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# Cloning of six cherry self-incompatibility alleles and development of allele-specific PCR detection

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**Abstract** Reverse transcription of stylar RNA from three cherry cultivars representing six self-incompatibility (*S*) alleles, Early Rivers ($S_1S_2$), Napoleon ($S_3S_4$) and Colney ($S_5S_6$), followed by 3′ RACE using degenerate primers based on conserved regions of *Prunus S* ribonucleases (*S* RNases), gave six classes of partial putative *S* RNase clones. These were sequenced, and specific primers were designed for each class and, by using them in genomic PCR on 28 cultivars previously genotyped, we were able to assign the classes to individual *S* alleles. The primers for $S_3$ amplified the allele reported previously as $S_8$, and a controlled cross showed that these two alleles are functionally the same. Analysis of three cherry progenies using the specific primers showed cosegregation with stylar *S* RNases for all six clones. This confirmed that the clones indeed represent cherry *S* RNases. The allele-specific primers for $S_5$ presented here provide the first PCR test for true $S_5$. In a fourth progeny, the amplification product of a mutant $S_4$ allele, $S_4'$, cosegregated with self-compatibility. Sixteen cultivars were genotyped for the first time using the allele-specific primers. Thus, this approach will be valuable for genotyping cultivars and seedlings that have the alleles $S_1$–$S_6$ and for detecting self-compatible seedlings from vegetative material. The sequences of five of the *S* RNases, including $S_5$, were completed by 5′ RACE.

**Keywords** Allele-specific PCR · Cherry · *Prunus avium* · Self-incompatibility · *S* RNase

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## Introduction

In commercial orchards of sweet cherry (*Prunus avium*) suitable pollinator cultivars must be planted to set an economic crop. Self-incompatibility and cross-incompatibility between various pairs of cultivars was attributed to the multi-allelic locus *S*, expressed gametophytically, by Crane and Lawrence (1929). Early studies at the John Innes Institute and elsewhere led to the classification of cultivars into various incompatibility groups, with ten of these being assigned various pairs of the alleles $S_1$–$S_6$ (Matthews and Dow 1969). Pollen irradiation work at the John Innes Institute led to the generation of some self-compatible selections, including JI 2420. In this selection, the ancestor of most self-compatible cultivars, self-compatibility is attributed to a mutant form of $S_4$, denoted $S_4'$, which has lost pollen activity (Lewis and Crowe 1954; Matthews 1970).

Recent studies at East Malling have indicated that the *S* alleles in cherry code for stylar ribonucleases that can be detected by the electrophoresis of stylar proteins and subsequent staining for activity (Boškovic and Tobutt 1996; Boškovic et al. 1997). These investigations followed up on work of Sassa et al. (1992), who reported some correlation of stylar RNases and *S* alleles in Japanese pear (*Pyrus serotina*), another rosaceous fruit species. Cherry cultivars in different incompatibility groups had distinct ribonuclease bands which corresponded to different *S* alleles – with the exception of the self-compatible mutant allele $S_4'$, the band of which appeared the same as $S_4$ – and segregated as expected in fully compatible and semi-compatible crosses. Stylar ribonuclease analysis has proved to be a valuable technique, e.g. for predicting incompatibility genotype in cultivars and seedlings and for detecting new *S* alleles; the alleles $S_7$–$S_{11}$ have recently been reported (Boškovic et al. 1997) and another three, $S_{12}$–$S_{14}$, detected (Boškovic and Tobutt 2001). However, these analyses require the availability of flower material; it would be convenient to have an assay that could be used on vegetative material, independently of age or season.

Recently, Tao et al. (1999b) have published complete sequences for two cherry *S* RNases, $S_3$ and $S_6$, and the partial sequence for a third, $S_2$. They reported that three primers based on conserved regions, when used in appropriate combinations, gave allele-specific amplification products from leaf material, reportedly for the six *S* alleles $S_1$–$S_6$. However, the two cultivars they used to represent $S_5$ were of an inappropriate *S* genotype (Boškovic and Tobutt 2001); therefore their interpretation for $S_5$ cannot be correct.

In the investigation reported here, we took an alternative approach by developing allele-specific primers for $S_1$–$S_6$, following the general strategy adopted by Janssens et al. (1995) in apple. At HRI East Malling many well-genotyped cherry cultivars and progenies were available from stylar ribonuclease analyses, and problems caused by mistakes and ambiguities in the literature, which are common in cherry, could be avoided. We isolated and sequenced partial cDNA clones of the six alleles by reverse transcription of stylar RNA and 3′ RACE (Rapid Amplification of cDNA Ends; Frohman et al. 1988) using degenerate primers based on conserved regions of *Prunus S* RNases (almond and cherry) (Ushijima et al. 1998b). Specific primers were designed from variable regions of these sequences. The primers were tested for allele specificity in polymerase chain reactions (PCR) using genomic DNA from a set of cultivars of known *S* genotype to correlate specific amplification products with particular *S* alleles. As the primers for $S_3$ also amplified one of the new alleles, a test cross was made to check if these two are functionally the same. To confirm the identity of the clones, we tested the cosegregation of amplification products with stylar *S* RNases in three progenies. In addition, a progeny segregating for $S_4'$ was analysed to see if the $S_4$-specific PCR product cosegregated with self-compatibility. We also used allele-specific PCR to assign *S* genotypes to a number of commercially important cultivars of known parentage for which the *S* alleles have not been reported previously. Finally, 5′ RACE provided the full-length sequences of five of the *S* RNases, including $S_5$, which has not been characterised previously.

## Materials and methods

### Plant material

Three cherry cultivars were chosen for cDNA cloning of cherry incompatibility alleles $S_1$–$S_6$: Early Rivers ($S_1S_2$), Napoleon ($S_3S_4$) and Colney ($S_5S_6$).

For checking that the allele-specific primers gave results consistent with known genotypes an additional 23 cultivars and two selections, listed in Table 3, were used, the genotypes of which had been confirmed by stylar ribonuclease analysis (Boškovic and Tobutt 1996; Boškovic et al. 1997). They include one to three representatives of incompatibility groups I–VII, IX and XIII, three self-compatible cultivars and the parents of the progenies tested. In addition to $S_1$–$S_6$, some of the parental cultivars have one or two of the more recently detected alleles $S_7$–$S_{11}$ (Boškovic et al. 1997).

For checking the identity of $S_3$ and $S_8$ a test cross was made of Gaucher ($S_5S_8$)×Bradbourne Black ($S_3S_5$) with viable pollen on an emasculated tree in an insect-proof glasshouse.

