progeny DNA samples were taken from the plate, written as the plant ID. The first letter and number showed the number of the plate, followed by the columns and the rows from which the DNA samples were taken.

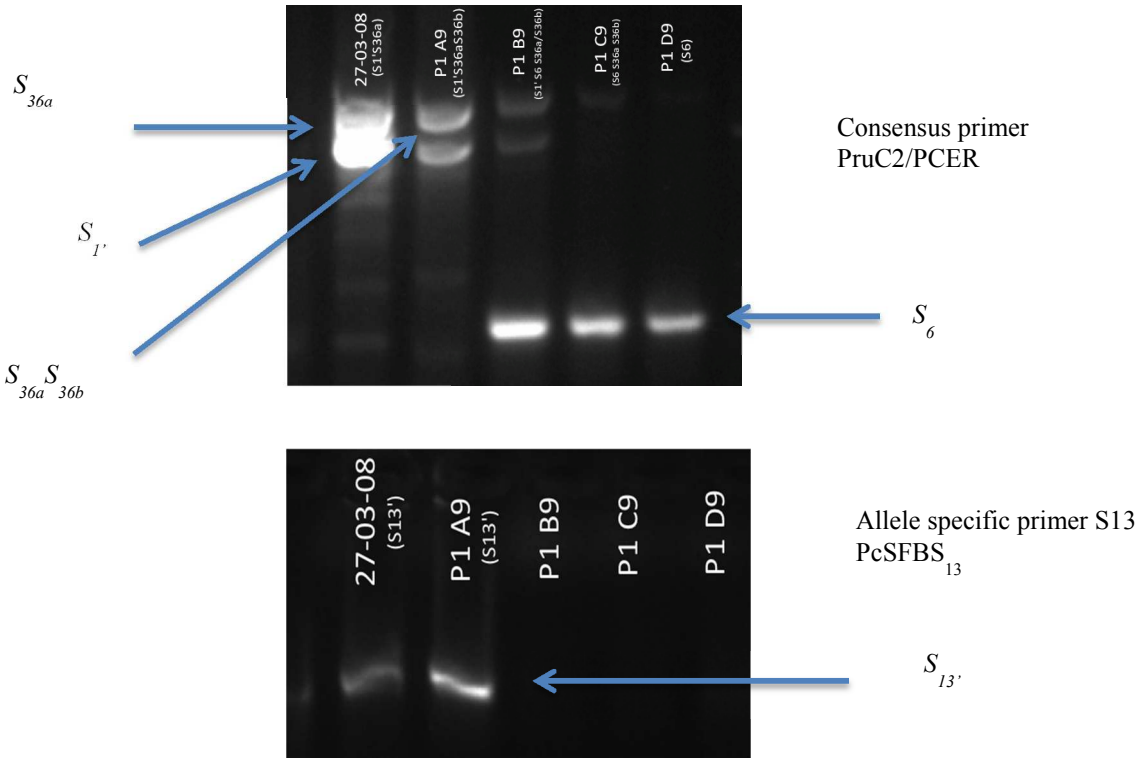


Table 3.4. Prediction of *S*-genotypes progeny generated from parental cross 25-14-20 × 27-03-08. The grey background column means the SI phenotypes of the progeny due to the presence of match *S*-functional or the absence of less than two non-functional *S*-haplotypes.

	25-14-20 $S_1S_6S_{36a}S_{36b}$ × 27-03-08 $S_1S_{13}S_{35}S_{36a}$					
	S_1S_{13}	S_1S_{35}	S_1S_{36a}	$S_{13}S_{35}$	$S_{13}S_{36a}$	$S_{35}S_{36a}$
S_1S_6	$S_1S_1S_6S_{13}$	$S_1S_1S_6S_{35}$	$S_1S_1S_6S_{36a}$	$S_1S_6S_{13}S_{35}$	$S_1S_6S_{13}S_{36a}$	$S_1S_6S_{35}S_{36a}$
S_1S_{36a}	$S_1S_1S_{13}S_{36a}$	$S_1S_1S_{35}S_{36a}$	$S_1S_1S_{36a}S_{36a}$	$S_1S_{13}S_{35}S_{36a}$	$S_1S_{13}S_{36a}S_{36a}$	$S_1S_{35}S_{36a}S_{36a}$
S_1S_{36b}	$S_1S_1S_{13}S_{36b}$	$S_1S_1S_{35}S_{36b}$	$S_1S_1S_{36a}S_{36b}$	$S_1S_{13}S_{35}S_{36b}$	$S_1S_{13}S_{36a}S_{36b}$	$S_1S_{35}S_{36a}S_{36b}$
S_6S_{36a}	$S_1S_6S_{13}S_{36a}$	$S_1S_6S_{35}S_{36a}$	$S_1S_6S_{36a}S_{36a}$	$S_6S_{13}S_{35}S_{36a}$	$S_6S_{13}S_{36a}S_{36a}$	$S_6S_{35}S_{36a}S_{36a}$
S_6S_{36b}	$S_1S_6S_{13}S_{36b}$	$S_1S_6S_{35}S_{36b}$	$S_1S_6S_{36a}S_{36b}$	$S_6S_{13}S_{35}S_{36b}$	$S_6S_{13}S_{36a}S_{36b}$	$S_6S_{35}S_{36a}S_{36b}$
$S_{36a}S_{36b}$	$S_1S_{13}S_{36a}S_{36b}$	$S_1S_{35}S_{36a}S_{36b}$	$S_1S_{36a}S_{36a}S_{36b}$	$S_{13}S_{35}S_{36a}S_{36b}$	$S_{13}S_{36a}S_{36a}S_{36b}$	$S_{35}S_{36a}S_{36a}S_{36b}$

[a] See the Appendix 3.1.1-3.1.4 for other parents with the *S*-allele genotypes.

DNA samples that run in this agarose gel were not amplified, either using the consensus primer PruC2 or specific primers $PcSFBS_{13}$. It can be assumed that the DNA samples used had poor DNA quality, due to long-term storage (since 2014).

For the next step, the paternity testing would be done for the remaining progeny population, using the specific primers, which can identify the specific *S*-allele from the paternal parent. For example, in the parental cross of 25-14-20 x 27e-04-54, the paternal parent was identified to have $S_{13m}S_{13'}S_{36a}S_{36a}$ (Table A3.1.1). To see the presence of the specific allele in the progeny derived from the paternal parent, specific primers pair of S_{13m} and $S_{13'}$ would be used. For the progeny of the parental cross 25-14-20 × 27e-05-33, the paternal parent was identified to have $S_6S_{13'}S_{36a}S_{36b}$ (Table A3.1.2), therefore, primers pair to identify S_6 an $S_{13'}$ should be used for this paternity testing. A specific primers pair to identify S_4 and $S_{13'}$ of the paternal parent would be used for the progeny derived from 25-14-20 ($S_1S_6S_{36a}S_{36b}$) × 27e-15-38 ($S_4S_{13'}S_{13'}S_{36a}$) (Table A3.1.3). Other specific primers would need to be used to identify the $S_{13'}$ and S_{35} allele of the paternal parent from the cross 25-14-20 ($S_1S_6S_{36a}S_{36b}$) × 27e-16-47 ($S_{13'}S_{35}S_{36a}S_{36b}$) (Table A3.1.4).

The identification of specific S-allele of the progeny based on the *S*-genotypes of the parent is necessary to detect SC to increase the efficiency of breeding program. Only seedlings with SC would be planted in the orchard, and would reduce the maintenance cost in the field. This also would lead to another advantage, for paternal testing, so the inheritance of *S*-allele in the progeny derived from the paternal parent would be identified.

This result revealed that the DNA test could be applied for various purposes of the breeding program, such as for MASS to identify the desired allele of specific traits for selection purposes; for identifying the *S*-locus to see the compatibility or incompatibility of the plants;

furthermore would give additional advantage for paternity testing in the progeny. Paternity testing using the *S*-locus information needs to be done to confirm the true parentage of the progeny thus can attain the accurate analysis of genetic result.

