Utilization of the *S*-locus as a Genetic Marker in Cherry to Differentiate Among Different Pollen Donors

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Abstract. Fruit set in sweet (*Prunus avium* L.) and sour cherry (*P. cerasus* L.) is frequently less than adequate for profitable production despite the availability of compatible pollen and abundant flowers. When fruit set consistently falls below acceptable levels, growers may attempt to increase fruit set by increasing the availability of compatible pollen. We describe the use of the self-incompatibility locus (*S*-locus) as a genetic marker to quantify the relative contributions of competing pollen sources in achieving fruit set in 'BalatonTM' sour cherry. Pollen race experiments were conducted to determine if nonself-pollen provided in a pollen mixture was more competitive than self-pollen in achieving fruit set in 'BalatonTM'. We further investigated what pollen set the 'BalatonTM' crop in two commercial 'BalatonTM' orchards where multiple potential pollinators were planted in adjacent orchards. *S*-allele genotyping using DNA extracted from the seed was done to discriminate among the competing pollen sources. The results suggest that in certain environmental conditions, nonself-pollen may be more competitive in achieving fruit set in 'BalatonTM' than self-pollen. These examples illustrate how seed genotyping can be used to further our understanding of the competitive abilities of different pollen sources in both controlled experiments and production orchards.

Sweet cherry and some sour cherry cultivars exhibit gametophytic self-incompatibility controlled by multiple alleles at the *S*-locus (de Nettancourt, 2001; Lansari and Iezzoni, 1990). Knowledge of the *S*-locus genotype and the resulting compatibility groups is used to identify cross-compatible cultivars for field planting (Matthews and Dow, 1969). Yet, despite the availability of compatible pollen and abundant flowers, fruit set in both sweet and sour cherry is frequently less than adequate resulting in poor yields and unprofitable production (Furukawa and Bukovac, 1989; Kuhn, 1988). For example, despite being self-compatible, the sour cherry cultivar BalatonTM frequently sets less than 10% of its flowers resulting in low yields per hectare. When fruit set consistently falls below acceptable levels, growers may attempt to increase fruit set by increasing the availability of compatible pollen. This can be accomplished by inter-planting with an additional pollenizer cultivar or use of commercially available pollen inserts. In these cases, the impacts on fruit set of the supplemental pollen sources cannot be individually quantified, yet it is

conceivable that the pollen sources would not equally contribute to fruit set.

The *S*-locus was selected as the genetic marker because over 35 different *S*-alleles have been identified in cherry to date (Bošković and Tobutt, 2001; Choi et al., 2000; Hauck et al., 2001; Sonneveld et al., 2001; Tao et al., 1999; Tsukamoto et al., 2006, 2008a, 2008b; Vaughan et al., 2008; Wiersma et al., 2001; Wünsch and Homaza, 2004). In addition, molecular markers that distinguish among these allelic variants have been designed to take advantage of the intron and sequence polymorphisms among the two completely linked genes that defined the *S*-locus, e.g., the stylar component *S*-ribonuclease (*S*-*RNase*) and pollen-component F-box protein (*SFB*) (Sonneveld et al., 2003; Tsukamoto et al., 2008b; Vaughan et al., 2008).

Herein, we demonstrate the use of the *S*-locus as a genetic marker in cherry to determine the relative fertilization success of competing pollen sources. In the first example, pollen race experiments were conducted to determine if nonself-pollen provided in a pollen mixture was more competitive than self-pollen in achieving fruit set in 'BalatonTM'. In the second example, what pollen set the 'BalatonTM' crop was investigated in two commercial 'BalatonTM' orchards where multiple potential pollinators were planted in adjacent orchards.

