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Genetic and molecular characterization of three novel S-haplotypes in sour cherry (Prunus cerasus L.)

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Abstract
Tetraploid sour cherry (Prunus cerasus L.) exhibits gametophytic self-incompatibility (GSI) whereby the specificity of self-pollen rejection is controlled by alleles of the stylar and pollen specificity genes, S-RNase and SFB (S haplotype-specific F-box protein gene), respectively. As sour cherry selections can be either self-compatible (SC) or self-incompatible (SI), polyploidy per se does not result in SC. Instead the genotype-dependent loss of SI in sour cherry is due to the accumulation of non-functional S-haplotypes. The presence of two or more non-functional S-haplotypes within sour cherry 2x pollen renders that pollen SC. Two new S-haplotypes from sour cherry, S33 and S34, that are presumed to be contributed by the P. fruticosa species parent, the complete S-RNase and SFB sequences of a third S-haplotype, S35, plus the presence of two previously identified sweet cherry S-haplotypes, S14 and S16 are described here. Genetic segregation data demonstrated that the S14, S33, S34, S35-haplotypes present in sour cherry are fully functional. This result is consistent with our previous finding that ‘hetero-allelic’ pollen is incompatible in sour cherry. Phylogenetic analyses of the SFB and S-RNase sequences from available Prunus species reveal that the relationships among S-haplotypes show no correspondence to known organismal relationships at any taxonomic level within Prunus, indicating that polymorphisms at the S-locus have been maintained throughout the evolution of the genus. Furthermore, the phylogenetic relationships among SFB sequences are generally incongruent with those among S-RNase sequences for the same S-haplotypes. Hypotheses compatible with these results are discussed.

Key words: Prunus cerasus, self-incompatibility, SFB, S-RNase.

Introduction
Gametophytic self-incompatibility (GSI) is a common genetic mechanism that promotes outcrossing in flowering plants (de Nettancourt, 2001). In GSI, self-incompatibility (SI) is determined by a single multi-allelic locus, called the S-locus, which contains a minimum of two genes, one (stylar-S) controlling stylar specificity and the other (pollen-S) controlling pollen specificity of the SI reaction. The stylar-S gene in three plant families, the Solanaceae, Plantaginaceae, and Rosaceae encodes a ribonuclease (S-RNase; Anderson et al., 1986; McClure et al., 1989; Sassa et al., 1992; Xue et al., 1996), which is expressed in the pistil and specifically degrades the RNA of incompatible pollen (McClure et al., 1990). The pollen-S gene encodes an F-box protein named S-locus F-box protein (SLF) in Antirrhinum (Lai et al., 2002), Petunia inflata (Sijacic et al., 2004), and Prunus mume (Entani et al., 2003), or S haplotype-specific F-box protein (SFB) in Prunus dulcis, P. mume, P. avium, P. spinosa, and P. cerasus (Ushijima et al., 2003; Yamane et al., 2003b; Ikeda et al., 2004a; Nunes et al., 2006). Despite having similar or even identical names in Solanaceae and Prunus the pollen gene is not orthologous (Wheeler and Newbiggin, 2007).

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Abbreviations: GSI, gametophytic self-incompatibility; SI, self-incompatible; SC, self-compatible; SFB, S-haplotype-specific F-box protein.
Within *Prunus* (Rosaceae), cherry represents a natural diploid–tetraploid series with the tetraploid sour cherry (*P. cerasus*) arising through hybridization between sweet cherry (*P. avium*) and the tetraploid ground cherry (*P. fruticosa*) (Olden and Nybom, 1968). Like sweet cherry, sour cherry exhibits an S-RNase-based GSI system (Yamane et al., 2001; Hauck et al., 2002; Tobutt et al., 2004), however, in contrast to sweet cherry, natural sour cherry selections include both SI and self-compatible (SC) types (Redalen, 1984; Lansari and Iezzoni, 1990). This genotypic-dependent loss of SI in sour cherry indicates that genetic changes, and not polyploidy per se, cause the breakdown of SI. The genetic switch from SI to SC in sour cherry results from the accumulation of non-functional S-haplotypes according to the ‘one-allele-match model’ (Hauck et al., 2006b). In this model, the match between a functional pollen-S in the 2x pollen and its cognate functional S-RNase in the style, results in an incompatible reaction. A similar reaction would occur regardless of whether the pollen contained a single functional pollen-S gene or two different pollen-S genes. The absence of any functional match results in a compatible reaction. Thus for successful self-fertilization, the absence of any functional match results in a compatible reaction in a SC selection of tetraploid *Prunus* pseudocerasus, raising the possibility that the SC mechanism between these two tetraploid *Prunus* species could be different. However, although the data in Huang et al. (2008) is consistent with hetero-allelic pollen being SC, homo-allelic pollen (e.g. *S1S1*, *S5S5*, or *S1S2*) was not shown to be successful in a compatible cross and unsuccessful in an incompatible cross. Therefore, it is possible that the SC in *P. pseudocerasus* could be caused by mutations in other genes critical for the SI reaction.

Six S-haplotypes present in sweet cherry (*S1*, *S3*, *S5*, *S9*, *S12*, and *S13*) have been shown to be present in sour cherry as well. However, three of these S-haplotypes (*S1*, *S5*, and *S13*) have also non-functional variants in sour cherry that have lost pollen and/or stylar function (Yamane et al., 2003a; Hauck et al., 2006a, b; Tsukamoto et al., 2006). Loss of function in these non-functional S-haplotypes was due to structural alterations of the S-RNase, SFB or S-RNase upstream sequences. Sour cherry also possesses S-haplotypes (*S26*, *S36a*, *S36b*, *S36c2*, and *S36d2*) that were presumably derived from the other species parent, *P. fruticosa*, as these S-haplotypes have not been identified in sweet cherry (Hauck et al., 2006b; T Tsukamoto et al., unpublished data). The extensive sour cherry germplasm collection at Michigan State University (Iezzoni, 2005) provides an excellent resource for the identification of previously undiscovered S-haplotypes for which information about their functionality would aid in the breeding of SC types. Therefore a germplasm survey was undertaken to search for novel S-haplotypes in sour cherry and to determine the functionality of these S-haplotypes. Here two new S-haplotypes identified in sour cherry are described that are presumed to be contributed by the *P. fruticosa* species parent. The first complete S-RNase and SFB sequences for a third S-haplotype that was previously identified in sour cherry are also reported. Furthermore, genetic segregation data presented demonstrates that all three of these sour cherry S-haplotypes are fully functional. In addition, the *S14* and *S16* haplotypes have been identified in sour cherry. Phylogenetic analyses of S-RNase and SFB sequences are used to investigate patterns of evolution of S-haplotypes in *Prunus*.

### Materials and methods

#### Plant material

One sweet cherry cultivar ‘Hedelfingen’ (*S5S5*) and 17 sour cherry cultivars were used: ‘Cigány 59’, ‘Crisana’, ‘Englaisse Timpurii’, ‘Erdi Botermo’, ‘Erdi Jubileum’, ‘Erdi Nagyyumolcsu’, ‘Meteor’, ‘Montmorency’, ‘Pandy 38’, ‘Pandy 114’, ‘Rheinische Schattenmorelle’, ‘Surefire’, ‘Tamaris’, ‘Targina’, ‘Tschermovorka’, ‘Újeřehrtói fürtős’, and ‘MSU III 18 (12)’. These cultivars were grown at the Michigan State University Clarksville Horticultural Experimental Station, Clarksville, MI, USA and a commercial orchard in Suttons Bay, MI, USA. To determine the inheritance of the S-haplotypes, self-pollinated progeny of ‘Meteor’, ‘Montmorency’ and ‘Tamaris’ plus cross-pollinated progeny between ‘Újeřehrtói fürtős’ × ‘Surefire’ were obtained.

#### DNA isolation

Young unfolded leaves were collected in the spring, frozen in liquid nitrogen, lyophilized, and stored at –20 °C. Genomic DNA was isolated from lyophilized leaves according to the method of Ikeda et al. (2004b). Extracted leaf DNA was treated with RNase A (Roche, Mannheim, Germany). To genotype the self-pollinated progeny of ‘Meteor’, ‘Montmorency’, and ‘Tamaris’, DNA was extracted from mature seed from which the testa was removed using the procedure of Hauck et al. (2006b). Extracted seed DNA was treated with RNase A (Roche).

#### PCR amplification

Total DNA was isolated from the cherry selections and used as template DNA for PCR. PCR procedures were identical to those used by Tao et al. (1999). The S-RNase gene specific primer set, Pru-C2 and PCE-R (Tao et al., 1999; Yamane et al., 2001) that correspond to the previously identified C2 and C3 conserved regions (Ushijima et al., 1998), respectively, were used. This primer pair can differentiate among most S-RNase alleles based on polymorphisms in the length of the second intron in the *Prunus* S-RNase. However, this primer pair cannot amplify *S35*-RNase. EM-PC2consFD and EM-PC5consRD (Sutherland et al., 2004) were used to amplify S35-RNase. In addition, the primer pair of Pru-C2 and PCE-R cannot differentiate *S36a* from *S36b*, *S36c2*, and *S36d2*. Instead, these variants of the *S36a*-haplotype were differentiated using the following primer pairs for detection of the *S36a*-haplotype [PcS36ab-F (5’-GCTAGCAAACACCTTACATCACG-3’) and PcS36a-spR (5’-GGATCCACCATGATAACAACTG-3’)] and detection of the *S36b*, *S36c2*, and *S36d2*-haplotypes [PcS36ab-F and PcS36b-spR (5’-ATACATTTGAGGCCAGTCTGTG-3’)]. PCR products were run on 2% agarose gels and the DNA bands were visualized by
ethidium bromide staining. Furthermore, PCR products were purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA), and transformed Escherichia coli JM109 (Promega). Plasmid DNA was prepared using Wizard Plus Minipreps DNA Purification Kit (Promega) and their sequences were determined as described below.