Consequences of MASS for D1

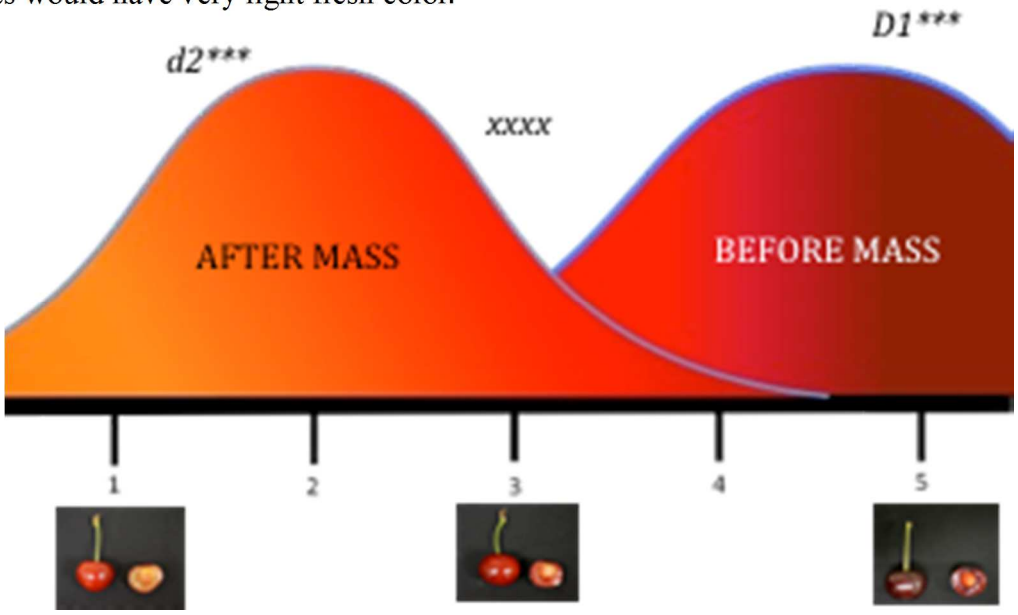
Of the 400 seedlings DNA tested from the cross 25-14-20 × 27-03-08, 178 plants (44%) were kept because they did not have the *D1* haplotype (Table 3.1). The MYB10 haplotypes that have significant effects on dark red flesh color are *D1, D2*, *D3*, and *D4*. Two haplotypes showed the significant effect to the light flesh color are *d1* and *d2*, while the other haplotype, *x1, x2, x3, x4*, and *x5* were used to indicate that there was no significant effect on flesh color. The only other *MYB10* haplotype segregating in these remaining individuals that has been shown to have a significant effect on flesh color was the *d2* haplotype from 27-03-08. The *d2* haplotype was associated with light flesh color (Stegmeir et al., submitted). Half the remaining progeny would be predicted to have the *d2* haplotype. The other haplotypes segregating in these progeny were four of the *x*-haplotypes (*x1*, *x2*, *x3,* and *x5*), none of which have been shown to be significantly associated with flesh color (Table 3.2). There was one cross over between haplotype *x1* to haplotype *x2* derived from the paternal parent 27-03-08 (Figure A3.3), and it would be segregated in the progeny. However, this crossover would not change the flesh color proportion of the progeny since these haplotypes did not have significant effect to the flesh color. Based on the flesh color predictions, the phenotype in the original seedling population (before MASS) would likely have been skewed to the dark red color due to ~ half the progeny individuals having the *D1* haplotype (Figure 3.4). In contrast, after MASS, half the progeny would be predicted to

have the *d2* light-fleshed haplotype, therefore suggesting that the mean color of the remaining progeny population would have shifted from 4-5 to 1-3 on 1-5 scale (Figure 3.4).

The DNA screening of the cross 25-14-20 × 27e-04-54 resulted in 32 plants (35%) of 91 total progeny could be kept due to the absence of *D1* haplotype (Table 3.1). Half of the remaining progeny populations were predicted to have *D2* haplotype from 27e-04-54, that cross over with *d1* haplotype (Table 3.2; Figure A3.4); while the other haplotypes segregating in these progeny were *x1, x2, x3,* and *x5,* which are not significantly associated with flesh color (Table 3.2). Based on the flesh color prediction, the phenotype before MASS implementation would be skewed to the dark red color due to the presence of the *D1* haplotype in about half of the total progeny population (Figure 3.5). After MASS implementation, approximately half of the remaining progeny would be predicted to have four *x* haplotypes (*x1, x2, x3,* and *x5*), which would not have a significant effect on the flesh color, and the mean color of the remaining progeny population would be predicted to shift from 4-5 to 3-4 on 1-5 scale (Figure 3.5).

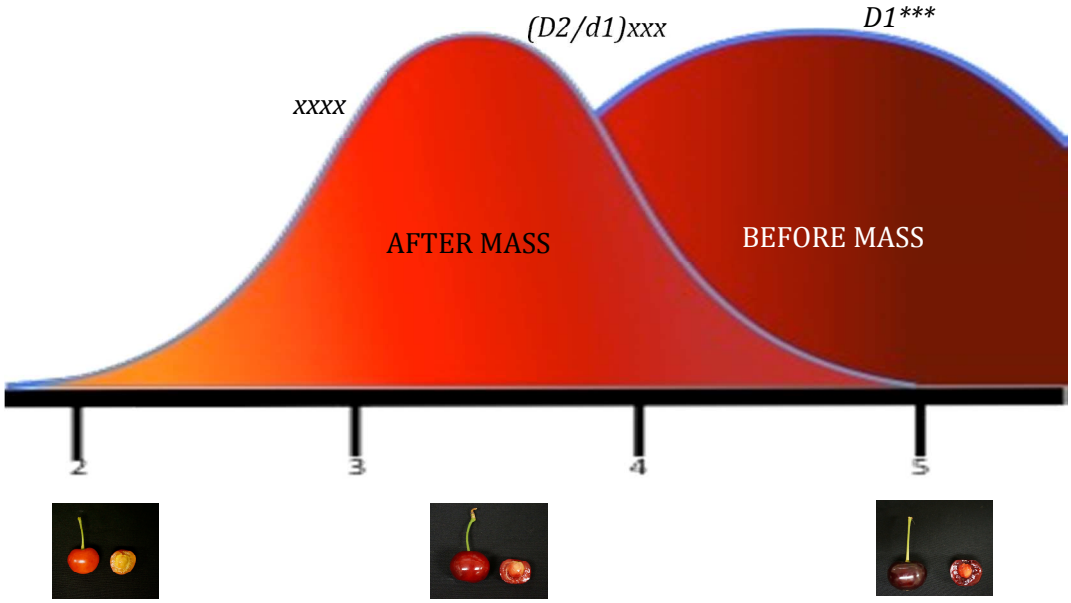
Eight plants (44%) of 18 seedlings in the progeny population generated from the parental cross 25-14-20 × 27e-05-33 were kept because they did not have *D1* haplotype (Table 3.1). Another *MYB10* haplotype in the remaining individuals, which has a significant effect on flesh color, was the *D4* from 27e-05-33 (Table 3.2; Figure A3.5). Half of the remaining progeny would be predicted to have *D4* haplotype that significantly associated with the dark color, while the other haplotypes segregating in this progeny were three of the *x*-haplotypes (*x1*, *x2*, and *x5*) and the crossover from *x2* to *D1* (Table 3.2). Based on the flesh color predictions, the phenotype in the original population before MASS implementation would likely skewed to dark red color due to half of the progeny individuals having the *D1* haplotype (Figure 3.6). After MASS, half of the progeny would be predicted to have the *D4* dark-fleshed haplotype and the rest of it would be

Figure 3.4. **Prediction of fruit color from progeny population derived from 25-14-20 × 27-03-08 after MASS implementation.** The progeny with *D1**** would have the darkest flesh color, and those with any *x* haplotype would be lighter than those with *D1* haplotype, but it does not significantly associated with either light or dark flesh color. The individuals with *d2**** haplotypes would have very light flesh color.



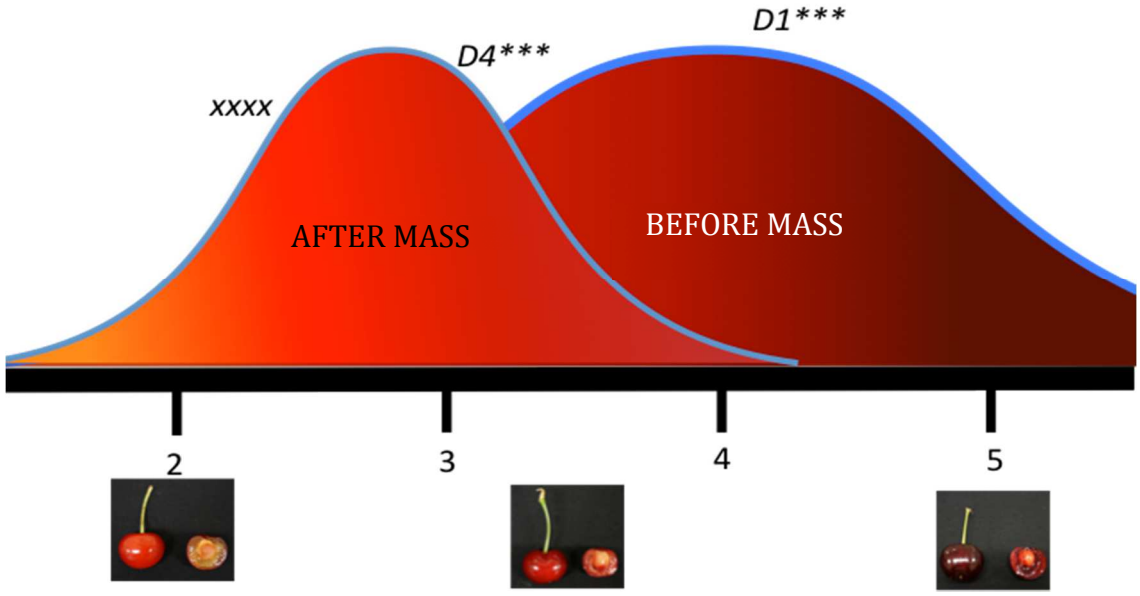
Flesh color rating

Figure 3.5. **Prediction of fruit color from progeny population derived from 25-14-20 × 27e-04-54 after MASS implementation.** The progeny with *D1**** would have the darkest flesh color. The individuals with crossover haplotype *D2/d1* and those with any *x*-haplotypes would be lighter than those with *D1* haplotype.