Materials and Methods

Pollen race experiment. Pollen samples from three sour cherry cultivars, BalatonTM,

Montmorency, and Cigany, and one sweet cherry cultivar, Sam, were collected and dried at room temperature using standard procedures (Table 1). Pollen collection was replicated two times to provide pollen sources to be used at two different locations and tested for adequate germination using established protocols (Brown et al., 1996). Approximately 600 'BalatonTM' flowers were emasculated in grower orchards at two locations in Michigan: Shelby and Northport. Equal quantities (weight) of pollen were combined to provide the following pollen mixtures: 'BalatonTM' + 'Montmorency', 'BalatonTM' + 'Sam', 'BalatonTM' + 'Montmorency' + 'Sam', and 'BalatonTM' + 'Cigany'. After emasculation, pollen from each mixture was applied to ≈150 stigmas per treatment using a glass rod. In July, a total of 285 fruit resulting from the pollination treatments were collected for *S*-allele genotyping of their seeds.

Fruit set in commercial orchards. Two commercial 'BalatonTM' orchards were chosen that had several potential pollenizer cultivars planted in adjacent rows. 'BalatonTM' Orchard A was bordered on the west side by two rows of the sweet cherry cultivars Emperor Francis and Schmidt (Fig. 1). To the south, a poplar wind break separated the 'BalatonTM' orchard from an orchard of the sour cherry cultivar Montmorency. 'BalatonTM' Orchard B was bordered on the west by a 21-row 'Montmorency' orchard. West of the 'Montmorency' orchard were six rows of six different sweet cherry cultivars (Fig. 1). Due to the relatively late bloom time of 'BalatonTM', all potential pollenizer cultivars were in bloom before 'BalatonTM'. For both Orchards A and B, fruit was harvested at maturity from six 'BalatonTM' trees in close proximity to the sweet cherry rows and six trees in rows distal to the sweet cherry rows. A total of 107 and 150 seeds were collected from Orchards A and B, respectively. The flesh was removed from the seeds in preparation for DNA extraction and *S*-allele genotyping. The *S*-allele genotypes of all the bordering sweet cherry cultivars had previously been reported (Table 1).

DNA extraction and *S*-allele genotyping. The endocarp (pit) from each fruit was cracked and the seed removed. Next, the maternally derived testa was peeled off the exterior of the cherry seed and the remaining embryo and cotyledons were ground and used for DNA extraction following the procedure of Hauck et al. (2006). The *S*-*RNase* gene-specific primer pair Pru-C2 and PCE-R (Tao et al., 1999; Yamane et al., 2001) was used for *S*-allele determination because it can differentiate among most *S*-*RNase* alleles based on length differences for the second intron in the *Prunus S*-*RNase*. However, the *S*₂- and *S*_{13m} alleles could not be reliably amplified using this primer pair; therefore, the primer pair PaS2-F and PaS2-R was used to identify *S*₂ (Sonneveld et al., 2003) and the primer pair PcS13-F and PcS13-R was used to identify *S*_{13m}

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(Tsukamoto et al., 2008b). Polymerase chain reaction (PCR) conditions were as previously described (Yamane et al., 2001) and the PCR products were separated on a 2% agarose gel in 1× TAE buffer.

Results

In the pollen race experiments, we tested whether nonself-pollen would be more competitive in the ‘Balaton™’ styles than self-pollen and used the *S*-locus as a genetic marker to discriminate among the competing pollen sources. Successful fertilization of ‘Balaton™’ by ‘Cigany’ pollen was determined by the presence of two of the following four ‘Cigany’ *S*-alleles in the seed: *S*_{6m2}, *S*₉, *S*₂₆, and/or *S*_{36b2} (Table 1). Successful fertilization of ‘Balaton™’ with ‘Montmorency’ pollen was determined by the presence of two of the following three *S*-alleles in the seed because ‘Montmorency’ pollen containing the *S*₃₅ allele would be incompatible: *S*₆, *S*_{13m}, and/or *S*_{36a}. In sour cherry, the match of one or two functional *S*-alleles in the 2x pollen with an *S*-allele in the style results in incompatibility (Hauck et al., 2006). Finally, successful fertilization by ‘Sam’ sweet cherry pollen was determined by the presence of an *S*₂ in the seed because the *S*₄ pollen from ‘Sam’ would be incompatible in a ‘Balaton™’ style.