**Construction and screening of genomic libraries**

Fosmid libraries were constructed using the Copy Control Fosmid library production kit (Epigenet Technology Support Facility, Madison, WI, USA). Fosmid libraries from ‘Meteor’, ‘Montermorey’, and ‘Tamaris’ were constructed to clone the S-RNase and SFB alleles from the S-haplotypes being investigated. The fosmid libraries were screened at 60 °C with a mixture of DIG-dUTP-labelled SfbR consensus primer and SFBR probes, as previously described (Ushijima et al., 2001). The DIG-labelled SfbR consensus and SFBR probes were obtained by PCR-labelling using the PCR DIG Probe Synthesis Kit (Roche) with Pru-C2 and PCE-R primers (Tao et al., 1999; Yamane et al., 2001), and SFB-C1F (Ikeda et al., 2004a) and SFB-C4R primers (Yamane et al., 2003c). Fosmid DNAs from positive clones were prepared using the Wizard Plus Minipreps DNA Purification Kit (Promega). The S-haplotypes of each positive fosmid clone were determined by PCR with the S-RNase consensus primer pair (Pru-C2 and PCE-R). Positive clones were also analysed by PCR with the SFB consensus primer pair (SFB-C1F and SFB-C2R) (Ikeda et al., 2004a) to check if the clone has SFB, S-RNase and SFB allele-specific primer pairs were also used to identify the S-haplotypes. For the S35-haplo (S351, S352, S353) positive clones were obtained by using a DIG-dUTP-labelled S35-RNase probe. Then more positive clones obtained by hybridization with a mixture of DIG-dUTP-labelled SFBR and SFBR probes were subjected to PCR with the SFB consensus primer (SFB-C1F and SFB-C2R) to confirm that the clones contain SFB. Then positive clones were analysed by PCR with SFB allele-specific (SFBg, SFB1g, and SFB16g/SFB2b) primer pairs: PaSFB6-F and PaSFB6-R (Ikeda et al., 2005), DdeS13-F (Tsukamoto et al., 2008) and SFB13-spR (Tsukamoto et al., 2006), and PsSFB36b-F (5’-GGCGGTCC-ATCTCTGATACGAC-3’) and PsSFB36b-R (5’-GTGCGCGAATACTGACGGC-3’), respectively. The positive clones from which a fragment could be amplified with the primer pair SFB-C1F and SFB-C2R, but not amplified with SFBg, SFB1g, and SFB16g/SFB2b-specific primers, were determined to contain SFB1g and one positive clone was sequenced.

**DNA sequencing**

DNA sequencing was carried out by using ABI PRISM 3100 Genetic Analyser at the Michigan State University Research Technology Support Facility. The plasmid clones were sequenced by using SP6 and T7 primers. The fosmid clones were sequenced by primer walking using Pru-T2, Pru-C2, Pru-C2R, PCE-F, PCE-R, Pru-C4R, and Pru-C5 (Tao et al., 1999; Yamane et al., 2001; Tsukamoto et al., 2006) for S-RNase, and SFB-C1F, SFB-C2R, SFBR-C5F, and FBR3 (Ikeda et al., 2005) for SFB. The EM-PC5consRD primer (Sutherland et al., 2004) was also used to sequence S35-RNase.

**Phylogenetic and variability analysis**

For phylogenetic analyses, available nucleotide sequences for S-RNase and SFB from Prunus armeniaca, P. avium, P. cerasus, P. domestica, P. dulcis, P. nana, P. salicina, P. spinosa, and P. tenella S-haplotypes were assembled (see Fig. 7). Only sequences whose predicted amino acid sequences covered at least 75% of the average length of available complete sequences of S-RNase (227 amino acids) and SFB (375 amino acids) from the two cherry species (P. avium and P. cerasus) were included. Nucleotide sequences were translated into deduced amino acid sequences using DAMBE (Xia and Xie, 2001); the amino acid sequences were then aligned using ClustalX (Thompson et al., 1997) and adjusted manually. Finally, nucleotide sequences were aligned to the amino acid alignments with DAMBE. Analyses of DNA variability were performed using DnaSP 4.1 (Rozas et al., 2003). Phylogenetic analyses of the aligned amino acid and nucleotide sequences based on maximum parsimony were implemented in PAUP* (Swofford, 2002) with heuristic searches using the TBR branch-swapping algorithm and 1000 random taxon addition replicates and maxtrees allowed automatic increases as necessary. Relative support for clades was assessed using 1000 bootstrap replicates with 10 random taxon addition replicates per bootstrap replicate and maxtrees set at 100.

Several approaches were used to test for significant incongruence between the topologies of phylogenetic trees supported by the SFB and S-RNase data sets, respectively, and all of these approaches were used for both the amino and the nucleotide sequences for each gene. First, a combined data set was constructed including only the 49 haplotypes for which sequences of both determinants were available. The partition homogeneity test, implemented in PAUP* with 1000 test replicates and heuristic searches using the TBR branch-swapping algorithm and 10 random taxon addition replicates per test replicate with maxtrees set to 100, was used to test for significant conflict between the S-RNase and SFB partitions within each data set. Second, each partition was analysed separately and all of the most parsimonious trees were saved to a single tree file. The Kishino-Hasegawa (K-H), Templeton, and winning-sites tests were implemented in PAUP* to test whether or not the topologies produced by the two data partitions were significantly different. Third, because bootstrap analyses showed that many relationships were only weakly supported by each of the two data partitions, constraint trees were constructed in which only groups supported with bootstrap values of 80% or more by each of the data partitions were resolved. Four such constraint trees were constructed; one each for SFB and S-RNase for the combined amino acid data set and one each for SFB and S-RNase for the combined nucleotide data set. Each data partition was analysed without constraints and the best trees were saved to a file. Then each data partition was analysed under the constraint corresponding to the well-supported groups from the other partition and the best trees were saved to the same file. The K-H, Templeton, and winning-sites tests were implemented in PAUP* to test whether or not the constrained trees were significantly longer than the unconstrained trees. In order to test whether or not the phylogenetic relationships among SFB and S-RNase alleles were significantly different from the phylogenetic relationships among species of Prunus based on other evidence, each data set was re-analysed with various topological constraints enforced (see Results). For each data set, all of the most parsimonious trees (MPT) from unconstrained and constrained analyses were saved to a single tree file. The K-H, Templeton, and winning-sites tests were implemented in PAUP* to test whether or not the constrained trees were significantly longer than the unconstrained trees.

**Results**

**Identification and cloning of novel S-haplotype genes (S-RNase and SFB) from sour cherry**

Genomic PCR with the S-RNase consensus genes (S-RNase and SFB) from sour cherry

Prunus cerasus (Tao et al., 1999; Yamane et al., 1999).
revealed three amplification products not previously characterized in three sour cherry cultivars (Fig. 1A). Fragments of ~ 480 bp, ~ 420 bp, and ~ 850 bp were identified in ‘Englaise Timpurii’, ‘Meteor’, and ‘Tamaris’, respectively.

The nucleotide sequence of the 481 bp S-RNase PCR product from ‘Englaise Timpurii’ revealed high similarity (99.6%) with the P. avium S14-RNase partial sequence (GenBank accession no. AJ635277; Sonneveld et al., 2003) and the P. avium S23-RNase complete sequence (GenBank accession no.AY259114; Wünsch and Hormaza, 2004; the two sequences are identical). The ‘Englaise Timpurii’ 481 bp S-RNase nucleotide sequence differed from the P. avium S14-/S23-RNase by only two base pairs within the second intron (data not shown). Therefore, the 481 bp PCR S-RNase product from ‘Englaise Timpurii’ was considered to be the S14-RNase, as the P. avium S14- and S23-RNases likely code the same specificity.

BlastN of the 424 bp partial nucleotide sequence of the S-RNase PCR product from ‘Meteor’ revealed high homology (98.3%) with the partial S10-RNase of Japanese apricot (P. mume) sequence (GenBank accession no. DQ011150; sequence upstream of the C2 conserved region and downstream of the RC4 conserved region is lacking). This novel S-haplotype was named S33 to follow the previously named functional cherry S-haplotypes (S1 to S7, S9, S10, S12 to S14, S16 to S32) (for a review see Vaughan et al., 2008).