To confirm the cosegregation of allele-specific amplification products with *S* alleles we analysed three progenies: F1/3 ($S_1S_2$)×Charger ($S_1S_7$), 46 seedlings, in which $S_1$ and $S_2$ segregate; Bradbourne Black ($S_3S_5$)×Merton Late ($S_1S_4$), 43 seedlings, in which $S_1$, $S_3$, $S_4$ and $S_5$ segregate; and Colney ($S_5S_6$)×Gaucher ($S_5S_8$), 34 seedlings, in which $S_5$ and $S_6$ segregate. All three progenies had been genotyped previously by stylar ribonuclease analysis (Boškovic et al. 1997).

In addition, the progeny Colney ($S_5S_6$)×Lapins ($S_1S_4'$), which should segregate for self-compatibility, was analysed with the primers specific for $S_4$. All 14 trees were tested for self-compatibility by selfing in the field, approximately 100–270 flowers per seedling. In the spring, insect-proof bags were put over several branches to prevent cross pollination, and subsequent fruit set was recorded.

We also genotyped several cherry cultivars, from crosses between parents having various pairs of $S_1$–$S_4$, for which the genotype has not been reported previously (Table 5). Some were self-incompatible and some were self-compatible; one was of unknown incompatibility status.

All cultivars and progenies were growing at HRI East Malling with the exception of Newstar and Vega, which were obtained from the National Fruit Collections, Brogdale, UK, and Salmo, which was supplied by the Agricultural University of Norway (NLH), Ås, Norway.

### RNA extraction

For the three cultivars chosen for cDNA cloning, total RNA was extracted from five styles (including stigmas) collected from unopened flowers at the balloon stage. The method was essentially a miniprep version of the method described by Prescott and Martin (1987), followed by a DNase treatment as follows. The RNA precipitated from five styles was resuspended in 95 µl sterile distilled water containing 36 U RNase inhibitor (RNAguard, Amersham Pharmacia Biotech) and 10 U DNase (Promega), and incubated at 37°C for 30 min. The volume was brought to 200 µl with sterile distilled water and extracted with an equal volume of phenol (equilibrated with citrate buffer pH 4.3); then the RNA was precipitated with 0.1 vol of 3 *M* sodium acetate (pH 5.2) and 2.5 vol of ethanol and resuspended in 25 µl sterile distilled water containing 18 U RNase inhibitor.

### 3′ RACE and 5′ RACE cloning

For reverse transcription the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech) was used. About 1.5 µg total RNA (2–8 µl) was used in a 15-µl reaction. An aliquot of this reaction was used directly for 3′ RACE (Frohman et al. 1988).

For 3′ RACE, two nested degenerate forward primers were designed from the conserved regions C1 and C2 of three *Prunus avium* and three *P. dulcis S* RNase amino acid sequences (Ushijima et al. 1998b): PC1-DF1, 5′-CA(A/G)(T/C)(T/C)NGTNCA(A/G)-CA(A/G)TGGCC-3′, and PC2-DF1, 5′-ACNAT(A/C/T)CA(C/T)-GGN(C/T)TNTGGC-3′. In the first PCR, PC1-DF1 was used as the forward primer and the oligo dT primer from the First-Strand cDNA Synthesis Kit as the reverse primer in a 50-µl reaction containing 20 m*M* Tris-HCl pH 8.3, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.4 m*M* dNTPs, 0.4 µ*M* PC1-DF1, 0.4 µ*M* oligo dT primer, 2.5 U *Taq* DNA polymerase (Life Technologies) and 2.5 µl of the heat-denatured first-strand synthesis reaction. PCR was carried out in a PTC-200 thermal cycler (MJ Research) for 35 cycles of 1 min at 94°C, 1 min 20 s at 42°C and 1 min at 72°C, with a final extension step of 10 min at 72°C. Five microliters of a 1/100 dilution of this first PCR reaction was used in a second round, half-nested PCR. PC2-DF1 was used as forward primer and again the oligo dT primer as reverse primer in a 50-µl reaction, set up as before, except for 0.2 m*M* instead of 0.4 m*M* dNTPs, and was amplified under the same conditions.

For each cultivar two 50-µl reactions were pooled and run on a 1.5% TAE agarose gel. A band approximately 650 bp in size,

**Table 1** Nucleotide sequences of nested allele-specific 5′ RACE primers for $S_1$–$S_6$ and cDNA position

| Primer | *S* allele and orientation | Sequence 5′→3′ | cDNA position[a] |
|---|---|---|---|
| PaS1-Ra | $S_1$ reverse | AGT ATT AGT TGC TGG ATC A | 555–573 |
| PaS1-Rb | $S_1$ reverse | GAT CTC TTC AGT TTG GAT CGC AAT | 273–296 |
| PaS1-Rc | $S_1$ reverse | TTA GGG TAC ACT TTC CTG TCC TCA T | 248–272 |
| PaS2-Ra | $S_2$ reverse | CAA CTG AGT CTT CTT ATC CT | 560–579 |
| PaS2-Rb | $S_2$ reverse | ATC TCT TCA GTT TGG CTC GCA AC | 279–301 |
| PaS2-Rc | $S_2$ reverse | TGA GGT GAC ACT TTC CTG CCA | 258–278 |
| PaS3-Ra | $S_3$ reverse | GTA TTC GCG TTA GGA TGT | 564–581 |
| PaS3-Rb | $S_3$ reverse | GAA TGG CTG ATA CTA TGT CCG AGT ACT | 488–514 |
| PaS3-Rc | $S_3$ reverse | GAG GGG ACA ATA GCT CTT TCT TAA ATC | 245–271 |
| PaS4-Ra | $S_4$ reverse | AAC TGA GTG CTC TTA TCG T | 551–569 |
| PaS4-Rb | $S_4$ reverse | GTA GGT CCA GTT TTT TGT CGC ACT | 466–489 |
| PaS4-Rc | $S_4$ reverse | CGC AAT TGA GGG TAC ACT TTC CT | 253–275 |
| PaS5-Ra | $S_5$ reverse | GCA TTT GGT ACG ATT TG | 451–467 |
| PaS5-Rb | $S_5$ reverse | GTA CCA TGT TTG TTC CAT TCG TCT C | 332–356 |
| PaS5-Rc | $S_5$ reverse | AAT CGG GGA TAC ACT TTA CTT TCG T | 245–269 |
| PaS6-Ra | $S_6$ reverse | TGT AAC AAC ACA GTA TTC TTG T | 554–575 |
| PaS6-Rb | $S_6$ reverse | TGG GTG CAA CTA TGT CCG AGT ACT | 485–508 |
| PaS6-Rc | $S_6$ reverse | ACA ATA TTC GCT TAA ATT GCG GT | 240–262 |

[a] The ‘A’ of the putative initiation codon (the first AUG) has been numbered +1

which was expected to correspond to *S* RNase cDNA, was excised from the gel and purified using the Qiagen II Gel Extraction Kit (Qiagen). In case the 3′ A overhang, created by *Taq* DNA polymerase, had been lost during the gel purification step, the DNA was incubated with *Taq* DNA polymerase and dATPs, allowing it to be cloned into the vector of the TA Cloning Kit (Invitrogen), according to the manufacturer’s instructions. The incubation with dATP and *Taq* DNA polymerase essentially followed the protocol from the TA Cloning Kit, with the addition of PCR buffer and $MgCl_2$ (concentrations as before).