When the mixture of ‘Balaton™’ and ‘Montmorency’ pollen was applied to ‘Balaton™’ styles, 89 seeds were obtained (Table 2). In Orchard 1, ‘Montmorency’ pollen was just slightly more competitive than ‘Balaton™’ pollen contributing to 53% and 47% of the fruit set, respectively. In Orchard 2, ‘Montmorency’ pollen was overwhelmingly more competitive than ‘Balaton™’ pollen resulting in 93% versus 7% successful fertilizations. When the mixture of ‘Balaton™’ and ‘Sam’ pollen was applied to the ‘Balaton™’ styles, 64 seed were obtained (Table 2). In both orchards, ‘Sam’ pollen was more competitive than ‘Balaton™’ pollen and resulted in 67% of the successful fertilizations. When a mixture of three pollen types, ‘Balaton™’, ‘Montmorency’, and ‘Sam’, was applied to ‘Balaton™’ styles, 53 seed were obtained (Table 2). The percentages of successful fertilizations by each pollen type were 43% (‘Balaton™’), 21% (‘Montmorency’), and 36% (‘Sam’). When a mixture of ‘Balaton™’ and ‘Cigany’ pollen was applied to ‘Balaton™’ styles, 79 seed were obtained (Table 2). The vast majority of the seeds (92%) resulted from fertilization with ‘Cigany’ pollen. For all pollen types, ‘Balaton™’ pollen was less competitive than nonself-pollen in Orchard 2 compared with Orchard 1. Collectively, these results demonstrate that outcross pollen can be more competitive than ‘Balaton™’ pollen in achieving fruit set on ‘Balaton™’. ‘Cigany’ pollen was the most successful at achieving fertilization on ‘Balaton™’ followed by ‘Sam’ and ‘Montmorency’ pollen. All nonself-pollen types were more competitive in Orchard 2 suggesting that environmental factors that

Table 1. *S*-allele genotypes of sweet and sour cherry selections used in the pollination experiments.

Cultivar	<i>S</i> -allele genotype ^z	Pollen type(s) compatible with Balaton™
<i>Sour cherry</i>		
Balaton™	<i>S</i> ₁ <i>S</i> ₄ <i>S</i> ₃₅ <i>S</i> _{36b}	<i>S</i> ₁ <i>S</i> _{36b}
Cigany	<i>S</i> _{6m2} <i>S</i> ₉ <i>S</i> ₂₆ <i>S</i> _{36b2}	<i>S</i> _{6m2} <i>S</i> ₉ , <i>S</i> _{6m2} <i>S</i> ₂₆ , <i>S</i> _{6m2} <i>S</i> _{36b2} , <i>S</i> ₉ <i>S</i> ₂₆ , <i>S</i> ₉ <i>S</i> _{36b2} , <i>S</i> ₂₆ <i>S</i> _{36b2}
Montmorency	<i>S</i> ₆ <i>S</i> _{13m} <i>S</i> ₃₅ <i>S</i> _{36a}	<i>S</i> ₆ <i>S</i> _{13m} , <i>S</i> ₆ <i>S</i> _{36a} , <i>S</i> _{13m} <i>S</i> _{36a}
<i>Sweet cherry</i>		
Cavalier	<i>S</i> ₂ <i>S</i> ₃	<i>S</i> ₂ , <i>S</i> ₃
Emperor Francis	<i>S</i> ₃ <i>S</i> ₄	<i>S</i> ₃
Gold	<i>S</i> ₃ <i>S</i> ₆	<i>S</i> ₃ , <i>S</i> ₆
Hedelfingen	<i>S</i> ₃ <i>S</i> ₅	<i>S</i> ₃ , <i>S</i> ₅
Sam	<i>S</i> ₂ <i>S</i> ₄	<i>S</i> ₂
Schmidt	<i>S</i> ₂ <i>S</i> ₄	<i>S</i> ₂

^z*S*-allele genotypes for sour cherry are from Tsukamoto et al. (2008a, 2008b). *S*-allele genotypes for sweet cherry are from Bošković and Tobutt (1996, 2001), Hauck et al. (2001), and Wiersma et al. (2001).

