Theor Appl Genet (2003) 107:1059–1070
DOI 10.1007/s00122-003-1274-4

T. Sonneveld · K. R. Tobutt · T. P. Robbins

Allele-specific PCR detection of sweet cherry self-incompatibility (S) alleles *S1* to *S16* using consensus and allele-specific primers

Received: 10 January 2003 / Accepted: 26 February 2003 / Published online: 14 June 2003
© Springer-Verlag 2003

Abstract PCR-based identification of all 13 known self-incompatibility (*S*) alleles of sweet cherry is reported. Two pairs of consensus primers were designed from our previously published cDNA sequences of S_1 to S_6 *S*-RNases, the stylar components of self-incompatibility, to reveal length variation of the first and the second introns. With the exception of the first intron of S_{13}, these also amplified S_7 to S_{14} and an allele previously referred to as S_x, which we now label S_{16}. The genomic PCR products were cloned and sequenced. The partial sequence of S_{11} matched that of S_7 and the alleles were shown to have the same functional specificity. Allele-specific primers were designed for S_7 to S_{16}, so that allele-specific primers are now available for all 13 *S* alleles of cherry (S_8, S_{11} and S_{15} are duplicates). These can be used to distinguish between *S* alleles with introns of similar size and to confirm genotypes determined with consensus primers. The reliability of the PCR with allele-specific primers was improved by the inclusion of an internal control. The use of the consensus and allele-specific primers was demonstrated by resolving conflicting genotypes that have been published recently and by determining genotypes of 18 new cherry cultivars. Two new groups are proposed, Group XXIII (S_3S_{16}), comprising 'Rodmersham Seedling' and 'Strawberry Heart', and Group XXIV (S_6S_{12}), comprising 'Aida' and 'Flamentiner'. Four new self-compatibility genotypes, S_3S_3', $S_4'S_6$, $S_4'S_9$ and $S_4'S_{13}$, were found. The potential use of the consensus primers to reveal incompatibility alleles in other cherry species is also demonstrated.

Communicated by H.F. Linskens

T. Sonneveld · K. R. Tobutt (✉)
Horticulture Research International, East Malling,
West Malling, Kent ME19 6BJ, UK
e-mail: ken.tobutt@hri.ac.uk
Tel.: +44-(0)-1732-843833
Fax: +44-(0)-1732-521557

T. Sonneveld · T. P. Robbins
University of Nottingham, Plant Science Division,
Sutton Bonington Campus, Loughborough,
Leicestershire LE12 5RD, UK

Keywords Cherry · Consensus and allele-specific primers · *Prunus avium* · Self-incompatiblity · *S*-RNase

Introduction

Self-incompatibility and incompatibility between cultivars in cherry (*Prunus avium* L.) is governed by the gametophytic, multi-allelic *S* locus (Crane and Lawrence 1929). Cultivars with the same *S* genotype are cross-incompatible. When cherries are grown commercially, compatible cultivars that flower simultaneously are planted together to ensure fruit set. Thus the *S* genotype of cherry cultivars is an agronomically important character. For many years just six *S* alleles, S_1 to S_6, were known; these had been detected via cross-pollination tests, in work carried out at the John Innes Institute (e.g. Crane and Brown 1937) and elsewhere. A classic table of cultivar genotypes was presented by Matthews and Dow (1969).

More recently, with the finding that *S* alleles in cherry code for stylar ribonucleases (*S*-RNases) (Bošković and Tobutt 1996; Bošković et al. 1997), new alleles have been reported. Bošković et al. (1997) and Bošković and Tobutt (2001) reported S_7 to S_{14} on the basis of zymograms of stylar proteins, though S_8 was subsequently found to be functionally the same as S_3 (Sonneveld et al. 2001).

PCR-based methods to detect *S* alleles in cherry have also been developed. Two papers described various pairs of consensus primers that amplify the two introns of cherry *S*-RNases. Tao et al. (1999) designed three primers from three cherry *S*-RNase sequences, which, in combination, could distinguish S_1 to S_4, S_6 and an allele they reported as S_5, but which is S_9 (Tobutt et al. 2001), because of the different lengths of the introns. Similarly, Wiersma et al. (2001) designed two pairs of consensus primers from cherry sequences to reveal length polymorphism of the two introns, digesting amplification products with restriction enzymes to distinguish between some alleles with introns of similar size. However, no data were given for amplifying an allele they labelled as S_{14}, and

only one primer pair worked for an allele they reported as S_{15}, but which is S_5 (Tobutt et al. 2001). With such approaches, various alleles with labels other than S_1 to S_6 have been reported, although whether they corresponded to the alleles already published was not ascertained (Choi et al. 2000; Yamane et al. 2000a; Wiersma et al. 2001).