To select colonies with an insert of the expected size of an *S* RNase from the C2 region to the 3′ end, we determined the insert size of various colonies by colony PCR using the universal M13 primers. Individual colonies were picked from the plate and transferred to a 0.5-ml tube containing 20 µl of LB (Luria-Bertani) broth. One microliter was denatured for 2 min at 94°C and used for PCR in a 20-µl reaction containing 20 m*M* TRIS-HCl pH 8.3, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.2 m*M* dNTP mix, 0.2 µ*M* of each of the primers and 0.5 U of *Taq* DNA polymerase. PCR was carried out for 35 cycles of 30 s at 94°C, 30 s at 50°C and 1 min 72°C, with a final extension step of 5 min at 72°C.

Colonies with an insert of the expected size of an *S* RNase from the C2 region to the 3′ end were sequenced using an automated sequencer (ABI 373 A, Perkin Elmer Applied Biosystems) at Sutton Bonington. Later, allele-specific primers were used to identify more clones of a particular class, so that in total three clones per class were sequenced.

For 5′ RACE a commercial kit was used (5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0, Life Technologies). For each allele three allele-specific, nested primers were designed and used (Table 1). The resulting 5′ RACE products were cloned, and three clones of each were sequenced as described for 3′ RACE.

### Sequence analysis and design of allele-specific primers

The DNA sequences obtained from 3′ RACE were analysed with the software program DNASTAR (DNAStar). The cDNA sequences of those showing the conserved *Prunus S* RNase amino acid regions described previously (Ushijima et al. 1998b) were aligned by the Clustal method. After 5′ RACE cloning the 5′ end was sequenced and added to provide the full length sequence.

Specific primer pairs for the six sequences were designed from the variable regions (Table 2). Primers were chosen at a position where at least the last base at the 3′ end was different from the other five sequences.

### DNA extraction and allele-specific PCR

Genomic DNA of cherry cultivars, selections and progenies was extracted from cherry buds or leaves principally according to a miniprep version of the CTAB extraction method described by Doyle and Doyle (1987). Modifications included the addition of 2% polyvinyl pyrollidine (PVP 40) to the extraction buffer and increasing the amount of β-mercaptoethanol to a final concentration of 1%.

Approximately 20–30 ng of genomic DNA was used for PCR amplification in a 25-µl reaction containing 20 m*M* TRIS-HCl pH 8.3, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 0.1 µ*M* of each primer and 0.625 U *Taq* DNA polymerase (Life Technologies). After the DNA was denatured for 2 min at 94°C, and cooled on ice, the mix, also kept on ice, was added. PCR was carried out in a PTC-100 or PTC-200 thermal cycler (MJ Research) for 35 cycles of 30 s at 94°C, 30 s specific annealing temperature (Table 2) and 1 min 72°C, with a 5-min final extension step at 72°C. Negative controls with distilled water instead of template genomic DNA were included in each PCR.

## Results

### 3′ RACE and 5′ RACE cloning

Total RNA was extracted from styles of three cherry cultivars, conveniently representing all six *S* alleles: Early Rivers ($S_1S_2$), Napoleon ($S_3S_4$) and Colney ($S_5S_6$). Reverse transcription was followed by 3′ RACE, which gave for all cultivars two fragments, one of approximately 300 bp and one of approximately 650 bp. The 650-bp band from all three cultivars was excised and cloned as described in the Materials and methods.

By sequencing on average six clones initially for each cultivar, we obtained two classes of ribonuclease clones from two of the cultivars, Early Rivers ($S_1S_2$) and Napo-

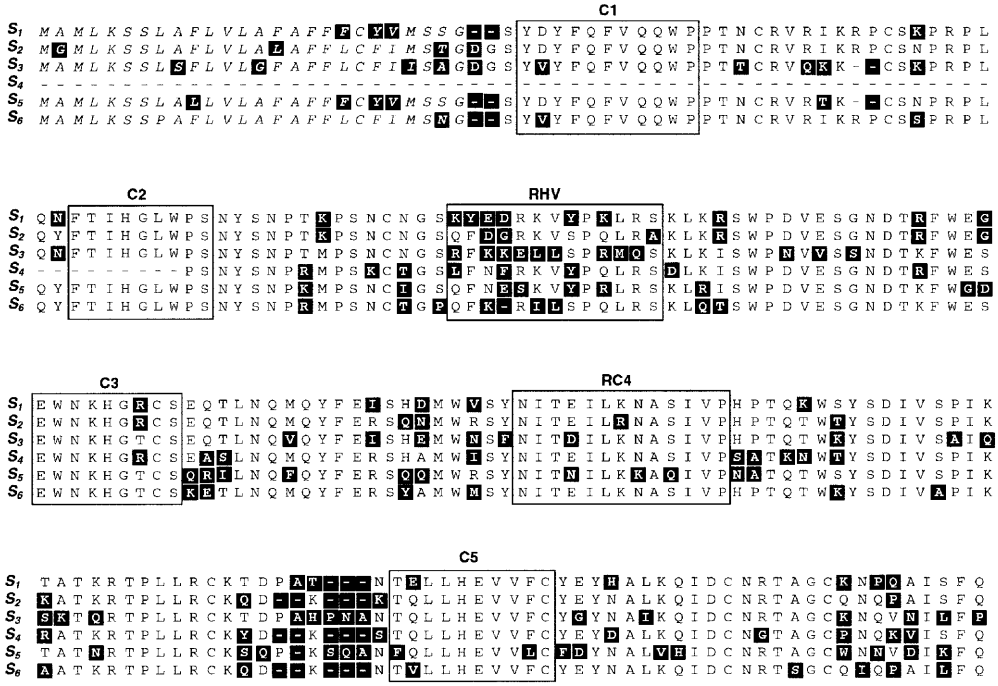


**Fig. 1** Alignment of amino acid sequences of cherry self-incompatibility alleles $S_1$–$S_6$ deduced from cDNA sequences. The sequences start with the methionine residue encoded by the first AUG codon as the putative initiation site. The predicted signal peptide sequence, based on N-terminal sequencing (Tao et al. 1999), is given in *italics*. For the annotation of conserved and variable regions (*boxed*) we have followed the convention of Ushijima et al. (1998b). Residues different from the consensus and gaps, which are represented by *dashes*, are *highlighted* in *black boxes*. Accession numbers of nucleotide sequences for $S_1$–$S_6$ are as follows: AJ298310 ($S_1$), AJ298311 ($S_2$), AJ298312 ($S_3$), AJ298313 ($S_4$), AJ298314 ($S_5$), and AJ298315 ($S_6$)