Fig. 1. Percentage of selected ‘Balaton™’ fruit pollinated with a pollen source other than ‘Balaton™’. R = row; T = tree. Percentages inside the circles represent the percent of ‘Balaton™’ fruit sampled that resulted from nonself-pollen.

influence fertilization and fruit set were more favorable to outcross pollen.

The pollen race experiments suggested that nonself-pollen can be more competitive in 'Balaton™' styles than self-pollen. If this were the case, it would be expected that some of the fruit set on 'Balaton™' in commercial blocks would result from nonself-pollen transferred by bees from other cherry cultivars in neighboring orchards. The percentage of nonself-pollen provided by neighboring cultivars that contributed to fruit set in 'Balaton™' was investigated in two commercial orchards by determining the *S*-allele genotypes of a sample of seeds. As in the pollen race experiments, successful fertilization of 'Balaton™' with 'Montmorency' pollen was determined by the presence of the following *S*-alleles in the seed because 'Montmorency' pollen containing the *S*₃₅ allele would be incompatible: *S*₆*S*_{13m}, *S*₆*S*_{36a}, or *S*_{13m}*S*_{36a}. Successful fertilization of 'Balaton™' by the sweet cherry cultivars Cavalier, Emperor Francis, Gold, Hedelfingen, Sam, or Schmidt was determined by the presence of an '*S*₆', '*S*₅', '*S*₃', or '*S*₂' allele, because the '*S*₄'-containing pollen would be incompatible (Table 1).

'Balaton™' Orchard A was bordered to the south by a 'Montmorency' orchard and to the west by a planting of two rows of two different sweet cherry cultivars, Emperor Francis and Schmidt (Fig. 1). A total of 107 seeds were sampled from 12 trees; six trees were from two rows adjacent to the sweet cherry rows and six trees were from two distal rows. When the results were averaged across the 12 'Balaton™' trees, 79% of the 'Balaton™' fruit resulted from self-pollination and 21% of the fruit resulted from cross-pollination. The percentages of fruit set with nonself-pollen were not significantly different between the six trees adjacent and the six trees distal to the sweet cherry rows, e.g., 23.1% and 19.8%, respectively, most likely as a result of the similar proximity of the 'Balaton™' trees to the 'Montmorency' orchard. However, there was a wide variation between individual trees in the percentage of 'Balaton™' fruit set with nonself-pollen. For example, 67% of the fruit on the sixth tree in Row 1 resulted from outcross pollen com-

pared with no fruit resulting from outcross pollen for the fifth tree in Row 2. It is possible that these widely different findings may reflect differences in the paths of the bee pollinators. All three potential non-'Balaton™' pollen sources were found to contribute to the 21% fruit set attributed to nonself-pollen. The percentage contributions to total fruit set for 'Montmorency', 'Schmidt', and 'Emperor Francis' were 11%, 6%, and 4%, respectively.

'Balaton™' Orchard B was bordered to the west by 21 'Montmorency' rows followed by six rows of six different sweet cherry cultivars (Fig. 1). A total of 150 'Balaton™' fruit were collected from 12 trees as described for Orchard A. When fruit set from all 12 trees was averaged, 62% of the fruit resulted from self-pollination and 38% resulted from nonself-pollen. In contrast to Orchard A, there was a significant difference in the percentage of fruit set with nonself-pollen based on proximity to the other cherry cultivars located on the west border ($P < 0.05$) (SAS Institute, 1999). The six trees adjacent to the 'Montmorency' rows had an average of 48% of their fruit set with nonself-pollen versus 23% for the six trees at the east side of the plot. *S*-allele genotyping confirmed the contribution of 'Montmorency' pollen to achieving fruit set in 'Balaton™' because 30% of the fruit evaluated resulted from 'Montmorency' pollination. By comparison, 2% of the fruit resulted from 'Sam' pollen, whereas 4% of the fruit resulted from one of the sweet cherry cultivars that have the *S*₃ allele, either 'Emperor Francis' or 'Gold'. An additional differential marker such as a polymorphic simple sequence repeat (SSR) marker would need to be used to distinguish among these two possibilities. *S*₆ pollen from 'Gold' contributed to only 1% of the fruit set.