Two S33 clones were obtained by screening a ‘Meteor’ fosmid library. Both clones contained the S-haplotype genes, S33-RNase and SFB33, and the complete nucleotide sequences of the S33-RNase (GenBank accession no. EU054325) and SFB33 (GenBank accession no. EU054328) were obtained by sequencing the genes from these two clones. The predicted amino acid sequence of the S33-RNase consisted of 238 residues and was aligned with that of functional S-RNases present in sour cherry (Fig. 2). When the nucleotide sequences of the coding regions of the sour cherry S33-RNase and P. mume S10-RNase were compared, there were four synonymous and one non-synonymous differences. In the second intron, there were four nucleotide differences plus two indels (148 bp and 1 bp long, respectively; see Supplementary Fig. 1 at JXB online).

SFB33 consisted of 376 amino acid residues that exhibited the characteristic F-box and variability patterns of previously identified SFBs (Fig. 3). S33-RNase and SFB33 specific primer pairs were designed (Table 1) that amplified the allele specific fragments of 819 bp and 860 bp, respectively (Fig. 4A, B).

BlastN with the 868 bp partial nucleotide sequence of the S-RNase PCR product from ‘Tamaris’ revealed high homology with the S1-RNase obtained from sweet cherry (GenBank accession no. AB031815), the P. tenella S8-RNase (GenBank accession no. DQ983367), and partial sequences of the P. dulcis S14-RNase (GenBank accession no. EU054324).
no. AM231660) and the *P. domestica S*R-Nase (GenBank accession no. AM746946). This novel S-haplotype was named *S*$_{34}$ (Fig. 5; Table 2). Sour cherry *S*$_{34}$-RNase falls in this previously described group of *S*-RNases where the sweet cherry *S*$_{1}$-RNase was found to be identical to the *P. tenella S*$_{8}$-RNase and to differ from the *P. dulcis S*$_{11}$-RNase by just one amino acid (Šurbanovski et al., 2007).

One *S*$_{34}$ clone was obtained by screening a 'Tamaris' fosmid library. This clone contained both of the *S*$_{34}$-RNase and *SFB*$_{34}$ permitting the complete nucleotide sequencing of the *S*$_{34}$-RNase (GenBank accession no. EU054326) and *SFB*$_{34}$ (GenBank accession no. EU054329). The predicted amino acid sequence of *S*$_{34}$-RNase consisted of 226 residues and was aligned with that of functional *S*-RNases obtained from sour cherry cultivars (Fig. 2). A comparison of the *S*$_{34}$-RNase with a partial sequence of the *P. domestica S*$_{5}$-RNase (PdoS$_{5}$-RNase lacks 30 and 27 amino acids at the N- and C-terminals, respectively) revealed high homology (97%) and only five amino acid differences (Fig. 5). When *PcS*$_{34}$-RNase was compared with *P. avium S$_{1}$-RNase, 15 amino acid differences were identified (Fig. 5). Nevertheless, the sweet cherry *S*$_{1}$-RNase specific primer pair (Sonneveld et al., 2001) amplified a product for the *S*$_{34}$-RNase. However, the amplification product from the *S*$_{34}$-RNase was the expected size of 850 bp instead of 615 bp as for the *P. avium S*$_{1}$-RNase (data not shown). The second intron of the *S*$_{34}$-RNase is 238 bp longer than that of the *P. avium S*$_{1}$-RNase (GenBank accession nos AB031815 and AB028153; see Supplementary Fig. 2 at *JXB* online).

*SFB*$_{34}$ was composed of 376 amino acid residues that exhibited the characteristic variability patterns of previously identified SFBs (Fig. 3). Due to the high homology of the *S*$_{34}$-RNase with the *P. avium S*$_{1}$-RNase, an alignment was performed with their respective SFBs. The predicted amino acid sequence of *P. cerasus SFB*$_{34}$...
was aligned with three cherry SFBI sequences: (i) the *P. cerasus* SFBI obtained from ‘Pandy 114’ (GenBank accession no. DQ827715), (ii) the *P. avium* SFBI cloned from ‘Skeena’ (GenBank accession no. AY805048), and (iii) the *P. avium* SFBI sequence obtained from ‘Seneca’ (GenBank accession no. AB111518) and *P. dulcis* SFBI11, and *P. tenella* SFBI8 (Fig. 6). The *P. cerasus* ‘Pandy 114’ SFBI, and *P. avium* cv. ‘Skeena’ SFBI1 sequences are identical, however the ‘Skeena’ sequence is not complete as 10 amino acid residues at the C-terminal are not determined. The amino acid sequence of the ‘Seneca’ SFBI is slightly different from that of SFBI1 of ‘Pandy 114’ and ‘Skeena’ (Fig. 6). Unlike their S-RNases, the predicted amino acid sequences for the sour cherry SFBI34...
and SFB1 differed by 69 amino acids (Fig. 6) and shared only 81.6% identity (Table 2). Similarly, the amino acid sequence of the *P. cerasus* SFB34 had only 81.0% homology with that of the identical *P. tenella* SFB8 and *P. dulcis* SFB11. By comparison, the *P. cerasus* SFB34 and the partial sequence of the *P. domestica* SFB5 (GenBank accession number. AM746955; PdoSFB5 is lacking 12 and 33 amino acid residues at the N- and C-terminals, respectively) differed at 15 amino acid residues (95.5% homology), compared with 69 different residues between SFB34 and SFB1 (Fig. 6).

To compare the S34 and SFB-haplotypes further, a partial sequence of the intergenic region between *S-RNase* and *SFB* of *P. cerasus* S34 was obtained. However, the intergenic sequence for the S34-haplotype was extremely divergent compared with that from the *P. avium* S1, *P. dulcis* S11, and *P. tenella* S8 and did not permit a conclusive alignment (see Supplementary Fig. 3 at JXB online).

To aid in the confirmation of the *S*34-haplotype in future germplasm surveys, *S34-RNase* and *SFB34* specific primer pairs were designed (Table 1) that amplified allele specific fragments of expected size (898 bp and 714 bp, respectively) (Fig. 4C). The *S1-RNase* specific primer pair (Sonneveld et al., 2001) amplified fragments for the *S1-RNase* (~615 bp) and *S34-RNase* (~850 bp) (data not shown), but the *S34-RNase* specific primer pair only amplified a product from the *S34-RNase*, not the *S1-RNase* (Fig. 4C).

To identify all the *S*-haplotypes in ‘Tamaris’, genomic PCR was performed with all available *S-RNase* specific primer pairs. A fragment of ~ 680 bp was amplified with the *S10-RNase* specific primer pair (Sonneveld et al., 2003). A comparison of the nucleotide sequence from the *S10-RNase* PCR product from ‘Tamaris’ with the *P. avium* *S10-RNase* revealed only one base pair difference in the second intron. Therefore ‘Tamaris’ was considered to have the *S10*-haplotype.

**Identification and cloning of the S35-RNase and SFB35**

Previously an *S*-haplotype survey in sour cherry cultivars was carried out using RFLP analysis, and *S-RNase* based PCR was also carried out using the consensus *S-RNase* gene-specific primer set, Pru-C2 and PCE-R (Tao et al., 1999; Yamane et al., 2001). Using these two approaches, only three different *S*-haplotypes were characterized in many cultivars, including the landrace sour cherry cultivar ‘Pandy’ (syn. ‘Köröser’, ‘Crisana’) and ‘Montmorency’. Yet, genetic studies with many of these cultivars led to the conclusion that none of the three *S*-haplotypes identified in each cultivar was present in a double dose (Hauk et al., 2006b). For example, the cultivar ‘Újfehértói fűrtös’ has the *S*-haplotypes *S1, S4, and S6b* of which *S1* and *S6b* are non-functional. Therefore, it is possible that ‘Újfehértói fűrtös’ could contain two copies for either of these two non-functional *S*-haplotypes. However, our genetic segregation data was consistent in rejecting this hypothesis (Hauk et al., 2006a, b). Therefore, it is postulated that these cultivars contained a fourth allele and termed it *Snull* as it was not possible to resolve this fourth allele on a Southern blot with either *S-RNase* or *SFB* probes.