leon ($S_3S_4$), as expected. For Colney ($S_5S_6$), initially only one class was obtained. So more colonies with an approximately 650-bp insert were screened using specific primers for the sequence of the class already obtained. Only inserts of colonies not giving amplification with these specific primers were sequenced, and a second ribonuclease class from this cultivar was obtained. These partial ribonuclease sequences obtained showed similarity to *Prunus S* RNases. Specific primers for each class were designed and used to identify more colonies of a particular class. In all, three clones per class were sequenced. In several cases clones of the same class varied in length at the 3′ end due to a difference in the start position of the poly A tail. The two classes of ribonuclease clones from Early Rivers were temporarily called A and B; from Napoleon, C and D; and from Colney, E and F. The proportion of the classes from Early Rivers and Napoleon was relatively even. For Colney the frequency of class-E colonies was about 15 times as high as that of class-F colonies. The assignment of class to allele was achieved later by allele-specific PCR and comparison with known genotypes.

Later, 5′ RACE products were obtained for five alleles. They showed overlapping sequences with the 3′ RACE products and provided the full-length cDNA sequence for $S_1$ (open reading frame (ORF) of 681 bp; 226 amino acids), $S_2$ (ORF of 681 bp; 226 amino acids), $S_3$ (ORF of 690 bp; 229 amino acids), $S_5$ (ORF of 681 bp; 226 amino acids) and $S_6$ (ORF of 672 bp; 223 amino acids). Amino acid identity of the sequences for $S_1$–$S_6$ ranges from 68.1% ($S_3$ and $S_4$) to 84.5% ($S_1$ and $S_2$). An alignment of the deduced amino acid sequences is given in Fig. 1.

## Allele-specific PCR

Testing the primers designed for each allele in genomic PCR using a range of genotyped cherry cultivars showed that all six pairs gave specific PCR amplification, after the annealing temperatures were optimised. Primer sequences and their positions for each of the six *S* alleles, together with the optimal annealing temperature for PCR and the estimated size of the resulting genomic amplification product, are given in Table 2. For $S_1$, $S_3$, $S_4$ and $S_6$ the amplification products were longer than the corresponding cDNA fragments, indicating the presence of an intron between the primer positions. The $S_4$ primers initially amplified a longer fragment (approx. 1200 bp) in cultivars not having $S_4$, but upon raising the annealing temperature, this band disappeared.

The position of the forward primers for $S_1$, $S_3$, $S_4$ and $S_6$ is in the hypervariable region between C2 and C3, upstream of the intron, which appeared to be in this region (data not shown), in a position similar to that for introns of other *S* RNases in members of the Rosaceae, notably apple (Broothaerts et al. 1995) and Japanese pear (Ushijima et al. 1998a). The position of the forward primer for $S_2$ and $S_5$ is between C3 and C4. Initially, forward primers designed upstream of the intron were used for $S_2$ and $S_5$ but, as they gave rather long amplification products, these primers did not amplify as reliably under standard conditions as the forward primers downstream of the intron. The position of the reverse primer for $S_1$ is upstream of, and partly includes, C5; for $S_2$, $S_3$ and $S_6$ the reverse primer is in the 3′ untranslated region; for $S_4$ it is between C3 and C4; and for $S_5$ it is in the coding region downstream of C5.

Considerable intron length variation was found between different alleles, as has been reported previously (Tao et al. 1999b). As just explained, a forward primer

**Table 2** Nucleotide sequences of allele-specific primers for $S_1$–$S_6$, cDNA position, optimal annealing temperature for PCR and estimated size of genomic amplification product

| Primer | *S* allele and orientation | Sequence 5′→3′ | cDNA position[a] | Annealing temperature (°C) | Estimated size genomic PCR product (bp) |
|---|---|---|---|---|---|
| PaS1-F | $S_1$ forward | GTA ATT GCA ACG GGT CAA AAT ATG AG | 227–252 | 56 | 820 |
| PaS1-R | $S_1$ reverse | ACA ACT CAG TAT TAG TTG CTG GAT CA | 555–580 | | |
| PaS2-F | $S_2$ forward | TAC TTC GAG CGA TCC CAA A | 400–418 | 50 | 350 |
| PaS2-R | $S_2$ reverse | AAG TGC AAT CGT TCA TTT G | 736–754 | | |
| PaS3-F | $S_3$ forward | GGG TCG CGA TTT AAG AAA GAG C | 238–259 | 60 | 960 |
| PaS3-R | $S_3$ reverse | AAC AAT CGT ACT TTG TGA TGA CTT CG | 704–729 | | |
| PaS4-F | $S_4$ forward | CAC TGG GTC GCT GTT TAA CTT TAG G | 231–255[b] | 60 | 820 |
| PaS4-R | $S_4$ reverse | TTG CAT TTG ATT AAG TGA GGC TTC A | 366–390[b] | | |
| PaS5-F | $S_5$ forward | ACA TGG TAC ATG TTC CCA ACG GAT C | 348–372 | 50 | 300 |
| PaS5-R | $S_5$ reverse | CTG CTG TTC GAT TAC AGT CAA TAT GTA C | 619–646 | | |
| PaS6-F | $S_6$ forward | ACT GGA CCG CAA TTT AAG CG | 235–254 | 53 | 470 |
| PaS6-R | $S_6$ reverse | AGT TGC TGC TTT AAT GGG TGC A | 501–522 | | |

[a] The 'A' of the putative initiation codon (the first AUG) has been numbered +1
[b] Position determined by using the sequence of Tao et al. (1999)