Sonneveld et al. (2001) sequenced cDNAs of S_1 to S_6 and developed allele-specific primers for these six alleles; these revealed the considerable polymorphism in the length of the second intron that lies in the hypervariable (RHV) region of the *S*-RNase. The S_4 specific primers also detect the self-compatibility allele S_4' (where $'$ denotes pollen-part mutation). The allele-specific primers, though useful for genotyping cultivars and seedlings with combinations of S_1 to S_6, do not detect "new" alleles. Moreover, the S_2-specific primers often give weak amplification (data not shown).

Recently, Bošković and Tobutt (2001) used *S*-RNase zymograms and, sometimes, test crossing to re-examine many of the cultivars genotyped in the table of Matthews and Dow (1969). Tobutt et al. (2001) published a 'Harmonization Table' by adding recent results from institutes in Canada, Germany, Japan and USA to the East Malling genotypes, and reconciling the various allele numbers and letters that had been published to a single system. They drew attention to several anomalies in which different institutes have assigned different genotypes to the same cultivar, even after due allowance for allele labelling differences. The self-incompatible cultivars in the Harmonization Table comprise 22 groups, i.e. distinct *S* genotypes, and a small group O, of unique genotypes. In this paper we follow the consensus allele labelling from the Harmonization Table.

The length variation of the introns and the conservation of various regions of the exons of S_1 to S_6 (Sonneveld et al. 2001) prompted us to design consensus primers that would amplify regions including the introns to distinguish not only those six alleles but also additional ones. These would be useful for determining the genotypes of untested cultivars and seedlings. The sequences of S_1 to S_6 allowed us to determine which DNA regions were most conserved, which was important for designing consensus primers that would amplify S_5. From the sequence of S_5 it was clear that the consensus primers of Tao et al. (1999) could not amplify this allele (Sonneveld et al. 2001); nor did the second intron primers of Wiersma et al. (2001) amplify S_5. To improve discrimination of alleles that appeared to have introns of similar length, we designed allele-specific primers for the new alleles S_7 to S_{14} and for one that had provisionally been labelled S_x (Bošković and Tobutt 2001), from their genomic sequences. We also designed a new, more reliable, forward primer for S_2 and optimised further the annealing temperatures of the previously published primers for S_1 to S_6 (Sonneveld et al. 2001). In addition, an internal control was used for PCRs with the allele-specific primers, greatly reducing the problem of false negatives. We used the consensus and allele-specific primers to investigate the anomalies in several cultivar genotypes revealed by Tobutt et al. (2001), and we determined the genotype of new cultivars in trials at East Malling. Furthermore, to investigate their general applicability in cherry, we tested the consensus primers on a range of cherry species.

Materials and methods

Plant material

Cultivars used as standards for S_1 to S_{16}

Ten standard cultivars were used for the cloning and sequencing of the genomic PCR products of S_1 to S_{14} and for S_x (Bošković and Tobutt 2001), that we later designate S_{16} (Table 1). Extra representatives of the alleles S_7 to S_{13} and S_x that were used to check the consistency of the PCR banding patterns are: 'Cryall's Seedling' (S_2S_7), 'Guigne d'Annonay' (S_2S_7), 'Burlat' (S_3S_9), 'Ramon Oliva' EM (S_6S_9), 'Black Tartarian E' (S_6S_9), 9129–67 (S_3S_{10}), 9129–72 (S_4S_{10}), 'Flamentiner' (S_6S_{12}), 'Wellington A' (S_3S_{13}), 'Goodnestone Black' (S_5S_{13}) and 'Rodmersham Seedling' (S_3S_x). Genotypes of cultivars are as given in Bošković and Tobutt (2001); two seedlings of progeny 9129 ('Napoleon' × Orleans 171) had been genotyped by Bošković and Russell (personal communication).

To check the possible functional identity of S_7 and S_{11}, 40 seedlings from the cross of 'Charger' (S_1S_7) × Orleans 171 ($S_{10}S_{11}$) (progenies 9232 and 9332) were genotyped for *S* alleles.

The cultivars and seedlings were growing at HRI East Malling, except for the following, which were supplied by the National Fruit Collection, Brogdale, UK: 'Dikkeloen', 'Cryall's Seedling', 'Guigne d'Annonay', 'Burlat', 'Ramon Oliva' EM, 'Black Tartarian E', 'Flamentiner', 'Wellington A' and 'Goodnestone Black'.