Discussion

Determining the biological basis of poor fruit set is frequently difficult because multiple factors are required for successful fruit set. These include 1) pollen availability at the onset of stigma receptivity; 2) successful transfer of genetically compatible pollen to the stigma; 3) pollen adherence to and ger-

mination on the stigma; 4) pollen tube growth in the style; 5) sufficient ovule longevity; 6) fertilization of at least one of the two ovules present in the *Prunus* megagametophyte; and 7) at least initial zygote viability. In cherry, poor fruit set is likely the result of deficiencies in a combination of these factors. For example, in individual 'Balaton™' flowers, the stigma is receptive before pollen dehiscence (A. Iezzoni, unpublished data). Therefore, the contribution of pollen from the earlier blooming sweet cherry cultivars and 'Montmorency' to fruit set in 'Balaton™' could in part be due to increased pollen availability at the onset of stigma receptivity. In addition, in a parallel project we compared fruit set between open-pollinated 'Balaton™' flowers with and without supplemental hand pollination in twelve orchards totaling over 15,000 flowers. This supplemental pollination increased percent fruit set across all orchards by an average of 14% (data not presented). This suggests that insufficient timely pollen transfer to the stigma is also contributing to the low fruit set in commercial orchards.

Our finding that non-'Balaton™' pollen can achieve a higher percent fruit set when competing with self-pollen could also be due to an increased growth rate of non-'Balaton™' pollen. In particular, an increased rate of pollen tube growth may result in more successful fertilizations because the pollen tubes reach the ovules before the ovules degenerate. For example, cherry ovules that were not fertilized became progressively senescent four to five days after anthesis, and about a week after flower opening a high percentage of ovules were not viable (Stosser and Anvari, 1982). Ovule longevity has also been shown to differ among sweet cherry cultivars with 'Schmidt' having a short period of ovule longevity compared to 'Bing' and 'Windsor' (Eaton, 1959, 1962). Therefore the availability of pollen at the onset of stigma receptivity and an accelerated rate of pollen tube growth may be especially important for cultivars that have a short period of ovule longevity. Finally, the competitive ability of the non-'Balaton™' pollen could instead result from enhanced zygote survival due to the absence of inbreeding

Table 2. Numbers and percentages of 'Balaton™' fruit that were set with 'Balaton™' pollen in competition with pollen from other sour and sweet cherry selections.

Pollen types	Orchard	No. of fruit from controlled pollinations	No. (%) of fruit set from Bal. pollen	No. (%) of fruit set from Mont. pollen	No. (%) of fruit set from Sam pollen	No. (%) of fruit set from Cigany pollen
Bal. + Mont.	1	62	29 (47%)	33 (53%)	—	—
	2	27	2 (7%)	25 (93%)	—	—
	Mean	Total = 89	31 (35%)	58 (65%)	—	—
Bal. + Sam	1	45	20 (44%)	—	25 (56%)	—
	2	19	1 (5%)	—	18 (95%)	—
	Mean	Total = 64	21 (33%)	—	43 (67%)	—
Bal. + Mont. + Sam	1	39	22 (56%)	7 (18%)	10 (26%)	—
	2	14	1 (7%)	4 (29%)	9 (64%)	—
	Mean	Total = 53	23 (43%)	11 (21%)	19 (36%)	—
Bal. + Cigany	1	58	6 (10%)	—	—	52 (90%)
	2	21	0 (0%)	—	—	21 (100%)
	Mean	Total = 79	6 (8%)	—	—	73 (92%)
Bal + other		Total = 285	81 (28%)			

Bal. = 'Balaton™'; Mont. = 'Montmorency'.

depression. Additional studies would be needed to determine the relative importance of these multiple factors.