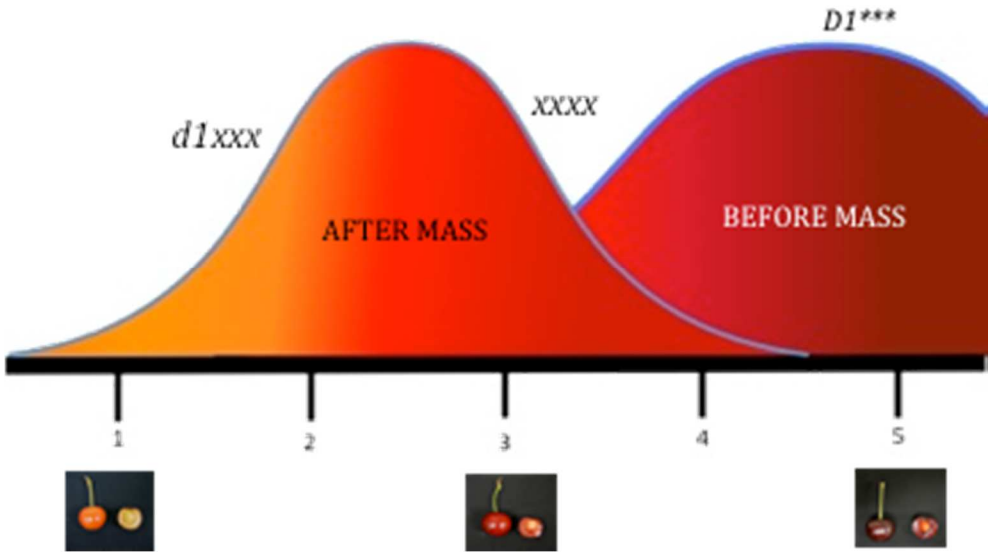
Flesh color rating

Figure 3.6. **Prediction of fruit color from progeny population derived from 25-14-20 × 27e-05-33 after MASS implementation**. The progeny with *D1**** would have the darkest flesh color, and those with *D4**** haplotypes would be lighter than individuals with *D1* haplotype, and those with any *x* haplotype would be lighter than those with *D1* or *D4* haplotype, but did not have significant difference from light or dark flesh color.



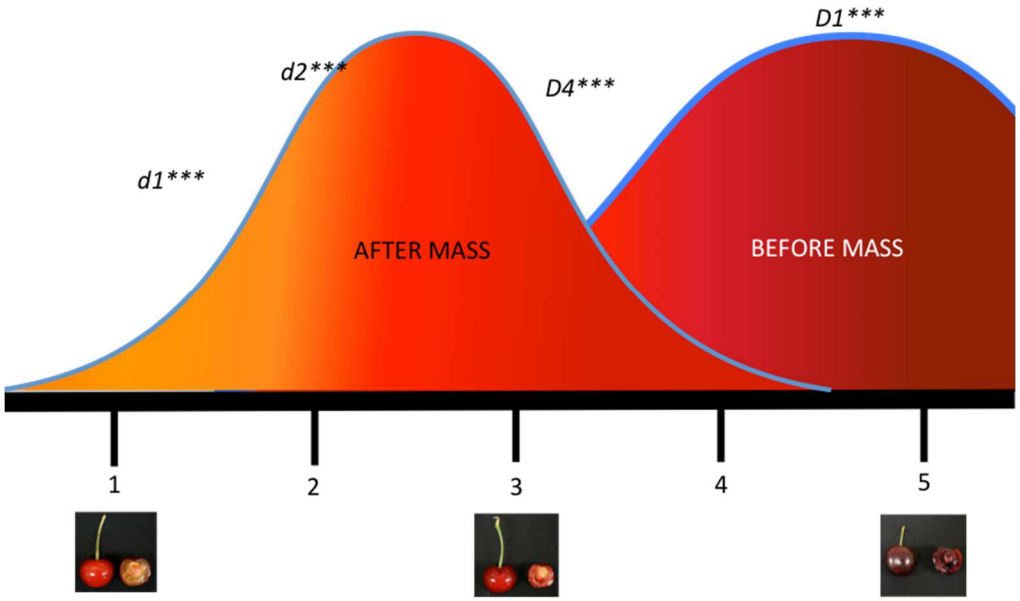
Flesh color rating

Figure 3.7. **Prediction of fruit color from progeny population derived from 25-14-20 × 27e-15-38 after MASS implementation.** The progeny with *D1**** would have the darkest flesh color, and those with *d1**** haplotypes would be very light color, and those with any *x* haplotype would be lighter than those with *D1* but it did not significant difference from individuals that have dark or light flesh color allele.



Flesh color rating

Figure 3.8. **Prediction of fruit color from progeny population derived from 25-14-20 × 27e-16-47 after MASS implementation.** The progeny with *D1**** would have the darkest flesh color, and those with *d2**** haplotypes would be have the light flesh color. Those with combination haplotypes of *x,d* and *D4*, would have lighter color than those with *D1* haplotype, but it did not significant difference from individuals that have dark or light flesh color allele.



Flesh color rating

predicted to have four *x*-haplotypes. Therefore the mean color of the remaining progeny population would be shifted from 4-5 to 2-4 scale shown in Figure 3.6.

Of the 26 seedlings DNA tested from the cross 25-14-20 × 27e-15-38, 14 plants (54%) were kept because they did not have the *D1* haplotype (Table 3.1). The *MYB10* haplotype segregating in these remaining individuals that had been shown to have a significant effect on flesh color was *d1* haplotype from 27-15-38 (Table 3.2; Figure A3.6). This *d1* haplotype was associated with the lightest flesh color (Stegmeir et al., submitted). Half of the remaining progeny would be predicted to have the *d1* haplotype, and the other haplotypes segregating for these progeny were four of the *x*-haplotypes (*x1, x2, x2, x6*), none of which have been shown to be significantly associated with flesh color (Table 3.2). The phenotype of the initial populations before MASS implementation were tend to be dark with score of flesh color rating from 4-5 due to the presence of *D1* haplotypes in the half of the progeny individuals. After the MASS, half of the progeny would be predicted to have the *d1* haplotype, which associated with very light flesh color, suggesting that the mean color of the remaining progeny population would be shifted from 4-5 to 1-3 on 1-5 scale (Figure 3.7).

Thirty-nine plants (39%) of the 84 seedlings progeny from the cross 25-14-20 x 27e-16-47 screened by the marker, were kept because they did not have the *D1* haplotype (Table 3.1). The other MYB10 haplotype that segregate in the remaining individuals were *D4* and *d1* from 27e-16-47 that have a significant effect on the flesh color (Table 3.2; Figure A3.7). The *D4* haplotype was associated with dark flesh color, while the *d1* was associated with the lightest flesh color (Stegmeir et al., submitted). Half of the remaining progeny would be predicted to have *D4* haplotype, and the other haplotype segregating in these progeny were *d1, d2, x1, x2*, and *x5*. The two *d*-haplotypes were significantly associated with clear flesh color, while the three *x*-

haplotypes were not significantly associated with flesh color (Table 3.2). Based on the flesh color prediction, the phenotype in the initial population (before MASS) would likely have been skewed to dark flesh color due to the presence of *D1* haplotype in the progeny. After MASS, half of the progeny would be predicted to have *D4* haplotype, which was associated with the dark flesh color, and the rest of the progeny population would be predicted to have *d1* and or *d2*-light fleshed haplotypes. Therefore, the mean color of the remaining progeny population would have shifted from 4-5 to 1-3 on 1-5 scale (Figure 3.8).