Bošković et al. (2006) reported the presence of a different *S-RNase*, *S*D, in ‘Köröser’, ‘Montmorency’, and ‘Bruine Waalse’ using a different *S-RNase* consensus primer pair (EM-PC2consFD and EM-PC5consRD). Therefore, this alternate primer pair was used to test the possibility that *S*D might be the *Snull* allele. Using this primer pair, a ~ 530 bp amplification product was amplified in eight selections (‘Crisana’, ‘Erdi Botermo’, ‘Montmorency’, ‘Pandy 38’, ‘Pandy 114’, ‘Surefire’, ‘Tscherhokorka’, and ‘Újfehértói fűrtös’; Fig. 1B), that had a similar size to the *S*D-*RNase* (Bošković et al., 2006). The 527 bp *S-RNase* PCR products amplified with the EM-PC2consFD and EM-PC5consRD primers from ‘Crisana’, ‘Pandy 114’, and ‘Újfehértói fűrtös’ were cloned and sequenced and the nucleotide sequences were

| Target gene | Primer name | Primer sequence 5’→3’ | Annealing temperature (°C) | Extension time (s) | Product size (bp) |
|-------------|-------------|------------------------|----------------------------|-------------------|------------------|
| *S33*-RNase | PcS33-F     | CACAGTTGCAAGAAATGC     | 66                         | 60                | 819              |
|             | PcS33-R     | ATOTGGCCACCTTGGTCCG    | 63                         | 60                | 860              |
|             | PcSFB33-F   | TCATTCACACTATGAAAGAGAT | 63                         | 60                | 802              |
|             | PcSFB33-R   | CAAAGTCTATTITTTTCATGCC | 63                         | 60                | 802              |
| *S34*-RNase | PcS34-F     | GTAATTGCAATGGGTCAAAAT | 66                         | 60                | 898              |
|             | PcS34-R     | CAGCCACATGGTCGAGGACT   | 66                         | 60                | 898              |
| *SFB33*    | PcSFB34-F   | TGTAGCATACGTTAACAGG    | 66                         | 60                | 802              |
|             | PcSFB34-R   | CACTCTCCACACAGGATAGTGTC | 66                        | 60                | 714              |
| *S35*-RNase | PcS35-F     | GACCCGAATTAGCAATGTGTT  | 66                         | 60                | 435              |
|             | PcS35-R     | GAGGCACTGCTAAATTGGAAG  | 66                         | 60                | 435              |
| *SFB35*    | PcSFB35-F   | AGCAATCAACGAAAATGCTTTC | 66                         | 60                | 557              |
|             | PcSFB35-R   | ATGCAAAACGAAATTCATTGCG | 66                         | 60                | 557              |

* Product size is based on the use of genomic DNA as a template.
identical. Unfortunately, these nucleotide sequences could not be compared with that of SD-RNase since the latter is not available in GenBank. Nevertheless, a comparison using the amino acid sequence available in Fig. 2 of Boskovic et al. (2006) revealed that our sequence differed from SD-RNase in two regions (see Supplementary Fig. 4 at JXB online). One region is just before the first intron. The SD-RNase has two additional glycine residues not present in our S-RNase sequence. The other region is the RHV region just after the second intron. The reasons for these discrepancies are not known. However, as the validity of our full length S-RNase sequences were verified multiple times from fosmid clones from three different genotypes (see below), and genetically verified...
in inheritance studies (see below), it has been tentatively postulated that our sequence indeed represents the previously identified S_{D}-haplotype. This S-haplotype is named S_{35}.

To clone the S_{35}-RNase and SFB_{35}, a fosmid library of 'Montmorency' (S_{6S13mS35S36a}) was first screened using a probe of DIG-dUTP-labelled with a 527 bp S_{35}-RNase fragment amplified by EM-PC2consFD and EM-PC5consRD from 'Crisana'. Sixteen positive clones were obtained. Among them, two clones (Mon37 and Mon46) were shown to contain the S_{35}-RNase since an 530 bp fragment was amplified by PCR with the S-RNase consensus primer pair (EM-PC2consFD and EM-PC5consRD) but no fragment was obtained with the other S-RNase consensus primer pair (Pru-C2 and PCE-R).

Unfortunately these two clones did not contain the SFB_{35}.

### Table 2. Identities of the derived amino acid sequences among the cherry S-locus genes whose complete sequence are available

The upper half presents amino acid sequence identities (%) between cherry SFBs; the lower half between the S-RNases. The sequence identity in a parenthesis was calculated by using partial amino acid sequence. n.a. means that sequence data is not available in GenBank. Pa, *Prunus avium*; Pc, *P. cerasus*. The sequences used are as follows; PaSFB 1 (AY805048 and DQ983372), PaSFB 2 (AB111519), PaSFB 3 (AB096857), PaSFB 4 (AB111521), PaSFB 5 (AB111520), PaSFB 6 (AB096858), PaSFB 7 (EU035976), PaSFB 8 (DQ422809), PaSFB 9 (AY805054), PaSFB 12 (DQ385844), PaSFB 13 (EU035977), PcSFB 26 (EU035975), PcSFB 33 (EU054328), PcSFB 34 (EU054329), PcSFB 35 (EU054330), PaS-RNase (AB028153), PaS-RNase (AB010304), PaS-RNase (AB010306), PaS-RNase (AB028154), PaS-RNase (AJ298314), PaS-RNase (AY259115), PaS-RNase (AY259114), PaS-RNase (AY259112), PaS-RNase (AY259113), PcS-RNase (EU035975), PaS-RNase (EU054325), PaS-RNase (EU054326), and PaS-RNase (EU054327).

| PaS1 | PaS2 | PaS3 | PaS4 | PaS5 | PaS6 | PaS7 | PaS9 | PaS12 | PaS13 | PaS23 | PaS24 | PaS25 | PaS26 | PaS33 | PaS34 | PaS35 |
|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| 76.6 | 76.8 | 77.6 | 75.3 | 76.0 | 75.4 | 77.1 | (73.2) | 77.1 | n.a. | n.a. | n.a. | 77.3 | 76.3 | 81.6 | 70.6 |
| 84.3 | 77.5 | 77.5 | 80.1 | 78.3 | 78.1 | (75.8) | 75.5 | n.a. | n.a. | n.a. | 80.4 | 80.4 | 73.3 | 73.9 |
| 73.9 | 77.6 | 77.5 | 80.1 | 78.3 | 78.1 | (75.8) | 75.5 | n.a. | n.a. | n.a. | 80.4 | 80.4 | 73.3 | 73.9 |
| 81.4 | 78.7 | 82.1 | 78.1 | 79.7 | (79.1) | 79.2 | n.a. | n.a. | n.a. | 81.0 | 80.9 | 78.7 | 74.8 |
| 75.4 | 74.0 | 76.9 | 80.5 | 77.9 | (75.7) | 76.7 | n.a. | n.a. | n.a. | 81.7 | 77.7 | 73.9 | 72.3 |
| 80.1 | 73.5 | 78.6 | 73.2 | 77.3 | 80.5 | (79.9) | 79.3 | n.a. | n.a. | n.a. | 81.1 | 80.5 | 79.9 | 73.7 |
| 77.9 | 70.4 | 77.7 | 78.5 | 81.7 | 79.4 | (74.6) | 76.2 | n.a. | n.a. | n.a. | 79.4 | 76.8 | 75.1 | 71.9 |
| 80.5 | 79.7 | (72.3) | 75.2 | 78.5 | 81.7 | (74.6) | (76.4) | 81.3 | n.a. | n.a. | 79.5 | 79.8 | 77.1 | 74.6 |
| 79.4 | 73.5 | 71.1 | 70.4 | 78.9 | 79.8 | (73.7) | (73.9) | n.a. | n.a. | n.a. | (76.2) | (79.5) | (74.3) | (72.1) |
| 81.9 | 72.1 | 76.0 | 71.4 | 77.9 | 74.3 | (76.7) | 74.1 | n.a. | n.a. | n.a. | 80.9 | 77.7 | 76.3 | 73.5 |
| 79.2 | 71.3 | 78.6 | 75.4 | 81.3 | 79.5 | (75.4) | 76.8 | 73.5 | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| 75.7 | 72.9 | 76.2 | 69.2 | 92.8 | 77.2 | (77.6) | 74.1 | 77.8 | 75.9 | n.a. | n.a. | n.a. | n.a. | n.a. |
| 77.8 | 76.9 | 72.5 | 73.7 | 73.7 | 73.7 | (79.5) | 75.6 | 69.1 | 75.4 | 69.1 | n.a. | n.a. | n.a. | n.a. |
| 75.9 | 78.1 | 69.3 | 75.3 | 71.9 | 78.9 | 78.5 | (78.8) | 72.4 | 75.3 | 73.7 | 78.0 | 68.5 | n.a. | 78.1 | 77.9 | 74.3 |
| 68.1 | 81.1 | 71.0 | 74.5 | 68.2 | 75.6 | 77.2 | (72.0) | 71.8 | 67.9 | 73.1 | 76.6 | 73.4 | 72.0 | – | 82.7 | 74.1 |
| 93.4 | 82.7 | 72.6 | 80.1 | 73.2 | 80.1 | 78.7 | (79.7) | 79.4 | 79.6 | 78.3 | 75.7 | 78.4 | 74.6 | 70.1 | – | 72.7 |
| 58.3 | 57.1 | 58.3 | 54.7 | 59.4 | 58.1 | 59.1 | (58.6) | 61.2 | 59.0 | 56.6 | 57.9 | 59.3 | 56.8 | 59.6 | 55.7 | – |