The pollen race experiments and analysis of “what set the crop” illustrate how seed genotyping can be used to differentiate among different pollen sources in both controlled experiments and production orchards. For example, Hedhly et al. (2004) compared the pollen tube growth rate for two sweet cherry cultivars of Spanish and Canadian origins, and found that the Spanish cultivar had a higher optimum temperature for pollen tube growth. The ability to use seed genotyping to determine the successful pollen donor would allow this finding to be validated in the orchard. In addition, in commercial orchards where growers have provided multiple pollinators whether by inter-planting different pollinators, or providing pollen inserts, seed genotyping can be used to quantify the relative contributions of the different pollen sources to fruit set. In those cases where the *S*-locus alone is insufficient to genetically distinguish among the array of pollen sources, additional DNA markers can be utilized. For example, an extensive number of SSR markers and dCAPs markers are available from linkage mapping studies in sweet cherry (Olmstead et al., 2008; Clarke et al., 2009).

The use of the *S*-locus as a genetic marker to distinguish among different pollen donors was illustrated using a sour cherry cultivar as the target cultivar (e.g., ‘Balaton™’) and two sour cherry cultivars as two of the of the pollen donors (e.g., ‘Montmorency’ and ‘Cigany’). The two *S*-alleles in sour cherry 2x pollen increases the complexity of the genetic analysis compared with an analysis using only sweet cherry selections. Thus, in sweet cherry, *S*-locus results are more easily interpreted. As an example, one could have evaluated the relative success of ‘Rainier’ (*S*₇*S*₄) versus ‘Gold’ (*S*₃*S*₆) pollen in achieving fruit set on ‘Emperor Francis’ (*S*₃*S*₄). As the *S*₄ and *S*₃ pollen types from ‘Rainier’ and ‘Gold’, respectively, are incompatible on ‘Emperor Francis’, successful ‘Rainier’ pollen would be identified by the presence of an *S*₇ in the seed and successful ‘Gold’ pollen would be identified by an *S*₆ in the seed. The four *S*-*RNase* alleles in this example can be differentiated using the Pru-C2 and PCE-R primer pair (Tao et al., 1999; Yamane et al., 2001) (Fig. 2).

We have used this sweet cherry example to develop a wet laboratory exercise entitled “What pollen source set the crop?” targeting horticultural students to illustrate the application of PCR and gel electrophoresis to address real-world horticulture problems. In the first part of the laboratory exercise, the students performed PCR using genomic DNA previously extracted from ‘Emperor Francis’, ‘Gold’, and ‘Rainier’ and ‘Emperor Francis’ seeds that were collected from the orchard. In the second session, the students separated the PCR products on an agarose gel, stained the gel, and interpreted their results. For example, an analysis of the gel image in Figure 2, revealed that seven

Fig. 2. Agarose gel image of the *S*-*RNase* alleles from the “What set your crop?” laboratory exercise. ‘Emperor Francis’ fruit could either have resulted from fertilization by an *S*₇ or *S*₆ containing pollen grain from ‘Rainier’ or ‘Gold’, respectively. The *S*-*RNase* alleles are amplified with the Pru-C2 and PCE-R primer pair (Tao et al., 1999; Yamane et al., 2001).

‘Emperor Francis’ seeds resulted from pollination with ‘Rainier’ pollen, whereas three seeds resulted from pollination with ‘Gold’ pollen. The students collectively compiled their results to test the hypothesis that the ‘Gold’ and ‘Rainier’ pollen were not equally successful in achieving fruit set in ‘Emperor Francis’. This provides a real-life example of how the growing body of genomics and genetics information can have an impact in production horticulture.

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