Cultivars for genotyping

Two accessions of each of six cultivars that had been reported with conflicting genotypes by different institutes (Tobutt et al. 2001) were used for genotyping with consensus and allele-specific primers. The accessions we analysed are the ones that had been used in the relevant publications. Table 5 includes origins, and accession numbers where known. For each of groups VIII, XXI and XXII, of which only one cultivar had previously been genotyped at East Malling, another representative was included. Eighteen cultivars currently in trials at East Malling that had not previously been genotyped were also analysed (Table 6).

Table 1 Cherry cultivars used as standards for alleles S_1 to S_{16}

S allele	Standard cultivar[a]	*S* genotype
S_1, S_2	Early Rivers	S_1S_2
S_3, S_4	Napoleon	S_3S_4
S_5, S_6	Colney	S_5S_6
S_7	Charger	S_1S_7
S_9	Inge	S_4S_9
S_{10}	Orleans 171	$S_{10}S_{11}$[b]
S_{12}	Schneiders Späte Knorpelkirsche	S_3S_{12}
S_{13}	Noble	S_6S_{13}
S_{14}	Dikkeloen	S_5S_{14}
S_{16}	Strawberry Heart	S_3S_x[c]

[a] Sources of all cultivars are as shown in Bošković and Tobutt (2001)
[b] Reported as $S_{10}S_{11}$ (Bošković et al. 1997), but this paper finds $S_{11} = S_7$ (see Results)
[c] S_x (Bošković and Tobutt 2001) re-labelled later in this paper as S_{16}

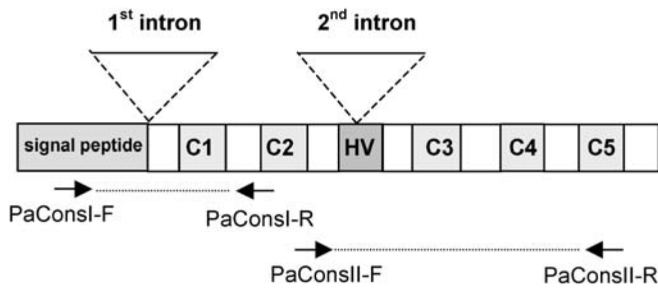

Fig. 1 Structure of a *Prunus S*-RNase (Ushijima et al. 1998; Tao et al. 1999) with intron locations and position of consensus primers (not to scale)

Species

To investigate whether the cherry consensus primers could be used in other cherry species, they were tested on two accessions of each of six diploid, presumably self-incompatible, cherry species, belonging to various sections of the subgenus Cerasus (Adans.) Focke, available at HRI East Malling. These were *Prunus canescens* Bois, *Prunus incisa* Thunb., *Prunus mahaleb* L., *Prunus nipponica* Matsum., *Prunus sargentii* Rehd. and *Prunus subhirtella* Miq. Accession numbers are given in the legend to Fig. 5.

Consensus primers – design and PCR

Design

Full-length cDNA sequences of S_1 to S_6 (Tao et al. 1999; Sonneveld et al. 2001) [EMBL accession numbers: AJ298310 (S_1), AJ298311 (S_2), AJ298312 (S_3), AJ298313 and AB028154 (S_4), AJ298314 (S_5) and AJ298315 (S_6)] were aligned using the Clustal method of the Megalign computer program (DNAStar Inc.). Two pairs of consensus primers were designed from three regions that were highly conserved among the six alleles, the signal peptide C2 and C5 (Table 2; Fig. 1). The first pair, PaConsI-F and PaConsI-R, was designed to amplify the first intron, located between the regions coding for the signal peptide region and the mature protein. The second pair, PaConsII-F and PaConsII-R, was designed to amplify the second intron, located in the RHV region between C2 and C3 (Fig. 1).