Summary

In summary, MASS implemented using a DNA test was able to determine the seedlings predicted with favorable color by selecting against those individuals identified to have the darkest haplotype (*D1*). Paternity testing by identifying *S*-locus inheritance in the progeny was required to verify the true cross and to examine the genetic hypothesis of 1:1 ratio. The DNA test through MASS and paternity testing were highly beneficial to increase the efficiency of sour cherry breeding program for fruit color.

APPENDIX

Figure A3.1. **Washington State University flesh color card rating scale used to determine flesh color rating for sour cherry individuals.**

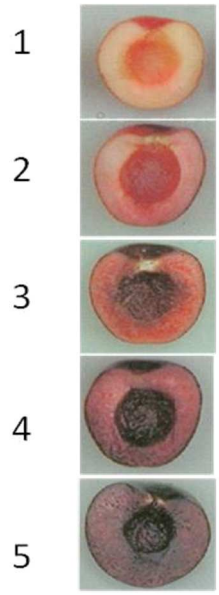


Figure A3.2. **Four haplotypes identified in 25-14-20 for G3 region containing MYB10**. This individual was used as the female parent in 2013 crosses (Summarized from Stegmeir, 2013).

Name		25-14-20			
		x1	*x2*	*D1*	*x5*
RB_S_3_09729116	ABBB	B	A	B	B
RB_T_3_09782875	AABB	A	A	B	B
RB_S_3_09789199	ABBB				
RB_S_3_10022424	ABBB	B	B	B	A
RB_S_3_10105783	AABB	A	A	B	B
RB_S_3_10162979	AAAB	B	A	A	A
RB_S_3_10264563	AABB	A	A	B	B
RB_S_3_10573974	AABB	A	A	B	B
RB_T_3_10590166	AABB	A	A	B	B
RB_S_3_10626205	ABBB	A	B	B	B
RB_S_3_10675150	AABB	A	A	B	B
RB_S_3_10822211	AABB	A	A	B	B
RB_T_3_10908880	AABB	A	A	B	B
RB_T_3_12115409	AABB	A	A	B	B
RB_S_3_12383977	AABB	B	B	A	A
RB_S_3_12474678	ABBB	B	A	B	B
RB_S_3_12500413	ABBB	A	B	B	B
RB_T_3_12503462	AABB	A	A	B	B
RB_T_3_12539794	BBBB	B	B	B	B
LG3_12.71Mb		3	2	2	2
3 MYB 10 homologs					
RB_S_3_12944437	AABB	A	A	B	B
RB_S_3_12987920	ABBB	B	B	B	A
RB_S_3_13025963	AAAB	A	A	A	B
RB_T_3_13063792	AABB	A	A	B	B
Marker LG3_13.146					
RB_S_3_13144730	ABBB	B	B	B	A
RB_S_3_13208005	AABB	B	B	A	A
RB_T_3_13369328	AAAB	A	B	A	A
RB_S_3_13406263	AABB	A	B	B	A
RB_S_3_13433848	ABBB	B	B	B	A
RB_S_3_13466702	ABBB	B	B	B	A
RB_S_3_13520194	ABBB	B	B	B	A
RB_S_3_13563908	AABB	A	A	B	B
RB_S_3_13567593	AABB	A	A	B	B

Figure A3.2. (cont'd).

Name		25-14-20			
		x1	*x2*	*D1*	*x5*
RB_S_3_13724726	AABB	A	A	B	B
RB_S_3_13754793	AABB	B	B	A	A
RB_S_3_13795019	AABB	B	B	A	A
RC3766-391_3_13878008	AABB	A	A	B	B
RB_T_3_13881088	AABB	A	A	B	B
RB_S_3_14024780	AAAB	A	B	A	A
RB_S_3_14146853	ABBB	B	A	B	B
RB_S_3_14316165	AABB	B	B	A	A
RB_T_3_14442011	ABBB	B	B	B	A
RB_S_3_14521488	AABB	B	B	A	A
RB_S_3_14599590	ABBB	B	B	B	A
RB_T_3_15171728	ABBB	A	B	B	B
RB_T_3_15305145	AABB	B	B	A	A
RB_S_3_15309954	AAAB	A	A	B	A
RB_S_3_15357433	AABB	B	B	A	A
RB_S_3_15455662	AAAB	A	A	B	A

Figure A3.3. Four haplotypes identified in 27-03-08 for G3 region containing MYB10. This individual was used as the male parent in 2013 crosses (Summarized from Stegmeir, 2013).

Name		27-03-08			
		x1/x2	*d2*	*x3*	*x5*
RB_S_3_09729116	BBBB	B	B	B	B
RB_T_3_09782875	AAAB	A	A	A	B
RB_S_3_09789199	AAAB				
RB_S_3_10022424	ABBB	B	B	B	A
RB_S_3_10105783	AABB	A	B	A	B
RB_S_3_10162979	AAAB	B	A	A	A
RB_S_3_10264563	AAAB	A	A	A	B
RB_S_3_10573974	AABB	A	B	A	B
RB_T_3_10590166	AABB	A	B	A	B
RB_S_3_10626205	AABB	A	B	A	B
RB_S_3_10675150	AABB	A	B	A	B
RB_S_3_10822211	AABB	A	B	A	B
RB_T_3_10908880	AABB	A	B	A	B
RB_T_3_12115409	AABB	A	B	A	B
RB_S_3_12383977	AABB	B	A	B	A
RB_S_3_12474678	BBBB	B	B	B	B
RB_S_3_12500413	AABB	A	B	A	B
RB_T_3_12503462	AABB	A	B	A	B
RB_T_3_12539794	ABBB	B	B	A	B
LG3_12.71Mb		3	1	5	2
3 MYB 10 homologs					
RB_S_3_12944437	AABB	A	B	A	B
RB_S_3_12987920	AABB	B	A	B	A
RB_S_3_13025963	AAAB	A	A	A	B
RB_T_3_13063792	AABB	A	B	A	B
Marker LG3_13.146					
RB_S_3_13144730	ABBB	B	B	B	A
RB_S_3_13208005	ABBB	B	B	B	A
RB_T_3_13369328	AAAB	B	A	A	A
RB_S_3_13406263	AABB	B	B	B	A
RB_S_3_13433848	AABB	B	A	B	A
RB_S_3_13466702	ABBB	B	B	B	A
RB_S_3_13520194	AABB	B	A	B	A
RB_S_3_13563908	AABB	A	B	A	B
RB_S_3_13567593	AABB	A	B	A	B

Figure A3.3. (cont'd).

Name		27-03-08			
		x1/x2	*d2*	*x3*	*x5*
RB_S_3_13724726	AABB	A	B	A	B
RB_S_3_13754793	AABB	B	A	B	A
RB_S_3_13795019	AABB	B	A	B	A
RC3766-391_3_13878008	AAAB	A	A	A	B
RB_T_3_13881088	AAAB	A	A	A	B
RB_S_3_14024780	AABB	B	B	A	A
RB_S_3_14146853	ABBB	A	B	B	B
RB_S_3_14316165	AABB	B	A	B	A
RB_T_3_14442011	AABB	B	A	B	A
RB_S_3_14521488	AABB	B	A	B	A
RB_S_3_14599590	AABB	B	A	B	A
RB_T_3_15171728	ABBB	B	B	A	B
RB_T_3_15305145	AABB	B	A	B	A
RB_S_3_15309954	AAAB	A	B	A	A
RB_S_3_15357433	AABB	B	A	B	A
RB_S_3_15455662	AAAB	A	B	A	A

Figure A3.4. **Four haplotypes identified in 27e-04-54 for G3 region containing MYB10**. This individual was used as the male parent in 2013 crosses (Summarized from Stegmeir, 2013).