Fig. 5. The deduced amino acid sequence alignment for the *Prunus cerasus* S_{34}-RNase (PcS34-RNase), *P. domestica* S_{5}-RNase (PdS5-RNase), *P. avium* S_{5}-RNase (PaS1-RNase), *P. dulcis* S_{11}-RNase (PdS11-RNase), and *P. tenella* S_{x}-RNase (PtS8-RNase). The asterisks indicate the five amino acid residues that are different between *P. cerasus* S_{34}-RNase and *P. domestica* S_{5}-RNase. Conserved nucleotides are shown on a darkened background. The five conserved regions, C1, C2, C3, RC4, and C5 (Ushijima et al., 1998) are marked with solid boxes, and the hypervariable region, RHV (Ushijima et al., 1998) reported in the rosaceous S-RNases, is marked with a dotted box.
since the SFB band was not amplified by PCR with the SFB consensus primer pair (SFB-C1F and SFB-C2R). Next the ‘Montmorency’ fosmid library was screened again with a mixture of DIG-dUTP-labelled SFB6 and SFB36a probes at lower stringency (55°C/17°C) and obtained 25 positive clones. Among them, six clones (Mon64, Mon71, Mon74, Mon76, Mon122, and Mon132) were considered to contain the novel SFB since these six clones amplified a SFB fragment by PCR with the SFB consensus primer (SFB-C1F and SFB-C2R) but not with allele specific primer pairs for SFB6, SFB13, and SFB36a/SFB36b. This novel SFB was named SFB35. These six clones were also analysed by PCR with the S-RNase primer pair EM-PC2consFD and EM-PC5consRD, but no fragment was amplified from any of the six clones. Therefore these six clones were considered to contain SFB35 but not to contain S35-RNase. This suggests that the intergenic distance between the S35-RNase and SFB35 is larger than that observed in the majority of cherry S-haplotypes (380 bp to 38 kb) identified to date (Ikeda et al., 2005) as the average insert size of our fosmid clones was 40 kb. Mon37 and Mon64 were sequenced to determine the complete nucleotide sequences of the S35-RNase (GenBank accession no. EU054327) and SFB35 (GenBank accession no. EU054330), respectively. The S35-RNase, which consists of 232 amino acid residues (Fig. 2), is very different from other S-RNases identified in cherry. The second intron of S35-RNase is extremely short, consisting of only 82 bp (see Supplementary Fig. 4 at JXB online). The S-RNase consensus primer pair (Pru-C2 and PCE-R) could not amplify S35-RNase because the Pru-C2 primer was designed based on the amino acid
sequence LWPSNYSN and the S_{35}-RNase has LWPSNYSK. The PCE-R primer was designed based on the amino acid sequence EXEWNK, but the deduced amino acid sequence for S_{35}-RNase is GREWKNK.

SFB_{35} is composed of 371 amino acid residues that exhibited the characteristic variability patterns of previously identified SFBs (Fig. 3). Using these sequences, S_{35}-RNase and SFB_{35} specific primers were designed (Table 1). The S_{35}-RNase specific primer pair amplified a fragment of the expected size (435 bp) in all eight selections from which the 527 bp PCR product was amplified with the EM-PC2consFD and EM-PC5consRD primer pair (Fig. 4E). The SFB_{35} specific primer pair also amplified fragments of the expected size (557 bp) in all eight selections that had a 527 bp fragment following amplification using the EM-PC2consFD and EM-PC5consRD primer pair (Fig. 4F). It was further confirmed that the ~560 bp fragment was amplified by the SFB_{35} specific primer pair in all six fosmid clones (Mon64, Mon71, Mon74, Mon76, Mon122, and Mon132) (data not shown).

**S-haplotype functionality**

The functionality of the S-haplotypes identified in sour cherry was tested using populations derived from self- and cross-pollination. In the parents of these populations the S-haplotype being tested would segregate in both the eggs and pollen grains in an expected 1:1 ratio assuming that these gametes are viable and equally probable of occurring in a successful gamete. Therefore, if the S-haplotype is functional in the style and pollen, the pollen that contains that S-haplotype would be incompatible and the ratio of that S-haplotype would be 1:1 to represent its expected ratio in the eggs. However, if the S-haplotype being tested has lost either pollen or stylar function, the pollen carrying that S-haplotype would be compatible and that S-haplotype would be expected to be present in the progeny in a 3:1 ratio representing the additional contribution of the pollen S-haplotype. Using this strategy, multiple functional and non-functional S-haplotypes were previously identified in sour cherry (Hauck et al., 2006b).

To determine if the S_{35}-haplotype in ‘Meteor’ was fully functional, the self-pollinated progeny of ‘Meteor’ were genotyped for their S-haplotypes. The progeny segregation ratio for S_{35} fit the expected 1:1 ratio and rejected the 3:1 ratio indicating that S_{35} is a functional S-haplotype (Table 3). Self-pollinated progeny of ‘Tamaris’ also segregated according to a 1:1 rejecting the 3:1 ratio for the S_{34}-haplotype indicating that this S-haplotype is functional (Table 3). As ‘Tamaris’ is the first report of the presence of the sweet cherry S_{16}-haplotype in sour cherry, the functionality of this S-haplotype was also tested. S_{16} segregated according to a 1:1 ratio indicating that the S_{16}-haplotype is also functional.

S_{35} was identified in several cultivars with diverse geographic origin; therefore, three different cultivars with diverse origins were used to test the functionality of S_{35}. Segregation of S_{35} in the self-pollinated progeny of ‘Montmorency’ fit a 1:1 ratio and rejected the 3:1 ratio indicating that S_{35} is a functional S-haplotype in this cultivar (Table 3). As S_{35} is also present in ‘Ujfehértói fürtős’ and ‘Surefire’ it allowed us to test whether the S_{35} containing pollen from ‘Surefire’ is compatible in ‘Ujfehértói fürtős’ styles. The segregation of S_{35} in the progeny from this cross segregated according to the 1:1 ratio, rejecting the 3:1 ratio confirming that S_{35} is a functional S-haplotype.

‘Montmorency’, ‘Ujfehértói fürtős’, and ‘Surefire’ had previously been determined to have the Snull-haplotype as our genetic data predicted the presence of a fourth S-haplotype (Hauck et al., 2006b). To test the hypothesis that Snull is the newly identified S_{35}, the progeny from self-pollinated ‘Montmorency’ and ‘Ujfehértói fürtős’×‘Surefire’ were S-genotyped with the addition of

| Table 3. Progeny segregation of the S_{16}, S_{33}, S_{34} and S_{35}-haplotypes to test the functionality of each S-haplotype |
|---------------------------------------------------------------|
| Population                                              | S-haplotype tested | Expected ratio<sup>a</sup> | Observed ratio + : – | Chi square | P-value |
|---------------------------------------------------------------|
| ‘Meteor’ (S_{13m}S_{35}S_{16}S_{36}b) self-pollinated   | S_{35}             | 3:1                          | 43 : 40             | 0.108      | 0.7419  |
| ‘Tamaris’ (S_{4}S_{35}S_{34}S_{36}b) self-pollinated    | S_{16}             | 3:1                          | 43 : 40             | 28.81      | <0.0001 |
| ‘Montmorency’ (S_{5}S_{13m}S_{35}S_{36}b) self-pollinated| S_{35}             | 3:1                          | 43 : 38             | 0.309      | 0.5785  |
| ‘Ujfehértói fürtős’ (S_{1}S_{35}S_{34}S_{36}b) self-pollinated | S_{35}     | 3:1                          | 43 : 38             | 20.74      | <0.0001 |
| ‘Surefire’ (S_{5}S_{13m}S_{35}S_{36}b) self-pollinated  | S_{35}             | 3:1                          | 43 : 52             | 1.241      | 0.2652  |

<sup>a</sup> A 1:1 ratio is expected if the S-haplotype is fully functional. Therefore the progeny would only be able to obtain this S-haplotype from the egg. A 3:1 ratio is expected if the S-haplotype is fully functional as this S-haplotype could also be contributed to the progeny through the pollen.
the $S_{35}$. In all cases $S_{35}$ co-segregated with the previous prediction of $S_{null}$ (data not presented) confirming that $S_{35}$ is the fourth $S$-haplotype in these selections.

**Phylogenetic analyses**

Phylogenetic analyses of amino acid and nucleotide sequences of $S$-RNase and SFB sequences from eight species of *Prunus* (Fig. 7) produced trees in which the closest relatives of many alleles were alleles from other species. This pattern, first reported for Solanaceae species, was named trans-specific evolution by Richman *et al.* (1996). In *Prunus* this pattern has been described before (see references in Vieira *et al.*, 2008), but in contrast with the observation made for the Solanaceae species, in this genus trans-specific evolution cannot be taken as evidence for the very old age of alleles (Vieira *et al.*, 2008). Although this pattern has been known for more than a decade, no proper phylogenetic analyses have so far been performed to show conclusively that this pattern is not simply due to lack of phylogenetic resolution. In *Prunus*, bootstrap support values are generally weak. Nevertheless, in no case were all the alleles of any one gene from any one species supported as monophyletic.