PCR amplification of the second intron

Approximately 25–100 ng of genomic DNA, prepared as described previously (Sonneveld et al. 2001), was used for PCR amplification in a 25-μl reaction, containing 1 × PCR buffer (Qiagen) with unspecified concentrations of Tris-HCl, KCl and $(NH_4)_2SO_4$, pH 8.7, a final concentration of 2.0 mM $MgCl_2$, 0.25 × 'Q solution' (Qiagen), 0.2 mM of dNTPs, 0.2 μM of each primer and 1.25 U of *Taq* DNA polymerase (Qiagen). PCR reactions were set up on ice and the tubes were transferred to a PTC-200 thermal cycler (MJ Research) once the block had reached 94 °C (simple hot start) and run for 2 min at 94 °C initial denaturing, 10 cycles of 10 s at 94 °C, 2 min at 58 °C and 2 min at 68 °C, followed by 25 cycles of 10 s at 94 °C, 2 min at 58 °C and 2 min at 68 °C with 10 s added each cycle to the 68 °C extension step. The PCR products were separated on a 1.3% agarose gel (20 cm wide × 25 cm long) for about 16 h at 60 V until the bromophenol blue front was approximately 2 cm from the end of the gel. 1-kb + molecular weight ladders (Invitrogen) were included. After staining for 1 h in a 0.5 μl/ml solution of ethidium bromide, a photograph was taken of the gel on an ultraviolet transilluminator.

The particular $MgCl_2$ concentration and cycling conditions (based on a Qiagen protocol for the amplification of long PCR products) for the second intron PCR were found to be critical for the amplification of certain alleles and need to be followed carefully. For example, bands for S_1, S_2, S_5, S_{10}, S_{13} and S_{16} did not amplify when 'normal' PCR cycles (3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 58 °C, 1 min and 30 s at 72 °C, followed by 5 min at 72 °C) were used. The addition of the Qiagen 'Q solution' (which changes the melting behaviour of nucleic acids) also helps consistent amplification, but may not be essential.

PCR amplification of the first intron

The 25-μl PCR contained final concentrations of 1 × PCR buffer (Qiagen), 2.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.2 μM of each primer and 0.625 U of *Taq* DNA polymerase, and was set up as described above. PCR was carried out for 2 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 54 °C and 1 min at 72 °C, with a 5-min final extension step at 72 °C. The products were run as described above, but on a 2% agarose gel, for about 17 h at 60 V. After staining, a photograph of the gel was taken immediately, as the bands tended to fade quickly due to small product size.

Cloning and sequencing of genomic PCR products

Genomic amplification products of the alleles S_1 to S_{14} and S_x from the standard cultivars for both pairs of consensus primers were cloned using a TA cloning kit (Invitrogen). Cloning and screening of colonies were as described in Sonneveld et al. (2001). Two clones of each were sequenced (Qiagen).

Allele-specific primers – design and PCR

The genomic sequences of the 13 known alleles were aligned, with coding regions and introns separately, using the Clustal method of the Megalign software (DNAStar Inc.). Using coding and intron sequence information as appropriate, allele-specific primers for S_7, S_9, S_{10}, S_{12}, S_{13}, S_{14} and S_x, later named S_{16}, were designed (19–26-bp long) to give genomic amplification products between approximately 300 and 700 bp. In addition, the intron sequence information for S_2 allowed the design of a new, more reliable, S_2-specific forward primer.

Allele-specific primer sequences with recommended annealing temperatures for PCR and the size of the genomic amplification products are given in Table 3. PCR reactions with the allele-specific primers were set up as described for the consensus primers, with final concentrations and cycles as given in Sonneveld et al. (2001). For each PCR two cultivars positive for the appropriate *S*

Table 2 Nucleotide sequences of the consensus primers for PCR amplification of the first and the second intron of cherry *S*-RNases

Primer[a]	Sequence 5′ → 3′	Annealing temp. (°C)	Region amplified
PaConsI-F	(C/A)CT TGT TCT TG(C/G) TTT (T/C)GC TTT CTT C	54	Signal peptide region to:
PaConsI-R	CAT G(A/G)A TGG TGA A(A/G)T (T/A)TT GTA ATG G		C2, incl. 1st intron
PaConsII-F	G GCC AAG TAA TTA TTC AAA CC	58	C2 to:
PaConsII-R	CA(T/A) AAC AAA (A/G)TA CCA CTT CAT GTA AC		C5, incl. 2nd intron

[a] F = forward, R = reverse

Table 3 Nucleotide sequences of allele-specific primers designed for S_7 to S_{16} and re-designed for S_2, optimal annealing temperature for PCR and size of the genomic amplification product