Name		27e-04-54			
		x2	*D2/d1*	*x3*	*x5*
RB_S_3_09729116	ABBB	A	B	B	B
RB_T_3_09782875	AAAB	A	A	A	B
RB_S_3_09789199	ABBB				
RB_S_3_10022424	AABB	B	A	B	A
RB_S_3_10105783	AABB	A	B	A	B
RB_S_3_10162979	AAAA	A	A	A	A
RB_S_3_10264563	AABB	A	B	A	B
RB_S_3_10573974	AABB	A	B	A	B
RB_T_3_10590166	AABB	A	B	A	B
RB_S_3_10626205	ABBB	B	B	A	B
RB_S_3_10675150	AAAB	A	A	A	B
RB_S_3_10822211	AABB	A	A	A	B
RB_T_3_10908880	AABB	A	B	A	B
RB_T_3_12115409	AABB	A	B	A	B
RB_S_3_12383977	AABB	B	A	B	A
RB_S_3_12474678	ABBB	A	B	B	B
RB_S_3_12500413	ABBB	B	B	A	B
RB_T_3_12503462	AABB	A	B	A	B
RB_T_3_12539794	ABBB	B	B	A	B
LG3_12.71Mb		2		5	2
3 MYB 10 homologs					
RB_S_3_12944437	AABB	A	B	A	B
RB_S_3_12987920	AABB	B	A	B	A
RB_S_3_13025963	AABB	A	B	A	B
RB_T_3_13063792	AABB	A	B	A	B
Marker LG3_13.146					
RB_S_3_13144730	AABB	B	A	B	A
RB_S_3_13208005	AABB	B	A	B	A
RB_T_3_13369328	AAAB	B	A	A	A
RB_S_3_13406263	AABB	B	A	B	A
RB_S_3_13433848	AABB	B	A	B	A
RB_S_3_13466702	AABB	B	A	B	A
RB_S_3_13520194	ABBB	B	B	B	A
RB_S_3_13563908	AABB	A	B	A	B
RB_S_3_13567593	AAAB	A	A	A	B

Figure A3.4. (cont'd).

Name		27e04-54			
		x2	*D2/d1*	*x3*	*x5*
RB_S_3_13724726	AABB	A	B	A	B
RB_S_3_13754793	AABB	B	A	B	A
RB_S_3_13795019	AABB	B	A	B	A
RC3766-391_3_13878008	AAAB	A	A	A	B
RB_T_3_13881088	AAAB	A	A	A	B
RB_S_3_14024780	AAAB	B	A	A	A
RB_S_3_14146853	ABBB	A	B	B	B
RB_S_3_14316165	ABBB	B	B	B	A
RB_T_3_14442011	AABB	B	A	B	A
RB_S_3_14521488	AABB	B	A	B	A
RB_S_3_14599590	ABBB	B	B	B	A
RB_T_3_15171728	ABBB	B	B	A	B
RB_T_3_15305145	AABB	B	A	B	A
RB_S_3_15309954	AAAB	A	B	A	A
RB_S_3_15357433	ABBB	B	B	B	A
RB_S_3_15455662	AAAA	A	A	A	A

Figure A3.5. Four haplotypes identified in 27e-05-33 for G3 region containing MYB10. This individual was used as the male parent in 2013 crosses (Summarized from Stegmeir, 2013).

Name		27e-05-33			
		x2	*D4*	*x2/D1*	*x5*
RB_S_3_09729116	AABB	A	B	A	B
RB_T_3_09782875	AABB	A	B	A	B
RB_S_3_09789199	BBBB				
RB_S_3_10022424	ABBB	B	B	B	A
RB_S_3_10105783	AABB	A	B	A	B
RB_S_3_10162979	AAAA	A	A	A	A
RB_S_3_10264563	AABB	A	B	A	B
RB_S_3_10573974	AABB	A	B	A	B
RB_T_3_10590166	AABB	A	B	A	B
RB_S_3_10626205	BBBB	B	B	B	B
RB_S_3_10675150	AABB	A	B	A	B
RB_S_3_10822211	AABB	A	B	A	B
RB_T_3_10908880	AABB	A	B	A	B
RB_T_3_12115409	AABB	A	B	A	B
RB_S_3_12383977	AABB	B	A	B	A
RB_S_3_12474678	AABB	A	B	A	B
RB_S_3_12500413	BBBB	B	B	B	B
RB_T_3_12503462	AABB	A	B	A	B
RB_T_3_12539794	BBBB	B	B	B	B
LG3_12.71Mb					
3 MYB 10 homologs					
RB_S_3_12944437	AABB	A	B	A	B
RB_S_3_12987920	ABBB	B	B	B	A
RB_S_3_13025963	AABB	A	B	A	B
RB_T_3_13063792	AABB	A	B	A	B
Marker LG3_13.146					
RB_S_3_13144730	ABBB	B	B	B	A
RB_S_3_13208005	AABB	B	A	B	A
RB_T_3_13369328	AABB	B	A	B	A
RB_S_3_13406263	AABB	B	A	B	A
RB_S_3_13433848	AABB	B	A	B	A
RB_S_3_13466702	AABB	B	A	B	A
RB_S_3_13520194	AABB	B	A	B	A
RB_S_3_13563908	AABB	A	B	A	B
RB_S_3_13567593	AABB	A	B	A	B

Figure A3.5 (cont'd)

Name		27e-05-33			
		x2	*D4*	*x2/D1*	*x5*
RB_S_3_13724726	ABBB	A	B	B	B
RB_S_3_13754793	AAAB	B	A	A	A
RB_S_3_13795019	AAAB	B	A	A	A
RC3766-391_3_13878008	AABB	A	A	B	B
RB_T_3_13881088	AABB	A	A	B	B
RB_S_3_14024780	AABB	B	B	A	A
RB_S_3_14146853	ABBB	A	B	B	B
RB_S_3_14316165	AAAB	B	A	A	A
RB_T_3_14442011	AABB	B	A	B	A
RB_S_3_14521488	AAAB	B	A	A	A
RB_S_3_14599590	AABB	B	A	B	A
RB_T_3_15171728	BBBB	B	B	B	B
RB_T_3_15305145	AAAB	B	A	A	A
RB_S_3_15309954	AABB	A	B	B	A
RB_S_3_15357433	AAAB	B	A	A	A
RB_S_3_15455662	AABB	A	B	B	A

Figure A3.6 Four haplotypes identified in 27e-15-38 for G3 region containing MYB10. This individual was used as the male parent in 2013 crosses (Summarized from Stegmeir, 2013).

Name		27e-15-38			
		x2	*x6*	*x2*	*d1*
RB_S_3_09729116	AABB	A	B	A	B
RB_T_3_09782875	AAAB	A	A	A	B
RB_S_3_09789199	AABB				
RB_S_3_10022424	BBBB	B	B	B	B
RB_S_3_10105783	AABB	A	B	A	B
RB_S_3_10162979	AAAA	A	A	A	A
RB_S_3_10264563	AAAB	A	A	A	B
RB_S_3_10573974	AABB	A	B	A	B
RB_T_3_10590166	AABB	A	B	A	B
RB_S_3_10626205	BBBB	B	B	B	B
RB_S_3_10675150	AABB	A	B	A	B
RB_S_3_10822211	AABB	A	B	A	B
RB_T_3_10908880	AABB	A	B	A	B
RB_T_3_12115409	AAAB	A	B	A	A
RB_S_3_12383977	ABBB	B	B	B	A
RB_S_3_12474678	AABB	A	B	A	B
RB_S_3_12500413	BBBB	B	B	B	B
RB_T_3_12503462	AABB	A	B	A	B
RB_T_3_12539794	BBBB	B	B	B	B
LG3_12.71Mb		2	2	2	2
3 MYB 10 homologs					
RB_S_3_12944437	AABB	A	B	A	B
RB_S_3_12987920	AABB	B	A	B	A
RB_S_3_13025963	AABB	A	B	A	B
RB_T_3_13063792	AABB	A	B	A	B
Marker LG3_13.146					
RB_S_3_13144730	AABB	B	A	B	A
RB_S_3_13208005	AABB	B	A	B	A
RB_T_3_13369328	AABB	B	A	B	A
RB_S_3_13406263	AABB	B	A	B	A
RB_S_3_13433848	AABB	B	A	B	A
RB_S_3_13466702	AABB	B	A	B	A
RB_S_3_13520194	ABBB	B	A	B	B
RB_S_3_13563908	AABB	A	B	A	B
RB_S_3_13567593	AAAB	A	B	A	A

Figure A3.6 (cont'd).

Name		27e-15-38			
		x2	*x6*	*x2*	*d1*
RB_S_3_13724726	AABB	A	B	A	B
RB_S_3_13754793	AABB	B	A	B	A
RB_S_3_13795019	AABB	B	A	B	A
RC3766-391_3_13878008	AAAB	A	B	A	A
RB_T_3_13881088	AAAB	A	B	A	A
RB_S_3_14024780	AABB	B	A	B	A
RB_S_3_14146853	AABB	A	B	A	B
RB_S_3_14316165	ABBB	B	A	B	B
RB_T_3_14442011	AABB	B	A	B	A
RB_S_3_14521488	AABB	B	A	B	A
RB_S_3_14599590	ABBB	B	A	B	B
RB_T_3_15171728	BBBB	B	B	B	B
RB_T_3_15305145	AABB	B	A	B	A
RB_S_3_15309954	AAAB	A	A	A	B
RB_S_3_15357433	ABBB	B	A	B	B
RB_S_3_15455662	AAAA	A	A	A	A

Figure A3.7. Four haplotypes identified in 27e-16-47 for G3 region containing MYB10. This individual was used as the male parent in 2013 crosses (Summarized from Stegmeir, 2013).