**Table 3** Presence (+) or absence of genomic PCR amplification in 26 cherry cultivars and two selections of known *S* constitution (Matthews and Dow 1969; Boškovic and Tobutt 1996; Boškovic et al. 1997) using specific primers designed from the sequences of the six classes of *S* ribonuclease clones (A–F) obtained from cvs. Early Rivers ($S_1S_2$), Napoleon ($S_3S_4$) and Colney ($S_5S_6$) by 3′ RACE cloning

| Cultivar/selection | Incompatibility group and S genotype | | Early Rivers | | Napoleon | | Colney | |
|---|---|---|---|---|---|---|---|---|
| | | | Class A | Class B | Class C | Class D | Class E | Class F |
| Early Rivers | I | $S_1S_2$ | + | + | | | | |
| F1/3a | I | $S_1S_2$ | + | + | | | | |
| Roundel | I | $S_1S_2$ | + | + | | | | |
| Summit | I | $S_1S_2$ | + | + | | | | |
| Merton Crane | II | $S_1S_3$ | + | | + | | | |
| Van | II | $S_1S_3$ | + | | + | | | |
| Napoleon | III | $S_3S_4$ | | | + | + | | |
| Bing | III | $S_3S_4$ | | | + | + | | |
| Ulster | III | $S_3S_4$ | | | + | + | | |
| Victor | IV | $S_2S_3$ | | + | + | | | |
| Late Black Big | V | $S_4S_5$ | | | | + | + | |
| Turkey Heart | V | $S_4S_5$ | | | | + | + | |
| Governor Wood | VI | $S_3S_6$ | | | + | | | + |
| Merton Heart | VI | $S_3S_6$ | | | + | | | + |
| Bradbourne Black | VII | $S_3S_5$ | | | + | | + | |
| Hedelfingen | VII | $S_3S_5$ | | | + | | + | |
| Hookers Black | VII | $S_3S_5$ | | | + | | + | |
| Merton Late | IX | $S_1S_4$ | + | | | + | | |
| Peggy Rivers | XIII | $S_2S_4$ | | + | | + | | |
| Vic | XIII | $S_2S_4$ | | + | | + | | |
| Lapins | (SC) | $S_1S_4'$ | + | | | + | | |
| Stella | (SC) | $S_3S_4'$ | | | + | + | | |
| Sunburst | (SC) | $S_3S_4'$ | | | + | + | | |
| Colney | | $S_5S_6$ | | | | | + | + |
| Gaucher | | $S_5S_8$ | | | + | | + | |
| Charger | | $S_1S_7$ | + | | | | | |
| Inge | | $S_4S_9$ | | | | + | | |
| Orleans 171 | | $S_{10}S_{11}$ | | | | | | |

designed upstream of the intron was also used initially for $S_2$ and $S_5$. Using these primers a PCR fragment of over 2 kb was amplified for $S_2$, but for $S_5$ two fragments were obtained, approximately 1600 bp and 2100 bp long, so the $S_5$ intron length could not be determined accurately. Estimated intron sizes are 460 bp ($S_1$), 1760 bp ($S_2$), 470 bp ($S_3$), 660 bp ($S_4$) and 180 bp ($S_6$).

## Testing in cultivars

Table 3 shows specific PCR amplification for 26 cherry cultivars and two selections of known *S* genotype with the six primer pairs designed for clones of classes A–F. By comparing amplification patterns with *S* genotype, we were able to assign the classes A–F to the alleles $S_1$–$S_6$.

The primers designed from the class-A *S* RNase sequence from Early Rivers ($S_1S_2$) gave amplification in the seven other cultivars and a selection which share $S_1$, so the sequence is likely to correspond to $S_1$. Likewise class B from Early Rivers ($S_1S_2$) could be assigned to $S_2$.

Primers from Napoleon ($S_3S_4$) class-C clones gave amplification in 12 other cultivars sharing $S_3$, and the sequence is likely to correspond to $S_3$. In addition, they gave amplification in Gaucher ($S_5S_8$), which is discussed later. Primers designed from Napoleon ($S_3S_4$) class-D clones gave amplification in 11 other cultivars which share $S_4/S_4'$ and therefore, the sequence should correspond to $S_4$.

Primers designed from Colney ($S_5S_6$) class-E clones gave amplification in the six other cultivars which share $S_5$, so it is likely that the sequence corresponds to $S_5$. Likewise, class-F from Colney ($S_5S_6$) could be assigned to $S_6$.

Orleans 171 ($S_{10}S_{11}$) did not give amplification with any of the primers. Inge ($S_4S_9$) and Charger ($S_1S_7$) gave amplification only with the $S_4$ and $S_1$ primers, respectively. Gaucher ($S_5S_8$), as just noted, gave amplification with the $S_3$ primers in addition to the expected amplification with the $S_5$ primers.

## Test cross

The cross of Gaucher ($S_5S_8$)×Bradbourne Black ($S_3S_5$) failed, 0 fruit from 200 flowers, showing that these two cultivars are cross-incompatible. In comparison, the control cross of Gaucher ($S_5S_8$)×Van ($S_1S_3$) set 47 fruit from 168 flowers. Thus, $S_3$ and $S_8$ appear to be functionally identical.

## Testing in progenies

Use of the specific primers in three progenies segregating variously for all six *S* alleles, as determined by stylar ribonuclease analysis (Boškovic et al. 1997), established cosegregation of specific amplification products with particular *S* alleles. Figure 2 shows segregating amplification products of allele-specific PCR for the six alleles. Each gel shows a representative sample of ten seedlings from the three progenies analysed. For each seedling the stylar ribonuclease score is given below the gel lane.

The primer pairs found to amplify $S_1$ and $S_2$ specifically in genotyped cultivars, when used for PCR amplification of 46 seedlings of the semi-compatible cross F1/3 ($S_1S_2$)×Charger ($S_1S_7$), gave amplification products cosegregating with $S_1$ and $S_2$, respectively (Fig. 2a). Likewise, amplification products of primer pairs found to amplify specifically $S_1$, $S_3$, $S_4$ and $S_5$ in the analysed cultivars cosegregated with $S_1$, $S_3$, $S_4$ and $S_5$ respectively, in 43 seedlings of the fully compatible cross Bradbourne Black ($S_3S_5$)×Merton Late ($S_1S_4$) (Fig. 2b). In 34 seedlings of the progeny Colney ($S_5S_6$)×Gaucher ($S_5S_8$) primer pairs that were specific for $S_5$ and $S_6$ in the cultivar survey cosegregated with $S_5$ and $S_6$, respectively (Fig. 2c).