S allele	Primer[a]	Sequence 5′ → 3′	Annealing temp. (°C)[b]	Size of genomic PCR product (bp)
S_2	PaS2-Fnew	CC TGC TTA CTT TGT CAC GCA	57–61	640
	PaS2-R[c]	AAG TGC AAT CGT TCA TTT G		
S_7	PaS7-F	AGC TTC TTT AGC GAC GTT AGA TG	55–60	584
	PaS7-R	TGC ATT TGG TTT AGT TTC TCT ACA		
S_9	PaS9-F	TT TGT TAC GTT ATG AGC AGC AG	58–62	495
	PaS9-R	ATG AAA CAA TAC ATA CCA CTT TGC TA		
S_{10}	PaS10-F	GTT TGA CGA TGC TCA GTA TCA C	58–62	505
	PaS10-R	GT ACT TCC ATC TTT GTC TTG CAC		
S_{12}	PaS12-F	ATT CTG ATG CTG GTC CTA TAG	59–63	562
	PaS12-R	AAC TCA GGC TTA TTA GGG TG		
S_{13}	PaS13-F	CA ATG GGT CGC AAT TTG ACG A	62–66	306
	PaS13-R	GGA GGA GGT GGA TTC GAA CAC TTG		
S_{14}	PaS14-F	G CAG AAT TTG GTA TGT GTT GGA	61–65	468
	PaS14-R	GG ATC GCT GGA AGT ATT GCA TTA T		
S_{16}	PaS16-F	T CAT CAA TTG CGT GAT TAG CAG	57–61	429
	PaS16-R	TGT ACC ATG TTT GTT CCA TTC CAT		

[a] F = forward, R = reverse
[b] The primers work at a range of temperatures; if internal control primers are included in the PCR a lower temperature is recommended, if internal control primers are not included a higher temperature is recommended. The new range of recommended annealing temperatures for the previously published allele-specific primers for S_1 to S_6 are: 61–65 °C (S_1), 63–67 °C (S_3), 60–64 °C (S_4), 50–53 °C (S_5) and 62–66 °C (S_6)
[c] Reported by Sonneveld et al. (2001)

allele and two cultivars negative for that *S* allele were included in each PCR as controls.

Internal control for PCR with allele-specific primers

To be able to identify false negatives in PCR reactions with allele-specific primers, the primers IC-F (5′-C AAA TTG AAG CTG CAG CAA TTA TGG A-3′) and IC-R (5′-GG TAA GAC CTG CAT TCC GTA ATC CTG TT-3′) were included in the PCR at 0.1 mM as an internal control. They were designed from the cherry DNA sequence of the nuclear gene for phenylalanine ammonia-lyase (PAL1) (Wiersma and Wu 1998; EMBL accession number AF036948) and are described elsewhere (Sonneveld et al., in preparation). They amplify a product of about 1,036 bp in cherry cultivars, which is longer than any of the *S* allele-specific products. For the approximate 960-bp product of S_3 the gels were run long enough to separate the band from the PAL band. As the *S* allele-specific bands tended to be weaker when the internal control was included, lower annealing temperatures are recommended (Table 3). When PAL primers were included in the PCR with S_{13}-specific primers, the PAL band did not always amplify in samples in which the S_{13} band amplified. However, the primers still work as internal control, as samples not amplifying the S_{13} band amplified the PAL band consistently.

Genotyping cultivars and cherry species

The *S* alleles present in the various cultivars of known, uncertain or unknown genotype were amplified with consensus primers for the first and the second intron, and the provisional genotypes were confirmed with appropriate allele-specific primers. Both sets of consensus primers were tested on the species accessions.

Results

Consensus primers and sequencing

The two pairs of consensus primers, designed from conserved coding regions flanking the two introns of cherry *S*-RNases that are variable in length (Fig. 1), were tested on cherry cultivars of known *S* genotype, representing the *S* alleles S_1 to S_{14} and the S_x allele of 'Strawberry Heart', later labelled S_{16}. In most of the standard cultivars tested, each of the two pairs of consensus primers amplified two bands of various sizes (Fig. 2). By comparison of the banding pattern with the known *S* genotype of cultivars, the bands could be correlated with particular *S* alleles. This indicates that the consensus primers recognise not only S_1 to S_6, from which they are designed, but also S_7 to S_{14} and S_x/S_{16}.

The sequences appeared to have the primary structural features of rosaceous *S*-RNases (Ushijima et al. 1998) and showed homologies with the published cherry *S*-RNase sequences, indicating that they were indeed *S*-RNases (data not shown). The two partial sequences for each allele have been submitted to the EMBL database.