Name		27e-16-47			
		d2	*D4*	*x2*	*d1*
RB_S_3_09729116	ABBB	B	B	A	B
RB_T_3_09782875	AABB	A	B	A	B
RB_S_3_09789199	ABBB				
RB_S_3_10022424	BBBB	B	B	B	B
RB_S_3_10105783	ABBB	B	B	A	B
RB_S_3_10162979	AAAA	A	A	A	A
RB_S_3_10264563	AABB	A	B	A	B
RB_S_3_10573974	ABBB	B	B	A	B
RB_T_3_10590166	ABBB	B	B	A	B
RB_S_3_10626205	BBBB	B	B	B	B
RB_S_3_10675150	ABBB	B	B	A	B
RB_S_3_10822211	ABBB	B	B	A	B
RB_T_3_10908880	ABBB	B	B	A	B
RB_T_3_12115409	AABB	B	B	A	A
RB_S_3_12383977	AAAB	A	A	B	A
RB_S_3_12474678	ABBB	B	B	A	B
RB_S_3_12500413	BBBB	B	B	B	B
RB_T_3_12503462	ABBB	B	B	A	B
RB_T_3_12539794	BBBB	B	B	B	B
LG3_12.71Mb		1	1	2	2
3 MYB 10 homologs					
RB_S_3_12944437	ABBB	B	B	A	B
RB_S_3_12987920	AABB	A	B	B	A
RB_S_3_13025963	AABB	A	B	A	B
RB_T_3_13063792	ABBB	B	B	A	B
Marker LG3_13.146					
RB_S_3_13144730	ABBB	B	B	B	A
RB_S_3_13208005	AABB	B	A	B	A
RB_T_3_13369328	AAAB	A	A	B	A
RB_S_3_13406263	AABB	B	A	B	A
RB_S_3_13433848	AAAB	A	A	B	A
RB_S_3_13466702	AABB	B	A	B	A
RB_S_3_13520194	AABB	A	A	B	B
RB_S_3_13563908	AABB	B	B	A	B
RB_S_3_13567593	AAAB	B	B	A	A

Figure A3.7 (cont'd)

Name		27e-16-47			
		d2	*D4*	*x2*	*d1*
RB_S_3_13724726	AABB	B	B	A	B
RB_S_3_13754793	AABB	A	A	B	A
RB_S_3_13795019	AABB	A	A	B	A
RC3766-391_3_13878008	AAAA	A	A	A	A
RB_T_3_13881088	AAAA	A	A	A	A
RB_S_3_14024780	ABBB	B	B	B	A
RB_S_3_14146853	AABB	B	B	A	B
RB_S_3_14316165	ABBB	A	A	B	B
RB_T_3_14442011	AABB	A	A	B	A
RB_S_3_14521488	AABB	A	A	B	A
RB_S_3_14599590	ABBB	A	A	B	B
RB_T_3_15171728	BBBB	B	B	B	B
RB_T_3_15305145	AABB	A	A	B	A
RB_S_3_15309954	AABB	B	B	A	B
RB_S_3_15357433	ABBB	A	A	B	B
RB_S_3_15455662	AAAB	B	B	A	A

Table A3.1. Four progeny with *S*-genotypes generated from five parental crosses: (**1**) 25-14-20 × 27-03-08; (**2**) 25-14-20 × 27e-04-54; (**3**). 25-14-20 × 27e-05-33; (**4**). 25-14-20 × 27e-15-38; (**5**). 25-14-20 × 27e-16-47.The grey background column means the SI phenotypes of the progeny due to the presence of match *S*-functional or the absence of less than two non-functional S-haplotypes.

(**1**) 25-14-20 × 27e-04-54

$S_1S_6S_{36a}S_{36b}$ $S_{13m}S_{13'}S_{36a}S_{36a}$

	$S_{13m}S_{13'}$	$S_{13m}S_{36a}$	$S_{13m}S_{36a}$	$S_{13'}S_{36a}$	$S_{13'}S_{36a}$	$S_{36a}S_{36a}$
S_1S_6	$S_1S_6S_{13m}S_{13'}$	$S_1S_6S_{13m}S_{36a}$	$S_1S_6S_{13m}S_{36a}$	$S_1S_6S_{13'}S_{36a}$	$S_1S_6S_{13'}S_{36a}$	$S_1S_6S_{36a}S_{36a}$
S_1S_{36a}	$S_1S_{13m}S_{13'}S_{36a}$	$S_1S_{13m}S_{36a}S_{36a}$	$S_1S_{13m}S_{36a}S_{36a}$	$S_1S_{13'}S_{36a}S_{36a}$	$S_1S_{13'}S_{36a}S_{36a}$	$S_1S_{13'}S_{36a}S_{36a}$
S_1S_{36b}	$S_1S_{13m}S_{13'}S_{36b}$	$S_1S_{13m}S_{36a}S_{36b}$	$S_1S_{13m}S_{36a}S_{36b}$	$S_1S_{13'}S_{36a}S_{36b}$	$S_1S_{13'}S_{36a}S_{36b}$	$S_1S_{36a}S_{36a}S_{36b}$
S_6S_{36a}	$S_6S_{13m}S_{13'}S_{36a}$	$S_6S_{13m}S_{36a}S_{36a}$	$S_6S_{13m}S_{36a}S_{36a}$	$S_6S_{13'}S_{36a}S_{36a}$	$S_6S_{13'}S_{36a}S_{36a}$	$S_6S_{13'}S_{36a}S_{36a}$
S_6S_{36b}	$S_6S_{13m}S_{13'}S_{36b}$	$S_6S_{13m}S_{36a}S_{36b}$	$S_6S_{13m}S_{36a}S_{36b}$	$S_6S_{13'}S_{36a}S_{36b}$	$S_6S_{13'}S_{36a}S_{36b}$	$S_6S_{13'}S_{36a}S_{36b}$
$S_{36a}S_{36b}$	$S_{13m}S_{13'}S_{36a}S_{36b}$	$S_{13m}S_{36a}S_{36a}S_{36b}$	$S_{13m}S_{36a}S_{36a}S_{36b}$	$S_{13'}S_{36a}S_{36a}S_{36b}$	$S_{13'}S_{36a}S_{36a}S_{36b}$	$S_{36}S_{36a}S_{36a}S_{36b}$

(**2**). 25-14-20 × 27e-05-33

$S_1S_6S_{36a}S_{36b}$ $S_6S_{13'}S_{36a}S_{36b}$

	$S_6S_{13'}$	S_6S_{36a}	S_6S_{36b}	$S_{13'}S_{36a}$	$S_{13'}S_{36b}$	$S_{36a}S_{36b}$
S_1S_6	$S_1S_6S_6S_{13'}$	$S_1S_6S_6S_{36a}$	$S_1S_6S_6S_{36b}$	$S_1S_6S_{13'}S_{36a}$	$S_1S_6S_{13'}S_{36b}$	$S_1S_6S_{36a}S_{36b}$
S_1S_{36a}	$S_1S_6S_{13'}S_{36a}$	$S_1S_6S_{36a}S_{36a}$	$S_1S_6S_{36a}S_{36b}$	$S_1S_{13'}S_{36a}S_{36a}$	$S_1S_{13'}S_{36a}S_{36b}$	$S_1S_{36a}S_{36a}S_{36b}$
S_1S_{36b}	$S_1S_6S_{13'}S_{36b}$	$S_1S_6S_{36a}S_{36b}$	$S_1S_6S_{36b}S_{36b}$	$S_1S_{13'}S_{36a}S_{36b}$	$S_1S_{13'}S_{36b}S_{36b}$	$S_1S_{36a}S_{36b}S_{36b}$
S_6S_{36a}	$S_6S_6S_{13'}S_{36a}$	$S_6S_6S_{36a}S_{36a}$	$S_6S_6S_{36a}S_{36b}$	$S_6S_{13'}S_{36a}S_{36a}$	$S_6S_{13'}S_{36a}S_{36b}$	$S_6S_{36a}S_{36a}S_{36b}$
S_6S_{36b}	$S_6S_6S_{13'}S_{36b}$	$S_6S_6S_{36a}S_{36b}$	$S_6S_6S_{36b}S_{36b}$	$S_6S_{13'}S_{36a}S_{36b}$	$S_6S_{13'}S_{36b}S_{36b}$	$S_6S_{36a}S_{36b}S_{36b}$
$S_{36a}S_{36b}$	$S_6S_{13'}S_{36a}S_{36b}$	$S_6S_{36a}S_{36a}S_{36b}$	$S_6S_{36a}S_{36b}S_{36b}$	$S_{13}S_{36a}S_{36a}S_{36b}$	$S_{13'}S_{36a}S_{36b}S_{36b}$	$S_{36a}S_{36a}S_{36b}S_{36b}$

Table A3.1 (cont'd).