Phylogenetic analyses of nucleotide sequences of both *S*-*RNase* and *SFB* data resolved the *P. cerasus* $S_{35}$ along with the *P. dulcis* $S_a$ as highly divergent from the remaining $S$-haplotypes (Fig. 7). Therefore, $S_{35}$ was identified as not only divergent from other cherry $S$-haplotypes but also divergent from other *Prunus* $S$-haplotypes. For the *P. cerasus* $S_{35}$ and *P. cerasus* $S_{34}$, as well as for the alleles from several other $S$-haplotypes, the resolved relationships were different in the two data sets (Fig. 7). All data sets strongly supported the sister relationship between *P. cerasus* $S_{34}$ and *P. domestica* $S_5$. Both the amino acid and the nucleotide sequence data resolved the *S-RNases* of the last two haplotypes as sister to a clade including the *P. avium* $S_1$-RNase, the *P. dulcis* $S_{11}$-RNase, and the *P. tenella* $S_9$-RNase. The *SFB* data did not, however, show this relationship, and the position of *P. cerasus* $S_{34}$- and *P. domestica* $SFB_5$ was very weakly supported. The *P. cerasus* $S_{34}$-RNase was weakly supported as sister to *P. mume* $S_7$-RNase and *P. spinosa* $S_{10}$-RNase; however, *P. cerasus* $SFB_3$ was resolved, again with very weak support, as sister to the clade of *P. avium* $SFB_3$, *P. cerasus* $SFB_{34}$, and *P. domestica* $SFB_5$. The relationships resolved by the amino acid sequence data for both genes (not shown) differed in some details from those resolved by the nucleotide sequence data, especially for the more weakly supported relationships.

Results of the Partition Homogeneity test revealed strong differences in phylogenetic signal between the *S-RNase* and the *SFB* nucleotide data sets as well as between the amino acid data sets for the two genes ($P=0.001$ for all tests). Tests were carried out for significant differences in the tree topologies between the *S-RNase* and *SFB* data for both nucleotides and amino acids using the Templeton, K–H, and winning-sites tests, as described above. For all cases in which data from one gene were constrained to give topologies identical to those found to be optimal for the other gene, the resulting trees were significantly longer than unconstrained trees ($P < 0.0001$ for all tests). In contrast, when data from one gene were constrained to resolve only the groups supported with bootstrap values of 80% or greater by the other gene, the results were not significant for any data set or any test. Because resolutions of relationships among the *Prunus* *S-RNase* and *SFB* alleles were generally weak, the possibility was tested that topologies consistent with one or more constraints concordant with our understanding of organismal relationships based on other data (Bortiri *et al.*, 2001) were not significantly worse than the best (most parsimonious) topologies resolved for the two data sets containing just the *Prunus* sequences. Constraint trees with three distinct topologies were used to test these hypotheses. In the first topology, the ‘species’ constraint, all representatives of each species were constrained to form a monophyletic group, but no restrictions were placed on the relationships among species. In the second topology, the ‘subgenera’ constraint, each of several groups of 2–4 species (*P. avium*/*P. cerasus*, *P. armeniaca*/*P. mume*, *P. dulcis*/*P. tenella*, and *P. cerasifer*/*P. domestica*/*P. salicina*/*P. spinosa*) was constrained as monophyletic, but no other restrictions were placed on the relationships within or among species. These groups have been resolved as closely related in recent studies and they also correspond to previously recognized subgenera (Rehder, 1940). In the third topology, the ‘largeclades’ constraint, each of two major lineages resolved in recent studies, one including the two cherry species (*P. avium* and *P. cerasus*) and one including the rest, was constrained as monophyletic, but no other restrictions were imposed. For both *S-RNase* and *SFB* alleles, the shortest unconstrained trees were significantly better ($P < 0.0001$ in all cases) than any tree consistent with any of these constraints, as assessed by the K–H, Templeton, and winning-sites tests.

**Variability analyses**

When comparing the *S-RNase* and *SFB* genes from *P. tenella* $S_{c}$- and *P. avium* $S_1$-haplotypes, Šurbanovski *et al.* (2007), observed no amino acid differences between the *S-RNases* but 12 amino acid differences between the *SFB* sequences from eight species. This pattern, first reported for Solanaceae species, was named trans-specific evolution by Richman *et al.* (1996). In *Prunus* this pattern has been described before (see references in Vieira *et al.*, 2008), but in contrast with the observation made for the Solanaceae species, in this genus trans-specific evolution cannot be taken as evidence for the very old age of alleles (Vieira *et al.*, 2008). Although this pattern has been known for more than a decade, no proper phylogenetic analyses have so far been performed to show conclusively that this pattern is not simply due to lack of phylogenetic resolution. In *Prunus*, bootstrap support values are generally weak. Nevertheless, in no case were all the alleles of any one gene from any one species supported as monophyletic.

Phylogenetic analyses of nucleotide sequences of both *S-RNase* and *SFB* data resolved the *P. cerasus* $S_{35}$ along with the *P. dulcis* $S_a$ as highly divergent from the remaining *S* haplotypes (Fig. 7). Therefore, $S_{35}$ was identified as not only divergent from other cherry $S$-haplotypes but also divergent from other *Prunus* $S$-haplotypes. For the *P. cerasus* $S_{35}$ and *P. cerasus* $S_{34}$, as well as for the alleles from several other $S$-haplotypes, the resolved relationships were different in the two data sets (Fig. 7). All data sets strongly supported the sister relationship between *P. cerasus* $S_{34}$ and *P. domestica* $S_5$. Both the amino acid and the nucleotide sequence data resolved the *S-RNases* of the last two haplotypes as sister to a clade including the *P. avium* $S_1$-RNase, the *P. dulcis* $S_{11}$-RNase, and the *P. tenella* $S_9$-RNase. The *SFB* data did not, however, show this relationship, and the position of *P. cerasus* $S_{34}$ and *P. domestica* $SFB_5$ was very weakly supported. The *P. cerasus* $S_{34}$-RNase was weakly supported as sister to *P. mume* $S_7$-RNase and *P. spinosa* $S_{10}$-RNase; however, *P. cerasus* $SFB_3$ was resolved, again with very weak support, as sister to the clade of *P. avium* $SFB_3$, *P. cerasus* $SFB_{34}$, and *P. domestica* $SFB_5$. The relationships resolved by the amino acid sequence data for both genes (not shown) differed in some details from those resolved by the nucleotide sequence data, especially for the more weakly supported relationships.