The size of the genomic amplification products for S_1 to S_{14} and S_x/S_{16} with the consensus primers for the first and the second intron (Fig. 1) ranged from 303 to 523 bp for the former and from 577 to 2,383 bp for the latter (Fig. 2; Table 4). The primers for the first intron failed to amplify S_{13}, which was therefore not sequenced. The primers for the second intron gave weak amplification of S_5, and a second, lower band was consistently associated with this allele. Several other alleles occasionally gave weak, secondary bands on the gel with these primers, as indicated in Table 4. Attempts to clone (and sequence) the

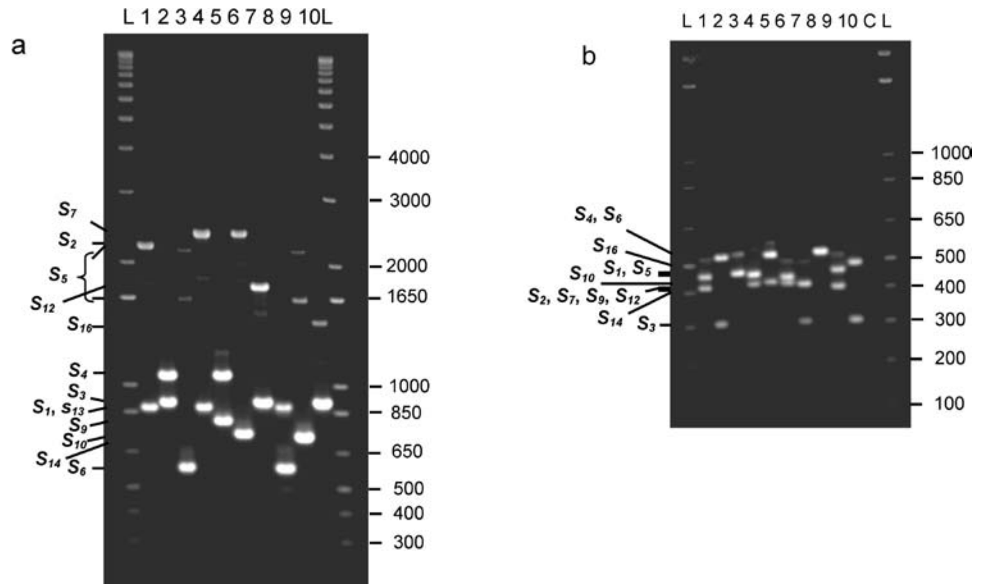

Fig. 2 PCR amplification of cultivars used as standards for S_1 to S_{16} with **a** consensus primers for the second intron, and **b** consensus primers for the first intron. Samples are (from the left): 1-kb + ladder, *1* 'Early Rivers' (S_1S_2), *2* 'Napoleon' (S_3S_4), *3* 'Colney' (S_5S_6), *4* 'Charger' (S_1S_7), *5* 'Inge' (S_4S_9), *6* Orleans 171 (S_7S_{10}), *7* 'Schneiders Späte Knorpelkirsche' (S_3S_{12}), *8* 'Noble' (S_6S_{13}), *9* 'Dikkeloen' (S_5S_{14}), *10* 'Strawberry Heart' (S_3S_{16}), [1st intron only: negative control (water) (C)], 1-kb + ladder

Table 4 Size of genomic amplification products for S_1 to S_{16} obtained with the consensus primers for the first and the second intron

S allele	Size of genomic PCR products (bp) with consensus primers	
	1st intron PCR product (PaConsI-F + -R)	2nd intron PCR product[a] (PaConsII-F + -R)
S_1	456	874 (+ ~800)
S_2	419	2,204 (+ ~1,800)
S_3	303	898 (+ ~825 + ~950)
S_4	523	1,064 (+ ~950 + ~1,200)
S_5	462	2,159[b] + ~1,650[b]
S_6	518	577
S_7	420	2,385 (+ ~1,850)
S_9	428	798
S_{10}	439	734
S_{12}	420	1,773 (+ ~1,500)
S_{13}	(Not amplified)	874[b] (+ ~490 + ~330)
S_{14}	407	719
$S_{x/16}$	485	1,454[b]

[a] Sizes in brackets are approximate and indicate very weak secondary bands that do not always appear
[b] Both S_5 bands, the S_{13} band and the S_{16} band are weak

lower band of S_5 failed. Sequencing the PCR product directly was also unsuccessful. A similar problem was encountered with the first intron PCR product for S_{11}.