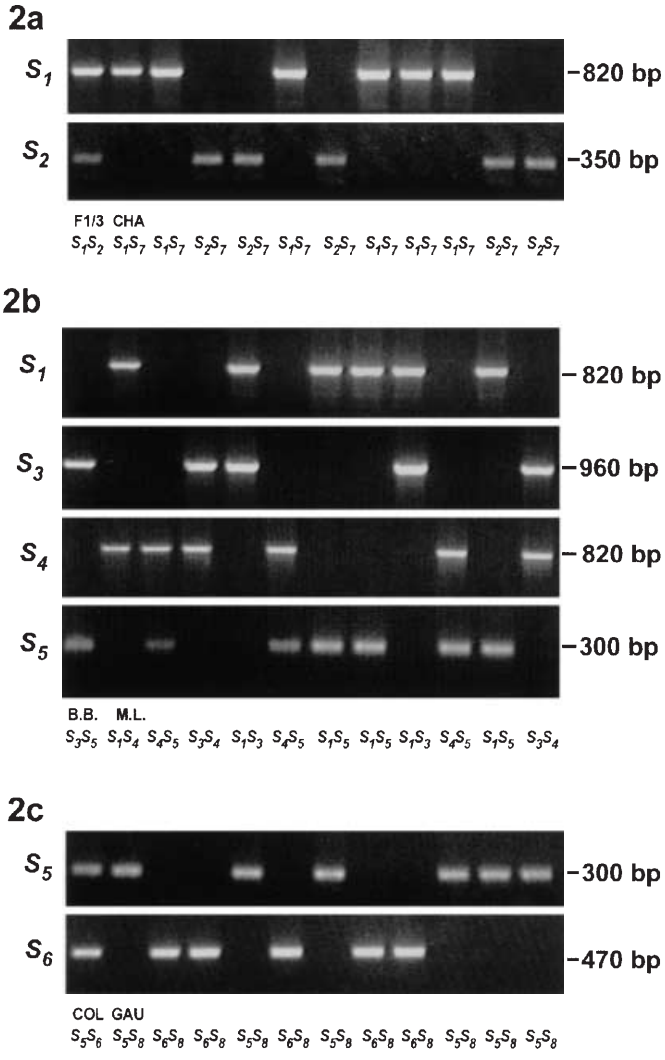


**Fig. 2** Allele-specific PCR analysis of progenies segregating for $S_1$–$S_6$. Stylar ribonuclease scores from the work by Boškovic et al. (1997) are given *below tracks*. **a** PCR amplification of genomic DNA with primers specific for $S_1$ and $S_2$ of the parents and ten seedlings of the semi-compatible cross F1/3 ($S_1S_2$)×Charger (*CHA*, $S_1S_7$), showing segregation for $S_1$ and $S_2$. **b** PCR amplification of genomic DNA with primers specific for $S_1$, $S_3$, $S_4$ and $S_5$ of the parents and ten seedlings of the fully compatible cross Bradbourne Black (*B.B.*, $S_3S_5$)×Merton Late (*M.L.*, $S_3S_5$), showing segregation for $S_1$, $S_3$, $S_4$ and $S_5$. **c** PCR amplification of genomic DNA with primers specific for $S_5$ and $S_6$ of the parents and ten seedlings of the semi-compatible cross Colney (*COL*, $S_5S_6$)×Gaucher (*GAU*, $S_5S_8$), showing segregation for $S_5$ and $S_6$

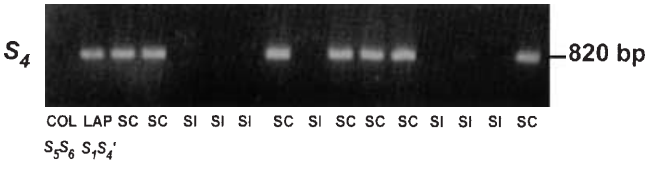


**Fig. 3** PCR amplification of genomic DNA with primers specific for $S_4$ of the parents and 14 seedlings of the cross Colney (*COL*, $S_5S_6$)×Lapins (*LAP*, $S_1S_4'$). Self-compatibility (*SC*) or self-incompatibility (*SI*) as determined by selfing is given *below tracks*

Analysis with primers specific for $S_4$ of the progeny Colney ($S_5S_6$)×Lapins ($S_1S_4'$), which should segregate for self-compatibility, gave $S_4$ amplification with 6 out of 14 seedlings. Upon selfing of the trees in the field the same six seedlings were found to be self-compatible, setting at least 4.5% fruit (Table 4). Figure 3 shows a gel with the 14 seedlings after PCR amplification with primers specific for $S_4$.

## Genotyping cultivars

The analysis by allele-specific PCR of 16 commercially important cultivars, of known parentage but for which the *S* alleles had not yet been reported, gave the scores shown in Table 5. Figure 4 shows PCR amplification of the cultivars for all *S* alleles involved. The genotype obtained for 15 cultivars was in accordance with their known parentage, but the score of $S_1S_4$ for Symphony [Lapins ($S_1S_4'$)×Bing ($S_3S_4$)] was unexpected. The ten cultivars reported as self-compatible, from crosses in which $S_4'$ is involved, showed amplification with the $S_4$ primers as expected, except for LaLa Star [Lambert Compact ($S_3S_4$)×Lapins ($S_1S_4'$)], the score of which was found to be $S_1S_3$. Sumele {Lapins ($S_1S_4'$)×[Van ($S_1S_3$)× Stella ($S_3S_4'$)]}, of unknown incompatibility status, was found to be $S_1S_3$ and should therefore be self-incompatible.

**Table 4** Presence (+) or absence (–) of $S_4$-specific PCR amplification of genomic DNA and percentage fruit set after selfing of 14 seedlings of Colney ($S_5S_6$)×Lapins ($S_1S_4'$)

| Seedling no. | $S_4$ amplification | Fruit set after selfing (%) |
|---|---|---|
| 1 | + | 16.8 |
| 2 | – | 0 |
| 3 | – | 0 |
| 4 | – | 0 |
| 5 | + | 24.0 |
| 6 | – | 0 |
| 7 | + | 8.9 |
| 8 | + | 10.3 |
| 9 | + | 4.5 |
| 10 | – | 0 |
| 11 | – | 0 |
| 12 | – | 0 |
| 13 | + | 9.1 |
| 14 | – | 0 |

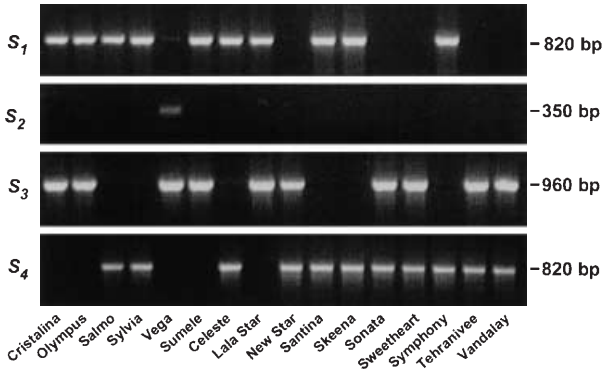


**Fig. 4** PCR amplification of genomic DNA with primers specific for $S_1$, $S_2$, $S_3$ and $S_4$ of cultivars of known parentage of which the *S* genotype has not been reported