(**3**). 25-14-20 × 27e-15-38

	$S_1,S_6S_{36a}S_{36b}$			S_4S_{13},S_{13},S_{36a}		
	S_4S_{13},	S_4S_{13},	S_4S_{36a}	S_{13},S_{13},	S_{13},S_{36a}	S_{13},S_{36a}
S_1,S_6	$S_1,S_4S_6S_{13}$,	$S_1,S_4S_6S_{13}$,	$S_1,S_4S_6S_{36a}$	S_1,S_6S_{13},S_{13},	S_1,S_6S_{13},S_{36a}	S_1,S_6S_{13},S_{36a}
S_1,S_{36a}	S_1,S_4S_{13},S_{36a}	S_1,S_4S_{13},S_{36a}	$S_1,S_4S_{36a}S_{36a}$	$S_1,S_{13},S_{13},S_{36a}$	$S_1,S_{13}S_{36a}S_{36a}$	$S_1,S_{13},S_{36a}S_{36a}$
S_1,S_{36b}	S_1,S_4S_{13},S_{36b}	S_1,S_4S_{13},S_{36b}	$S_1,S_4S_{36a}S_{36b}$	$S_1,S_{13},S_{13},S_{36b}$	$S_1,S_{13},S_{36a}S_{36b}$	$S_1,S_{13},S_{36a}S_{36b}$
S_6S_{36a}	$S_4S_6S_{13},S_{36a}$	$S_4S_6S_{13},S_{36a}$	$S_4S_6S_{36a}S_{36a}$	S_6S_{13},S_{13},S_{36a}	$S_6S_{13},S_{36a}S_{36a}$	$S_6S_{13},S_{36a}S_{36a}$
S_6S_{36b}	S_4S_6 S_{13},S_{36b}	$S_4S_6S_{13},S_{36b}$	$S_4S_6S_{36a}S_{36b}$	S_6S_{13},S_{13},S_{36b}	$S_6S_{13},S_{36a}S_{36b}$	$S_6S_{13},S_{36a}S_{36b}$
$S_{36a}S_{36b}$	$S_4S_{13},S_{36a}S_{36b}$	$S_4S_{13},S_{36a}S_{36b}$	$S_4S_6S_{36a}S_{36b}$	S_6S_{13},S_{13},S_{36b}	$S_6S_{13},S_{36a}S_{36b}$	$S_6S_{13},S_{36a}S_{36b}$

(**4**). 25-14-20 × 27e-16-47

	$S_1,S_6S_{36a}S_{36b}$			$S_{13},S_{35}S_{36a}S_{36b}$		
	S_{13},S_{35}	S_{13},S_{36a}	S_{13},S_{36b}	$S_{35}S_{36a}$	$S_{35}S_{36b}$	$S_{36a}S_{36b}$
S_1,S_6	S_1,S_6S_{13},S_{35}	S_1,S_6S_{13},S_{36a}	S_1,S_6S_{13},S_{36b}	$S_1,S_6S_{35}S_{36a}$	$S_1,S_6S_{35}S_{36b}$	$S_1,S_6S_{36a}S_{36b}$
S_1,S_{36a}	$S_1,S_{13},S_{35}S_{36a}$	$S_1,S_{13},S_{36a}S_{36a}$	$S_1,S_{13},S_{36a}S_{36b}$	$S_1,S_{35}S_{36a}S_{36a}$	$S_1,S_{35}S_{36a}S_{36b}$	$S_1,S_{36a}S_{36a}S_{36b}$
S_1,S_{36b}	$S_1,S_{13},S_{35}S_{36b}$	$S_1,S_{13},S_{36a}S_{36b}$	S_1,S_{13} ,$S_{36a}S_{36b}$	$S_1,S_{35}S_{36a}S_{36b}$	$S_1,S_{35}S_{36b}S_{36b}$	$S_1,S_{36a}S_{36b}S_{36b}$
S_6S_{36a}	$S_6S_{13},S_{35}S_{36a}$	$S_6S_{13},S_{36a}S_{36a}$	$S_6S_{13},S_{36a}S_{36b}$	$S_6S_{35}S_{36a}S_{36a}$	$S_6S_{35}S_{36a}S_{36b}$	$S_6S_{36a}S_{36a}S_{36b}$
S_6S_{36b}	$S_6S_{13},S_{35}S_{36b}$	$S_6S_{13},S_{36a}S_{36b}$	$S_6S_{13},S_{36b}S_{36b}$	$S_6S_{35}S_{36a}S_{36b}$	$S_6S_{35}S_{36b}S_{36b}$	$S_6S_{36a}S_{36b}S_{36b}$
$S_{36a}S_{36b}$	$S_{13},S_{35}S_{36a}S_{36b}$	$S_{13},S_{36a}S_{36a}S_{36b}$	$S_{13},S_{36a}S_{36b}S_{36b}$	$S_{35}S_{36a}S_{36a}S_{36b}$	$S_{35}S_{36a}S_{36b}S_{36b}$	$S_{36a}S_{36a}S_{36b}S_{36b}$

Table A3.2. Chi square analysis for 43 individuals progeny population generated from five parental crosses and the 1:1 prediction ratio for individuals with *D1* haplotype and without *D1* haplotype.

Parental Crosses	Trait	Expected ratio	Observed (O)	Expected (E)	Deviation (O-E)	Deviation^2 (d)^2	X^2
25-14-20 × 27-03-08	With *D1*	1/2	222	200	22	484	2.42
	Without *D1*	1/2	178	200	-22	484	2.42
Total		1	400	400			$X^2 = 4.84$ $p = 0.03$
25-14-20 × 27e-04-54	With D1	1/2	59	45.5	13.5	182.25	4.05
	Without *D1*	1/2	32	45.5	-13.5	182.25	4.05
Total		1	91	91			$X^2 = 8.1$ $p = 0.004$
25-14-20 × 27e-05-33	With *D1*	1/2	10	9	1	1	0.11
	Without *D1*	1/2	8	9	-1	1	0.11
Total		1	18	18			$X^2 = 0.22$ $p = 0.6$
25-14-20 × 27e-15-38	With *D1*	1/2	12	13	1	1	0.07
	Without *D1*	1/2	14	13	-1	1	0.07
Total			26	26			$X^2 = 0.14$ $p = 0.7$
25-14-20 × 27e-16-47	With *D1*	1/2	55	42	13	169	4.02
	Without *D1*	1/2	29	42	-13	169	4.02
Total			84	84			$X^2 = 8.04$ $p = 0.04$

REFERENCES

REFERENCES

Ban, Y., Honda, C., Hatsuyama, Y., Igarashi, M., Bessho, H., and Moriguchi, T. (2007). Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. Plant Cell Physiol. 48(7): 958-970.

Beaver, J. and A. F. Iezzoni. (1993). Allozyes inheritance in tetraploid sour cherry (*Prunus cerasus* L.). J. Amer. Soc. Hort. Sci. 118: 873-877.

Chagne, D., Carlisle, C. M., Blond, C., Volz, R. K., Whitworth, C. J., Oraguzie, N. C., Crowhurst, R. N., Allan, A. C., Espley, R. V., Hellens, R. P., and Gardiner, S. (2007). Mapping a candidate gene (MdMYB10) for red and flesh and foliage color in apple. BMC Genomics. 8: 212.

Chandra, A., Nair, M. G., and A. Iezzoni. (1992). Evaluation and characterization of the anthocyanin pigments in tart cherries (*Prunus cerasus* L.). J. Agric. Food Chem. 40: 967-969.

de Nettancourt, D. 2001. Incompatibility and incongruity in wild and cultivated plants. Springer, Berlin, Germany.

Edge-Garza, D. A. and Peace, C. P. (2010). Enabling marker-assisted seedling selection in the Washington apple breeding program. In Bassil, N.V. and Martin, R. (eds). Proc. International Society for Horticultural Science. Acta Hort. 859: 369-375.

Espley, R. V., Hellen, R. P., J. Putterill, D. E. Stevenson, Kutty-Amm, S., and Allan, A. C. (2007). Red coloration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J. 55(6): 954-967.

Gjertson, D.W., Brenner, C.H., Baur, M.P., Carracedo, A., Guidet, F., Luque, J.A., Lessig, R., Mayr, W.R., Pascali, V.L., Prinz, M., Schneider, P.M., and Morling, N. (2007). ISFG: Recommendations on biostatistics in paternity testing. Forensic Sci. Int. Genet. 1: 223-231.