Possible identity of S_7 and S_{11}

The alleles S_7 and S_{11} could not be distinguished with the consensus primers for the first and the second intron as the PCR products had the same size (Fig. 2). Moreover, we found no evidence that the S_{11} sequence is different from S_7. The (partial) coding sequence of the genomic product for S_{11}, amplified using the consensus primers for the second intron, appeared to be identical to the S_7 sequence. This suggested that S_{11} could be functionally the same as S_7. Genotyping of 40 seedlings of the progeny 'Charger' (S_1S_7) × Orleans 171 ($S_{10}S_{11}$) for S alleles using consensus primers for the second intron to test this showed that 18 seedlings were S_1S_{10}, 21 seedlings S_7S_{10} and one seedling S_1S_{11} (assignment of S_7 or S_{11} is on the basis of parentage, because they are indistinguishable by PCR). With the exception of the S_1S_{11} seedling, the segregation suggests that the cross was semi-compatible and that S_7 and S_{11} are indeed functionally the same. The S_1S_{11} seedling was checked with a polymorphic microsatellite marker, and the fingerprint was consistent with that of the parents (data not shown), suggesting that this seedling was not a contaminant, but that the S_7 style had not rejected an S_{11} pollen grain.

S_x of 'Strawberry Heart' re-labelled S_{16}

With consensus primers for both introns, 'Strawberry Heart', reported as S_3S_x (Bošković and Tobutt 2001), showed a band distinct from those for the alleles already numbered, S_1 to S_{14}. Sequencing confirmed that S_x represented indeed a new allele, which we propose labelling S_{16} (see Discussion). 'Rodmersham Seedling', also reported as S_3S_x (Bošković and Tobutt 2001), showed

the same banding pattern as 'Strawberry Heart' with the two pairs of consensus primers, and PCR amplification with the allele-specific primers for S_{16} confirmed that 'Rodmersham Seedling' has this allele (data not shown). 'Strawberry Heart' and 'Rodmersham Seedling' therefore form a new incompatibility group, XXIII, S_3S_{16}.

Discrimination of S alleles using consensus primers

Because the product sizes are so similar, the consensus primers for the first intron cannot satisfactorily distinguish S_4/S_6, S_1/S_5 and $S_2/S_7/S_9/S_{12}$, which are also difficult to be distinguished from S_{10} and S_{14}; nor, as mentioned, do they detect S_{13}. With the consensus primers for the second intron it is likewise difficult or impossible to distinguish $S_1/S_3/S_{13}$, S_{10}/S_{14} and S_2/S_5 (top band)/S_7. When combining results for the first and the second intron it is still difficult to distinguish S_2/S_7 and S_{10}/S_{14} and sometimes S_1/S_{13}.

Allele-specific primers

When the specificity of the allele-specific primers for S_7 to S_{16} (Table 3) was tested on cultivars of known genotype, PCR amplification was in accord with genotype. None of the allele-specific primers amplified the 'wrong' alleles, showing that the primers are indeed allele-specific. The new S_2 primer PaS2-Fnew (Table 3) was found to give stonger and more reliable amplification of the S_2 allele than the S_2 forward primer PaS2-F published earlier (Sonneveld et al. 2001). For each primer pair, the highest possible annealing temperature still giving strong amplification was determined (Table 3). It was important to use this temperature to avoid occasional non-specific amplification in negative samples (i.e. cultivars not having the allele). Testing the internal control primers with the S allele-specific primers showed that each pair could be successfully multiplexed with the internal control. However, a lower annealing temperature was necessary for these PCRs (Table 3). Apart from allowing the detection of false negatives, the internal control also much reduced the occurrence of very faint, non-specific bands (in the position of the S allele-specific band) in negative samples.

Resolving conflicting reported genotypes of cultivars and extra East Malling scores

We were able to explain the anomalies highlighted in the Harmonization Table (Tobutt et al. 2001) by genotyping the different accessions of the cultivars using consensus primers for the first and the second intron and allele-specific primers (Table 5). Amplification with consensus primers for the second intron is shown in Fig. 3a and for specific primers for S_1, S_{13}, S_2 and S_5 in Fig. 3b–e.