Results of the Partition Homogeneity test revealed strong differences in phylogenetic signal between the *S-RNase* and the *SFB* nucleotide data sets as well as between the amino acid data sets for the two genes ($P=0.001$ for all tests). Tests were carried out for significant differences in the tree topologies between the *S-RNase* and *SFB* data for both nucleotides and amino acids using the Templeton, K–H, and winning-sites tests, as described above. For all cases in which data from one gene were constrained to give topologies identical to those found to be optimal for the other gene, the resulting trees were significantly longer than unconstrained trees ($P < 0.0001$ for all tests). In contrast, when data from one gene were constrained to resolve only the groups supported with bootstrap values of 80% or greater by the other gene, the results were not significant for any data set or any test. Because resolutions of relationships among the *Prunus* *S-RNase* and *SFB* alleles were generally weak, the possibility was tested that topologies consistent with one or more constraints concordant with our understanding of organismal relationships based on other data (Bortiri *et al.*, 2001) were not significantly worse than the best (most parsimonious) topologies resolved for the two data sets containing just the *Prunus* sequences. Constraint trees with three distinct topologies were used to test these hypotheses. In the first topology, the ‘species’ constraint, all representatives of each species were constrained to form a monophyletic group, but no restrictions were placed on the relationships among species. In the second topology, the ‘subgenera’ constraint, each of several groups of 2–4 species (*P. avium*/*P. cerasus*, *P. armeniaca*/*P. mume*, *P. dulcis*/*P. tenella*, and *P. cerasifer*/*P. domestica*/*P. salicina*/*P. spinosa*) was constrained as monophyletic, but no other restrictions were placed on the relationships within or among species. These groups have been resolved as closely related in recent studies and they also correspond to previously recognized subgenera (Rehder, 1940). In the third topology, the ‘largeclades’ constraint, each of two major lineages resolved in recent studies, one including the two cherry species (*P. avium* and *P. cerasus*) and one including the rest, was constrained as monophyletic, but no other restrictions were imposed. For both *S-RNase* and *SFB* alleles, the shortest unconstrained trees were significantly better ($P < 0.0001$ in all cases) than any tree consistent with any of these constraints, as assessed by the K–H, Templeton, and winning-sites tests.
Fig. 7. Phylogenetic trees based on phylogenetic analysis of nucleotide sequences of S-RNase (left) and SFB (right) alleles for species of Prunus. Numbers above, below, or adjacent to branches are bootstrap support values greater than 50%; asterisks indicate branches that collapsed in the strict consensus tree from each analysis. The positions of Pcs33, Pcs34, and Pcs35 are indicated, respectively, by open boxes, lightly shaded boxes, and darkly shaded boxes. Left: Single most parsimonious tree (l = 2366, ci excluding autapomorphies = 0.3271, ri = 0.5263) based on S-RNase alleles. The nucleotide sequences for three novel P. cerasus (PcS-RNase) alleles presented in this study were aligned with 12 S-RNase alleles from P. avium (PaS1-RNase, AB028153; PaS2-RNase, AB010304; PaS3-RNase, AB010306; PaS4-RNase, AB028154; PaS5-RNase, AY298314; PaS6-RNase, AB010305; PaS7-RNase, EU035974; PaS12-RNase, AY259113; PaS13-RNase, AY259111; one from P. cerasus (Pcs26-RNase, EU035975); three from P. domestica (PdS5-RNase, AM746946; PdS6-RNase, AM746947; PdS9-RNase, AM746948); nine from P. dulcis (Pds5-RNase, AB026836; Pds8-RNase, AB011469; Pds11-RNase, AB011470; Pds12-RNase, AB011471; Pds13-RNase, AB252409; Pds14-RNase, DQ998895; Pds5-RNase, DQ998925; Pds11-RNase, AM231660; Pds12-RNase, AM746949); two from P. mume (Pms11-RNase, AB101438; Pms7-RNase, AB101439); four from P. armeniaca (ParS1-RNase, AY587561; ParS2-RNase, AY587562; ParS4-RNase, AY587564; ParS7-RNase, EU516388); three from P. cerasifera (PcsfS3-RNase, AM746943; PcsfS9-RNase, AM746944; PcsfS10-RNase, AM746945); ten from P. salicina (Psa-RNase, AB252411; Psa9-RNase, AB084102; PsS-RNase, AB084103; PsSf-RNase, AB280693; PsSj-RNase, DQ512911; PsSj-RNase, AM746950; Pslk-RNase, DQ512914; Pslk-RNase, AY781290; Pslk-RNase, DQ512913); seven from P. spinosa (PspS8-RNase, DQ677587; PspS9-RNase, DQ677588; PspS10-RNase, DQ677589; PspS11-RNase, DQ677584; PspS3-2-RNase, DQ677585; PspS7-1-RNase, DQ677586); and nine from P. tenella (PsT1-RNase, AM983373; PsT2-RNase, AM983374; Ps31-RNase, DQ983375; Ps34-RNase, AM983363; Ps35-RNase, DQ983364; Pslk-RNase, AM983365; Pslk-RNase, DQ983370). Data set contained 63 taxa and 744 characters, of which 220 were constant, 129 were variable but uninformative, and 395 were parsimony-informative. Right: One of 65 most parsimonious trees (l = 2366, ci excluding autapomorphies = 0.3913, ri = 0.4860) from phylogenetic analysis of nucleotide sequences of SFB alleles. The nucleotide sequences for three novel P. cerasus (PcSFb) alleles presented in this study were aligned with coding sequences for 12 SFB alleles from P. avium (PsfFb1, AY805048; PsFb2, AB111519; PsFb3, AB096857; PsFb4, AB111520; PsFb5, AB096858; PsFb7, EU035976; PsFb9, DQ422809; PsFb10, AY805053; PsFb12, AY805054; PsFb13, DQ385844; PsFb16, AY805056); two from P. cerasus (PsfFb1, DQ877755; PsFb26, EU035977); three from P. domestica (Pds5-RFb, AM746955; Pds8-RFb, AM746956; Pds12-RFb, AM746957); seven from P. dulcis (Pds5-RFb, AB092966; Pds8-RFb, AB092967; Pds12-RFb, AB081648; Pds13-RFb, AB252408; PsFb11, EF061758; PsFb12, AM746959); three from P. mume (PmsFb1, AB101440; PmsFb7, AB101441; PmsFb9, AB092645); four from P. armeniaca (ParS1-RFb, AY587562; ParS2-RFb, AY87565; ParS7-RFb, EU516388); three from P. cerasifera (PcsFb3, AM746952; PcsFb9, AM746953; PcsFb10, AM746954); nine from P. salicina (PscFbA, AB252410; PscFbB, AB252412; PscFbC, DQ849084; PscFbD, AM746962; PscFbE, AB280794; PscFbF, DQ849089; PscFbG, AM746963; PscFbH, DQ489118; PscFbI, DQ489085); seven from P. spinosa (PspFb8, DQ677587; PspFb9, DQ677588; PspFb10, DQ677589; PspFb12, DQ677586; PspFb3-1, DQ677616; PspFb3-2, DQ677615; PspFb7-1, DQ677595), and one from P. tenella (PsfFbB, AM983369). Data set contained 54 taxa and 1161 characters, of which 337 were constant, 230 were variable but uninformative, and 594 were parsimony-informative.
increased rate of non-synonymous or synonymous mutation at one of the genes compared with the other (Non-parametric Sign test, \( P > 0.05 \) in both cases).

Since there is no evidence for an increased rate of mutation at the \( SFB \) gene when compared with the \( S\text{-RNase} \) gene, it is conceivable that a rare recombination event could be responsible for the discrepancy observed by Šurbanovski et al. (2007). Using 22 \textit{Prunus} \( S \)-haplotypes, Nunes et al. (2006) noticed that the history of the two genes is positively correlated, although not highly correlated. When using all pair-wise \( K_s \) values, the Pearson correlation coefficient was +0.628 \((n=231; P < 0.01)\), and when using all pair-wise \( K_a \) values, the Pearson correlation coefficient was +0.759 \((n=231; P < 0.01)\). This observation is suggestive of recombination at one or both genes. Using the same methodology but a larger data set \((n=48)\), the correlation coefficient was now even lower. When using all pair-wise \( K_s \) values, the Pearson correlation coefficient was +0.346 \((n=1128; P < 0.01)\) and when using all pair-wise \( K_a \) values, the Pearson correlation coefficient was +0.673 \((n=1128; P < 0.01)\). Therefore recombination cannot be ruled out as one conceivable reason for the discrepancy observed by Šurbanovski et al. (2007). Nevertheless, it should be noted that for both the \( S\text{-RNase} \) and \( SFB \) gene there is no evidence for the clustering of synonymous or non-synonymous mutations, as expected under the hypothesis of a recent recombination event affecting only a portion of the \( SFB \) or \( S\text{-RNase} \) gene.

### Discussion

Sour cherry is a segmental allotetraploid (Beaver and Iezzoni, 1993) arising from the inter-mating of the diploid sweet cherry and tetraploid ground cherry. Previous studies have shown that six \( S \)-haplotypes present in sweet cherry \((S_1, S_4, S_6, S_9, S_{12}, \text{and } S_{13})\) are present in sour cherry as well. In this study, it is shown that the sweet cherry \( S_{14}\) and \( S_{15}\)-haplotypes are also found in sour cherry, bringing to eight the total number of \( S \)-haplotypes shared by the two species. The identification in this study of three \( S \)-haplotypes not previously identified in sour cherry and not found in sweet cherry suggests that these \( S \)-haplotypes were contributed by the ground cherry \((P. \textit{fruticosa})\). To address this question, an ongoing survey of the \( P. \textit{fruticosa} \) present in the Michigan State University cherry germplasm collection has resulted in the identification of multiple accessions possessing the \( S_{35} \) and \( S_{55} \)-haplotypes. However, no individual possessing the \( S_{46} \)-haplotype has been identified (T Tsukamoto et al., unpublished data).

The finding that the \( S_{35} \)-haplotype is functional has significant implications regarding the genetic control of SI and SC in sour cherry. The ‘one allele match model’ states that for any sour cherry cultivar to be SI it must possess a minimum of three functional \( S \)-haplotypes (Hauck et al., 2006b). This is because all pollen carrying at least one matching functional \( S \)-haplotype is predicted to be incompatible. The landrace cultivars ‘Pandy 38’, ‘Pandy 114’ (syn. ‘Crisiana’, ‘Köröser’), and ‘Tschernokorka’ are well documented examples of SI sour cherry cultivars (Redalen, 1984; Lansari and Iezzoni, 1990). Both these cultivars possess three functional \( S \)-haplotypes, thereby supporting the current hypothesis for the genetic control of SI and SC in sour cherry (Yamane et al., 2001; Hauck et al., 2006b). The three functional \( S \)-haplotypes in ‘Pandy 38’ and ‘Pandy 114’ are \( S_1, S_4, \text{and } S_{55} \) while the three functional \( S \)-haplotypes in ‘Tschernokorka’ are \( S_9, S_{13}, \text{and } S_{35} \). This also provides additional evidence that competitive interaction, for example, the compatibility of heteroallelic pollen that is associated with the breakdown of GSI in the Solanaceae, does not occur in tetraploid sour cherry as pollen containing two functional \( S \)-haplotypes is incompatible. Our result is in contrast to Huang et al. (2008) who reported a set of observations compatible with the ‘competitive interaction model’ in a SC selection of tetraploid \textit{Prunus pseudocerasus}. However, as not all predictions of the model were tested, further data are needed to understand the generality of the ‘one allele match model’ in \textit{Prunus}. 