**Table 5** Cultivars for which the *S* genotype had not been reported, and their parentage, analysed using allele-specific PCR. For each cultivar a reference for parentage and incompatibility status is given

| Cultivar | Parentage | Reference[a] | *S* alleles[b] |
|---|---|---|---|
| Reportedly self-incompatible | | | |
| Cristalina | Star ($S_3S_4$)×Van ($S_1S_3$) | E | $S_1S_3$ |
| Olympus | Lambert ($S_3S_4$)×Van ($S_1S_3$) | A | $S_1S_3$ |
| Salmo | Lambert ($S_3S_4$)×Van ($S_1S_3$) | A | $S_1S_4$ |
| Sylvia | Van ($S_1S_3$)×Sam ($S_2S_4$) | C | $S_1S_4$ |
| Vega | Bing ($S_3S_4$)×Victor ($S_2S_3$) | A | $S_2S_3$ |
| Incompatibility status not reported | | | |
| Sumele | Lapins ($S_1S_4'$)×[Van ($S_1S_3$)×Stella ($S_3S_4'$)] | B | $S_1S_3$ |
| Reportedly self-compatible | | | |
| Celeste | Van ($S_1S_3$)×Newstar ($S_3S_4'$) | E | $S_1S_4'$ |
| LaLa Star | Lambert Compact ($S_3S_4$)×Lapins ($S_1S_4'$) | F | $S_1S_3$ |
| Newstar | Van ($S_1S_3$)×Stella ($S_3S_4'$) | D | $S_3S_4'$ |
| Santina | Stella ($S_3S_4'$)×Summit($S_1S_2$) | E | $S_1S_4'$ |
| Skeena | [Bing ($S_3S_4$)×Stella ($S_3S_4'$)]×[Van ($S_1S_3$) ×Stella ($S_3S_4'$)] | E | $S_1S_4'$ |
| Sonata | Lapins ($S_1S_4'$)×[Van ($S_1S_3$)×Stella ($S_3S_4'$)] | E | $S_3S_4'$ |
| Sweetheart | Van ($S_1S_3$)×Newstar ($S_3S_4'$) | E | $S_3S_4'$ |
| Symphony | Lapins ($S_1S_4'$)×Bing ($S_3S_4$) | E | $S_1S_4$ |
| Tehranivee | Van ($S_1S_3$)×Stella ($S_3S_4'$) | E | $S_3S_4'$ |
| Vandalay | Van ($S_1S_3$)×Stella ($S_3S_4'$) | E | $S_3S_4'$ |

[a] A, Andersen 1997; B, Edin et al. 1997; C, Lane 1992; D, Lane and Sansavini 1988; E, Lang 1999; F, Sansavini and Lugli 1997

[b] The distinction between $S_4$ and $S_4'$ was based on the consideration of pedigree as the bands were identical

## Discussion

Using the approach described, we obtained cDNA clones of the cherry *S* RNases $S_1$–$S_6$. Correlations with known genotypes of 26 cultivars and two selections were established using allele-specific PCR primers. Their cosegregation with *S* RNases was demonstrated in three progenies segregating for $S_1$–$S_6$, and for self-compatibility in one segregating for $S_4'$. This confirmed the identity of the sequences reported here. Genotypes were assigned to 16 cultivars of previously unknown *S* genotype, including some self-compatible cherries. The sequences of five of the *S* RNases were completed by 5′ RACE. The availability of a range of plant material at HRI East Malling correctly genotyped from stylar ribonuclease analyses proved very useful, especially in the identification of $S_5$. Tao et al. (1999b) reported a PCR test for $S_1$–$S_6$, checked on just 16 cultivars, and in the case of $S_5$ this was based on the incorrect assumption that Burlat and Moreau have this allele, as discussed later.

### Sequence analysis

The deduced amino acid sequences possess the conserved regions of rosaceous *S* RNases, particularly of *Prunus* (Broothaerts et al. 1995; Norioka et al. 1995, 1996; Ushijima et al. 1998b). The amino acid sequences were 68–84% identical with each other. Alleles $S_3$ and $S_6$ had previously been sequenced by Tao et al. (1999b) and a third, $S_2$, had been partially sequenced. Our sequences were similar to those but were longer at the 5′ end and in some cases varied in length at the 3′ end; however, this did not alter the predicted protein sequence. The sequences for $S_6$ show four nucleotide differences, which affect the protein sequence for two amino acids at the 3′ end. Differences in the sequence found could be due to PCR artefacts or to variation in the sequence of the same allele from a different source. The length of the 5′ untranslated region obtained was dependent on the quality of the RNA sample used. Multiple polyadenylation signals which may occur at the 3′ end could account for transcripts of various lengths.

During preparation of this manuscript the sequences for $S_1$ and $S_4$ were submitted to the EMBL database (Tao et al. 1999a). These are similar to our sequences for $S_1$ and $S_4$. The sequences for $S_1$ differ for one nucleotide, not affecting the protein sequence. For $S_4$, our sequence has an extra base at two positions in the 3′ untranslated region. For $S_5$, no cDNA sequence has previously been reported.

The recent increase in the number of *S* alleles sequenced in cherry, and indeed in Rosaceae, should contribute to phylogenetic studies of the *S* locus, such as those conducted by Sassa et al. (1996), Richman et al. (1997) and Ushijima et al. (1998b).

### Design and specificity of allele-specific primers

Cloning and sequencing of cherry *S* RNases has allowed us to design six different pairs of allele-specific primers that amplify specifically the six *S* alleles $S_1$–$S_6$ in genomic PCR, and we have validated these in cultivars and progenies that had been previously genotyped.

The allele-specific primers are reliable when used on material carrying combinations of $S_1$–$S_6$, which are frequent amongst commercial cultivars (Matthews and Dow 1969). As sequences of all six alleles were available and as these were sufficiently different from each other, primers could be designed which amplify only one particular allele. Subsequent restriction enzyme digestions are not necessary to distinguish certain alleles, in contrast to the method developed by Janssens et al. (1996) for identifying various apple *S* alleles. The annealing temperatures given in Table 2 were found to be optimal under our conditions. It may be that when the primers are used under different conditions, slightly different annealing temperatures will be optimal.

The primers might also amplify alleles different from $S_1$–$S_6$. However, a check on $S_7$–$S_{11}$ showed no amplification, with the exception of $S_8$, which is amplified with the $S_3$ primers, as discussed later. And testing Schneiders ($S_3S_{12}$), Noble ($S_6S_{13}$) and Dikkeloen ($S_5S_{14}$) (Boškovic and Tobutt 2001) showed no amplification of the ‘new’ alleles $S_{12}$–$S_{14}$ (data not shown). To be able to design allele-specific primers for $S_7$–$S_{14}$, or other *S* alleles that might be detected, the *S* RNases of those need to be cloned and sequenced and compared with the known sequences. When more sequences become available, some of the primers for $S_1$–$S_6$ may have to be re-designed to make them specific also when used on material with alleles different from $S_1$–$S_6$, or restriction enzyme digestions may be necessary to discriminate between some alleles.