Hauck NR, Yamane H, Tao R, Iezzoni AF. (2002). Self-compatibility and incompatibility in tetraploid sour cherry (*Prunus cerasus* L.). Sexual Plant Reproduction. 15: 39–46.

Hauck, N. R., Ikeda, K., Tao, R., and Iezzoni, A. F. (2006a). The mutated S_1-haplotype in sour cherry has an altered *S*-haplotype specific F-box protein gene. J. Hered. 97:514-520.

Hauck, N. R., Yamane, H., Tao, R. and Iezzoni, A. F. (2006b). Accumulation of non-functional *S*-haplotypes results in the breakdown of gametophytic self-incompatibility in tetraploid *Prunus*. Genetics. 172: 1191-1198.

Hormaza, J.J. and Herrero, M. (1996). Male gametophytic selection as plant breeding tool. Scientia Horticulturae. 65: 321-333.

Hummer, K. H. and Janick, J. (2009). Rosaceae: taxonomy, economic importance and genomics. in Folta, K.M. and Gardiner, S.E. (eds.). Genetics and Genomics of Rosaceae, Plant Genetics and Genomics: Crops and Models 6. Springer Science. New York. DOI 10.1007/978-0-387-77491-6 1.

Iezzoni, A.F. (1988). 'Montmorency' sour cherry. Fruit Var. J. 42:74-75.

Iezzoni, A. F. (2005). Acquiring cherry germplasm from central and eastern Europe. HortScience. 40(2): 304-308.

Iezzoni, A. F. (2013). Jewels in the Genome. RosBREED Newsletter. 4(1): 9. (Available online at: www.rosbreed.org/sites/default/files/files/2013-3-RosBREED_Newsletter.pdf

Kumar, S., S. Gerber, T.E. Richardson, and L. Gea. 2007. Testing for unequal paternal contributions using nuclear and chloroplast SSR markers in polycross families of radiata pine. Tree Genet. Genomes 3:207–214.

Mulcahy, D.L., Mulcahy, G.B., Popp R., Fong, N., Pallais, N., Kalinowski, A. and Marien, J.N., 1988. Pollen selection for stress tolerance or the advantage of selecting before pollination. In: M. Cresti, P. Gori and E. Pacini (Editors). Sexual Reproduction in Higher Plants. Springer Berlin, pp. 43-50.

McClure, B., Cruz-Garcıa, F., and Romero, C. (2011). Compatibility and incompatibility in S-RNase-based systems. Annals of Botany 108: 647–658.

Olden, E.J. and Nybom, N. (1968). On the origin for *Prunus cerasus* L. Heriditas. 59:327-345.

Olmstead, J. W., Sebolt, A. M., Cabrera, A., Sooriyapathirana S.S., Hammar, S., Iriarte G., Wang, D., Chen, C. Y., van der Knaap, E., and Iezzoni, A.F. (2008). Construction of an intra-specific sweet cherry (*Prunus avium* L.) genetic linkage map and synteny analysis with the Prunus reference map. Tree Genetics & Genomes. 4:897–910

Peace, C. Bassil, N., Main, D., Ficklin, S., Rosyara, U. R., Stegmeir, T., Sebolt, A. M., Gilmore, B., Lawley, C., Mockler, T. C., Bryant, D. W., Wilhelm, L., 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.

Sebolt, A. M. and Iezzoni, A. F. 2009. Utilization of the S-locus as a Genetic Marker in Cherry to Differentiate Among Different Pollen Donors. Hortscience. 44(6):1542–1546.

Siddiq, M., Iezzoni, A., Khan, A., Breen, P., Sebolt, A. M., Dolan K. D., and Ravi, R. (2011). Characterization of new sour cherry *(Prunus cerasus* L.): selection based on fruit quality, total anthocyanin, and antioxidant capacity. International Journal of Food Properties. 14(2): 471-480.

Sonneveld, T, Tobutt, K. R., Vaughan, S. P., Robbins, T.P. (2005). Loss of pollen-S function in two self-compatible selections of *Prunus avium* is associated with deletion/mutation of an *S*-haplotype specific F-box gene. Plant Cell. 17:37–51.

Sooriyapathirana, S. S., Khan, A., Sebolt, A. M., Wang, D., Bushakra, J. M., Lin-Wang, K., Allan, A. C., Gardiner, S. E., Change, D., and Iezzoni, A. F. (2010). QTL analysis and candidate gene mapping for skin and flesh color in sweet cherry fruit (*Prunus avium* L.). Tree Genetics & Genomes. 6:821-832.

Stegmeir, T. (2013). Discovery of a QTL for cherry leaf spot resistance and validation in tetraploid sour cherry of QTLS for bloom time and fruit quality traits from diploid *Prunus* species. Dissertation. Michigan State University.

Stegmeir, T. Basundari, F. R. A., Cai, L., Sebolt, A. M., and Iezzoni. A. F. 201x. A DNA test for fruit flesh color in tetraploid sour cherry (*Prunus cerasus* L.). Submitted.

Takos, A.M., Jaffe, F. W., Jacob, S. R., Bogs, J., Robinson, S. P., and Walker, A. R. (2006). Light-induced expression of a MYB gene regulates anthocyanin biosynthesis in red apples. Plant Physiology. 142(3):1216-1232.

Tobutt KR, Boskovic R, Cerovic R, Sonneveld T, Ruzic D. (2004). Identification of incompatibility alleles in the tetraploid species sour cherry. Theoretical and Applied Genetics. 108: 775–785.

Tsukamoto T, Hauck N. R., Tao, R, Jiang, N, Iezzoni A. F. (2006). Molecular characterization of three non-functional *S*-haplotypes in sour cherry (*Prunus cerasus*). Plant Mol Biol. 62:371–383

Tsukamoto, T., Tao, R., and Iezzoni, A. F. (2008). PCR markers for mutated *S*-haplotypes enable discrimination between self-incompatible and self-compatible sour cherry selections. Mol. Breeding. 21:67-80.

Tsukamoto T, Hauck N. R., Tao, R, Jiang, N, Iezzoni A. F. (2010). Molecular and genetic analyses for nonfunctional S-haplotype variants derived from a common ancestral S-haplotype identified in sour cherry (*Prunus cerasus* L.). Genetics. 184: 411-427.

Verde I, A.G. Abbott, Scalabrin, S., Jung, S., Shu, S., Marroni, F., Zhebentyayeva, T., Dettori, M. T., Grimwood, J., Cattonaro, F., Zuccolo, A., Rossini, L., Jenkins, J., Vendramin, E. , Meisel, L. A., Decroocq, V., Sosinski, B., Prochnik, S., Mitros, T., Policriti, A., Cipriani, G., Dondini, L., Ficklin, S., Goodstein, D.M., Xuan, P., Del Fabbro, C., Aramini, V., Copetti, D., Gonzalez, S., Horner, D. S., Ralchi, R., Lucas, S., Mica, E., Maldonado, J., Lazzari, B., Bielenberg, D., Pirona, R., Miculan, M., Barakat, A., Testolin, R., Stella, A., Tartarini, S., Tonutti, P., Arús, P., Orellana, A., Wells, C., Main, D., Vizzotto, G., Silva, H., Salamini, F., Schmutz, J., Morgante, M., Rokhsar, D. S. (2013). The high-quality draft of peach (*Prunus persica* L.) identifies unique patterns of genetic diversity, domestication and genome evolution. Nature Genetics. 45: 487-494.

Wang, H., Nair, M. G., Iezzoni, A. F., Strasburg, G. M., Booren, A. M., and Gray J. I. (1997). Quantification and characterization of anthocyanins in balaton tart cherries. J. Agric. Food Chem. 45: 2556-2560.

Wang, X.-R., Torimaru, T., Lindgren, D., and Fries, A. (2010). Marker-based parentage analysis facilitates low input 'breeding without breeding' strategies for forest trees. Tree Genet. Genomes. 6: 227–235.

Yamane, H. and Tao, 2009. Molecular basis of self-(in)compatibility and current status of S-genotyping in Rosaceous fruit trees. J. Japan. Soc. Hort. Sci. 78(2): 137-137.

Yamane, H., Tao, R., Sugiura J., Hauck, N. R., and Iezzoni, A. F. (2001). Identification and Characterization of S-RNases in Tetraploid Sour Cherry (*Prunus cerasus*). Amer. Soc. Hort. Sci. 126 (6): 661–667.

Yamane H, Ikeda K, Hauck NR, Iezzoni AF, Tao R. 2003. Self-incompatibility (S) locus region of the mutated S6-haplotypes of sour cherry (*Prunus cerasus*) contains a functional pollen S-allele and a non-functional pistil *S*-allele. Journal of Experimental Botany. 54: 2431–2434.