It is possible that the \( S_{35}\text{-RNase} \) identified in ‘Meteor’ has the same specificity as the \( P. \textit{mume} S_{10}\text{-RNase} \) (subgenus \textit{Prunus}). Confirmation will require the complete sequence of the \( P. \textit{mume} S_{10}\text{-RNase} \). However, if these genes do indeed code for identical proteins, this

| \textit{Prunus} sequences | \textit{S-RNase} | \textit{SFB} |
|---------------------------|-----------------|-----------|
| \textit{P. avium} \( S_1 \) | \textit{P. dulcis} \( S_{14} \) | 0.0326 0.0000 | 0.0588 0.0151 |
| \textit{P. avium} \( S_1 \) | \textit{P. tenella} \( S_6 \) | 0.0551 0.0000 | 0.0519 0.0185 |
| \textit{P. avium} \( S_3 \) | \textit{P. domestica} \( S_{34} \) | 0.1134 0.0060 | 0.0975 0.0116 |
| \textit{P. armeniaca} \( S_{17} \) | \textit{P. spinosa} \( S_{14} \) | 0.0319 0.0030 | 0.0396 0.0083 |
| \textit{P. armeniaca} \( S_{17} \) | \textit{P. spinosa} \( S_{12} \) | 0.0319 0.0060 | 0.0464 0.0100 |
| \textit{P. cerasus} \( S_{26} \) | \textit{P. spinosa} \( S_9 \) | 0.0645 0.1366 | 0.2622 0.1125 |
| \textit{P. cerasus} \( S_{26} \) | \textit{P. mume} \( S_7 \) | 0.0912 0.1180 | 0.2194 0.0919 |
| \textit{P. cerasus} \( S_{24} \) | \textit{P. domestica} \( S_3 \) | 0.0979 0.0090 | 0.0683 0.0166 |
| \textit{P. dulcis} \( S_2 \) | \textit{P. dulcis} \( S_{12} \) | 0.0215 0.0030 | 0.0000 0.0000 |
| \textit{P. dulcis} \( S_2 \) | \textit{P. cerasus} \( S_3 \) | 0.0437 0.0030 | 0.0192 0.0050 |
| \textit{P. dulcis} \( S_{11} \) | \textit{P. tenella} \( S_6 \) | 0.0437 0.0030 | 0.0322 0.0033 |
| \textit{P. dulcis} \( S_{12} \) | \textit{P. cerasus} \( S_3 \) | 0.0215 0.0059 | 0.0192 0.0050 |
| \textit{P. mume} \( S_2 \) | \textit{P. spinosa} \( S_{10} \) | 0.0957 0.0374 | 0.2688 0.0910 |
| \textit{P. salicina} \( S_9 \) | \textit{P. domestica} \( S_6 \) | 0.0324 0.0000 | 0.0063 0.0134 |
| \textit{P. salicina} \( S_9 \) | \textit{P. cerasus} \( S_{10} \) | 0.0211 0.0090 | 0.0064 0.0017 |
| \textit{P. salicina} \( S_{16} \) | \textit{P. cerasus} \( S_{10} \) | 0.0215 0.0000 | 0.0131 0.0033 |
| \textit{P. salicina} \( S_7 \) | \textit{P. spinosa} \( S_{14} \) | 0.0883 0.0304 | 0.1907 0.0689 |
| \textit{P. salicina} \( S_7 \) | \textit{P. spinosa} \( S_{12} \) | 0.0883 0.0335 | 0.1991 0.0707 |
| \textit{P. spinosa} \( S_{11} \) | \textit{P. spinosa} \( S_{12} \) | 0.0000 0.0030 | 0.0065 0.0017 |
| \textit{P. spinosa} \( S_9 \) | \textit{P. spinosa} \( S_{14} \) | 0.0992 0.1262 | 0.2299 0.1060 |
would represent another example of ancestral specificities shared between Prunus subgenera. These findings are consistent with S-haplotype divergence predating speciation in Prunus (Nunes et al., 2006; Vieira et al., 2008). Prunus species from the same subgenus share, on average, a higher percentage of ancestral specificities than Prunus species from different subgenera (Vieira et al., 2008).

Nevertheless, identical amino acid sequences, as in this case (the comparison of the S-RNase from P. cerasus S$_{33}$ and P. mume S$_{10}$), were only found between P. tenella S$_{S}$-RNase and P. avium S$_{1}$-RNase (Šurbanovski et al., 2007), and P. tenella S$_{3}$-RNase and P. salicina S$_{F}$-RNase (although the latter two sequences are partial; Vieira et al., 2008). The SFB of P. tenella S$_{S}$- and P. avium S$_{1}$-haplotypes differ, however, by 12 amino acids scattered along the gene (Šurbanovski et al., 2007). Such comparison is not possible for P. cerasus S$_{33}$- and P. mume S$_{10}$- SFB alleles since the latter sequence is not available. Moreover, the SFB sequences of the P. tenella S$_{3}$- and P. salicina S$_{F}$-haplotypes are not available.

The inferred rate of synonymous and non-synonymous mutation for closely related haplotypes is not higher at the SFB than at the S-RNase gene. Therefore, recombination may be the cause of the pattern observed by Šurbanovski et al. (2007), although we failed to identify where the putative recombination event occurred.

Phylogenetic analyses provide strong support for the placement of the P. cerasus S$_{33}$ and P. dulcis S$_{D}$-RNases and SFBs as a sister group to all the other alleles examined. These specificities are among the oldest ones, and are 15–20 million years old (Vieira et al., 2008). The significant conflict between the S-RNase and SFB data sets, as revealed by the partition homogeneity and tree topology tests, suggest real differences in the underlying phylogenetic histories of the S-RNase and SFB genes (Fig. 7). These results echo those of Nunes et al. (2006). Two observations suggest that homoplasy within one or both data sets, which could result from rapid evolution and/or intragenic recombination involving either or both genes, may be the primary cause of the lack of phylogenetic congruence. First, in phylogenies from both genes, there is generally weak resolution of relationships among alleles, especially for the deeper branches in the trees (Fig. 7). Second, those relationships that are strongly supported tend either to be strongly supported by sequences from both genes (e.g. the sister relationship of P. cerasus S$_{33}$ and P. domestica S$_{3}$) or they are strongly supported by one data set and weakly resolved in the other (e.g. the position of P. spinosa S$_{7-1}$), as reflected by the non-significant results of the topology tests in which each data set was constrained to resolve only groups with 80% or better bootstrap support by the other gene. The fact that the relationships that are strongly supported by either data set, and especially those supported by both data sets, appear to involve recent divergences, while the deeper branches are generally weakly supported, suggests that whatever factors are causing phylogenetic incongruence between the two data sets are also acting to limit our ability to reliably construct more distant relationships among alleles of either gene alone. The lack of congruent topologies of relationship for S-RNase and SFB alleles does not, in any case, preclude the possibility that the two genes have coevolved to maintain self-incompatibility. Indeed, as expected, since amino acid sites responsible for specificity determination at the S-RNase and SFB must coevolve, the much higher correlation coefficient for non-synonymous divergence (+0.673) than for synonymous divergence (+0.346) suggests otherwise. As long as the history of amino acid sites important for specificity determination at the S-RNase and SFB genes is tightly linked, the S-haplotype will be functional. For instance, historical intragenic gene conversion may have preferentially occurred in regions where there are no amino acid sites important for specificity determination. It remains, nevertheless, to be determined how much intragenic recombination needs to be argued in order to account for the observed synonymous and non-synonymous correlation coefficients as well as for the overall topology incongruencies. It should be noted that features compatible with recombination have been previously reported at the S-RNase and SFB genes, although the evidence is still not unequivocal (Vieira et al., 2003; Nunes et al., 2006; Ortega et al., 2006).

The overall amounts of total change and the relative distribution of that change on internal and terminal branches are similar. In both cases, internal branches are generally short compared to many of the terminal branches, suggesting that cladogenic diversification in each gene may have occurred in a common ancestral species, followed by lineage sorting and recombination (not necessarily in that order). In agreement with this view, our phylogenetic analyses of S-RNase and SFB sequences produced gene trees in which alleles from a single species were not resolved as monophyletic. Such results are expected for genes involved in self-incompatibility among closely related species (Lu, 2001; Vieira et al., 2008). Given sufficient divergence time, however, the genes within a particular evolutionary lineage should eventually coalesce. It is known, for example, that within Rosaceae, the S-RNase genes of tribe Pyreae (Potter et al., 2007) form a clade distinct from those of Prunus (Igic and Kohn, 2001). In an attempt to ascertain the phylogenetic magnitude of the lack of correspondence between our gene trees and the presumed phylogeny for these species, a constraint tree approach was used (see Results). Constraining the topologies of the trees in any way to correspond to current understanding of organismal relationships resulted in significantly longer tree lengths. This was true at the level of species, groups of 2–4 closely related species, and two major clades that have been
resolved in recent phylogenetic studies of the genus. Thus, our sampling of five of the roughly 200 species of *Prunus*, which includes representatives of two of the three major lineages within the genus resolved in recent phylogenetic analyses, indicate that neither the *SFB* nor the *S-RNase* locus has attained coalescence at any level below that of the entire genus. Sampling of additional species of *Prunus*, and of species of related genera in Rosaceae, will be required to test this hypothesis and to determine the precise level at which coalescence has occurred.

**Supplementary data**

The following supplementary data for this article are available at *JXB* online.

Fig. S1. Nucleotide sequence and the deduced amino acid sequence alignments for the *Prunus cerasus S35-RNase* and *Prunus mume S10-RNase*.

Fig. S2. Nucleotide sequence alignment for the *Prunus cerasus S34-RNase*, *P. avium S1-RNase*, *P. dulcis S11-RNase*, and *P. tenella S8-RNase*.

Fig. S3. Comparison between downstream sequence of *Prunus cerasus S34-RNase* and intergenic sequence between *S-RNase* and SFB of *P. avium S1*, *P. dulcis S11*, and *P. tenella S8*.

Fig. S4. Nucleotide sequence and the deduced amino acid sequence alignment for the *Prunus cerasus S35-RNase* and *S7-RNase*.

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