The amplification of $S_8$ with the $S_3$ primers suggested that the two alleles are very similar with respect to the sequences from which the primers were designed. Moreover, the products of the two alleles have very similar pI values (Boškovic et al. 1997). However, the possibility that $S_3$ and $S_8$ are essentially the same is inconsistent with the John Innes Institute’s assignment of Gaucher ($S_5S_8$) not to Group VII ($S_3S_5$ according to Boškovic et al. 1997), the group to which it should belong if $S_3$ and $S_8$ are the same, but to Group O, the universal donor group (Matthews and Dow 1969). Nevertheless, the failure of the test cross Gaucher ($S_5S_8$)×Bradbourne Black ($S_3S_5$) shows that $S_3$ and $S_8$ have the same function. A slight electrophoretic difference between *S* RNases reportedly of the same function has been noted in apple for $S_{10}$ and $S_{10A}$ (Boškovic and Tobutt 1999) and may indicate heterogeneity of the glycan chains attached to the *S* RNases or a change in amino acid sequence not affecting the specificity.

### Application to cherry growing and breeding

The opportunity to genotype cultivars and selections from vegetative material will be useful for pomologists and breeders. It will allow appropriate pollinators to be

determined and the compatibility of planned crosses to be checked, or the semi-compatibility of those crosses aimed to give only self-compatible seedlings (Williams and Brown 1960).

When allele-specific primers are used for all *S* alleles that can be expected in a plant or progeny, on the basis of the parentage, false negatives, which are known to occur in PCR but which were encountered here only occasionally, can be detected and the tests repeated.

Selection for self-compatibility determined by $S_4'$ can be carried out soon after germination in appropriate progenies using the primers specific for $S_4$, if necessary in combination with primers for other *S* alleles involved in the cross. It is likely that the $S_3$ primers will likewise be useful for detecting $S_3'$ self-compatible seedlings derived from another self-compatible cherry selection, JI 2434 (Boškovic et al. 2000).

Using allele-specific PCR, the *S* genotype has been determined for 16 cultivars of known parentage for which the *S* alleles had not been reported. The genotype obtained for 15 cultivars was in accordance with their parentage (Table 5). However, the score of $S_1S_4$ for Symphony [Lapins ($S_1S_4'$)×Bing ($S_3S_4$)] casts doubts on its reported parentage. $S_4$ pollen of Bing would be expected to fail on the style of Lapins, as the $S_4'$ allele of Lapins, inherited from JI 2420, via Stella, is known to have lost pollen activity but not stylar function (Matthews 1970). Symphony is reported to be self-compatible (Lang 1999), and in that case may have resulted from selfing Lapins rather than from the cross Lapins×Bing. The ten cultivars reported as self-compatible, derived from crosses in which at least one of the parents has $S_4'$, showed amplification with the $S_4$ primers as expected, except for LaLa Star [Lambert Compact ($S_3S_4$)×Lapins ($S_1S_4'$)], the score of which was $S_1S_3$. This is in accordance with the given parentage but not with reports that it is self-compatible (Sansavini and Lugli 1997). Assuming that the tree we analysed is true to name, LaLa Star is unlikely to be self-compatible as it has inherited $S_1$ from Lapins instead of $S_4'$. An alternative explanation, that self-compatibility in derivatives of JI 2420 is not associated with $S_4$, has been discussed and dismissed by Boškovic et al. (2000).

## Comparison with other *S* allele detection methods

The advantage of the allele-specific PCR detection method described here over the method of Boškovic and Tobutt (1996), which involves the IEF of stylar proteins and staining for ribonuclease activity, is that flowers are not needed. The method can detect the alleles $S_1$–$S_6$ and $S_4'$ from vegetative material, for example from graftwood of a new cultivar or from cotyledons of seedlings. However, so far, no PCR-based method has been demonstrated for detecting alleles other than $S_1$–$S_6$.

The allele-specific primers for $S_5$ reported here provide the first PCR detection of the genuine $S_5$. Tao et al. (1999b) designed allele-specific tests for $S_1$–$S_6$ based on combinations of amplification patterns obtained after PCR amplification with three primers designed from conserved regions of three *S* RNase DNA sequences (to be used in two pairs). Differences in the size of the amplification products are due to significant intron length variation between the *S* alleles, which seems to be characteristic for rosaceous *S* alleles (Broothaerts et al. 1995; Ishimizu et al. 1999). However, as mentioned earlier, the results reported by Tao et al. (1999b) for $S_5$ need reinterpretation. Burlat and Moreau, the only two cultivars used as a representatives of $S_5$ in their work, had been assigned provisionally to Group VII, genotyped as $S_4S_5$ by the John Innes Institute (Matthews and Dow 1969). However, Boškovic and Tobutt (1996) and Boškovic et al. (1997) corrected the genotype of this group to $S_3S_5$, and Boškovic and Tobutt (2001) showed that Burlat and Moreau do not belong to Group VII and do not have $S_5$. Therefore, the band amplified in Burlat and Moreau, considered by Tao et al. (1999b) to correspond to $S_5$, does not represent this allele.

In addition, the primers of Tao et al. (1999b) do not amplify the real $S_5$ (data not shown). Their reverse primer, based on conserved DNA sequences of $S_2$, $S_3$ and $S_6$, does not match the $S_5$ sequence reported in this work at several positions, including the last nucleotide, at the 3′ end.

PCR methods using consensus primers, based on conserved regions, have been reported for *S* alleles in *Brassica oleracea* (Brace et al. 1993), cherry (Tao et al. 1999b) and Japanese pear (Ishimizu et al. 1999). These methods allow the detection of *S* alleles by carrying out only one or two PCR reactions, if necessary followed by restriction enzyme digestions. The complex patterns obtained, however, are not always easy to interpret. In addition, in *Brassica*, preferential amplification of one of the alleles appeared to be a problem, although this did not seem to occur in Japanese pear. For allele-specific PCR, individual PCR reactions have to be carried out for each *S* allele, but they provide, rather than fragment size, a straightforward plus/minus assay.

To enable the development of a PCR detection method for the new *S* alleles, $S_7$–$S_{14}$ or any other alleles that might be detected, DNA sequences of these alleles are needed, to enable not only the design of allele-specific primers from variable regions but also the design of reliable consensus primers from conserved regions. The consensus primer approach relies on intron length variation between the alleles, but it is not known whether there is enough intron length variation in cherry to distinguish all known alleles. The interpretation of results will certainly become more difficult. Once DNA sequences are available it will be straightforward to design allele-specific primers, if necessary to be applied in combination with restriction enzyme digestions.

## Final remarks

The allele-specific PCR method has already proved useful in genotyping cherry cultivars and in detecting self-

compatible seedlings. Using the same approach an allele-specific PCR detection method can be developed for the more recently detected alleles $S_7$–$S_{15}$. This should be useful for cherry growing and breeding as well as for cherry self-incompatibility studies. The sequence information presented here will be valuable for further work on cherry self-incompatibility, which could include studies of allele specificity, the nature of the intron, molecular evolution of the *S* locus and the molecular basis of self-(in)compatibility in cherry.

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