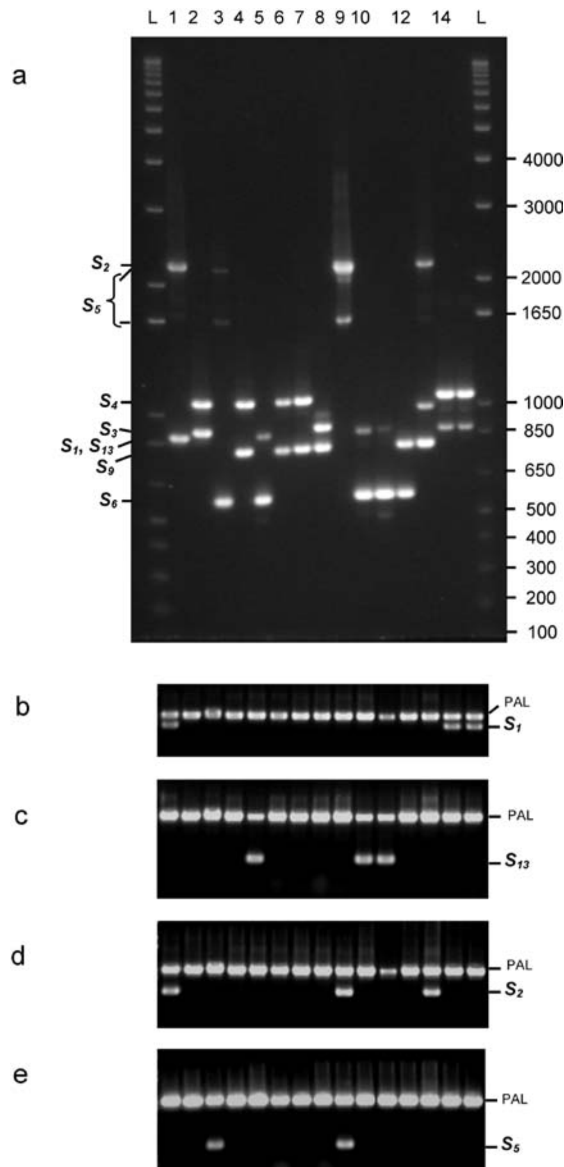

Fig. 3 PCR amplification of cultivars previously scored differently by different institutes listed in Table 5 and standards with **a** consensus primers for the second intron of cherry S-RNases, **b** specific primers for S_1, **c** specific primers for S_{13}, **d** specific primers for S_2, and **e** specific primers for S_5. Samples on the gel are (from the left): 1-kb + ladder, *1* 'Early Rivers' (S_1S_2), *2* 'Napoleon' (S_3S_4), *3* 'Colney' (S_5S_6), *4* 'Inge' (S_4S_9), *5* 'Noble' (S_6S_{13}), *6* 'Merchant' AH, *7* 'Merchant' BC, *8* 'Mona' MI, *9* 'Mona' BC, *10* 'Noble' EM, *11* 'Noble' NY, *12* 'Ramon Oliva' EM, *13* 'Ramon Oliva' BC, *14* 'Viscount' NY, *15* 'Viscount' BC, 1-kb + ladder

In 'Ramon Oliva' BC we found, unexpectedly, three alleles (confirmed by sequencing), S_2, S_9 and S_{new}, whereas Wiersma et al. (2001) had reported its genotype as S_9S_{new}. Cytometric analysis subsequently showed that 'Ramon Oliva' BC was tetraploid (data not shown).

Our genotyping of 'Merpet' (S_4S_9), 'Princess' (S_3S_{12}) and 'Vista' (S_2S_5) provided a second East Malling score for groups XXI, XXII and VIII respectively, groups in which we had previously genotyped only a single cultivar.

Table 5 Cultivars identified by Tobutt et al. (2001) as scored differently by different institutes, or included so that at least two members of each incompatibility group have been genotyped at East Malling, analysed using consensus primers for the first and the second intron and allele-specific primers

Accession	Used by[a]	Source	Reported genotype as standardised by Tobutt et al. (2001)	Our PCR score	Incompatibility group
Cultivars with conflicting scores					
'Merchant'	AH	HRI East Malling, UK	S_2S_4	S_4S_9	XXI
'Merchant'	BC	Summerland, BC, Canada	S_4S_9	S_4S_9	XXI
'Mona'	MI	Germplasm Repository, Davis, California, US	S_3S_9	S_3S_9	XVI
'Mona'	BC	Vineland Station, Ontario, Canada (Vic4 Row3 Tree7)	S_2S_{new}	S_2S_5	VIII
'Noble'	EM	HRI East Malling, UK	S_6S_{13}	S_6S_{13}	XII
'Noble'	NY	Cornell University, New York, US (RN6-2-79)	S_1S_6	S_6S_{13}	XII
'Ramon Oliva'	EM	HRI East Malling, UK	S_6S_9	S_6S_9	X
'Ramon Oliva'	BC	Import Station Saanich, Sydney, Canada (#1857-07E2)	S_9S_{new}	$S_2S_9S_{new}$[b]	
'Viscount'	NY	Cornell University, New York, US (RN5-2-31)	S_1S_4	S_1S_4	IX
'Viscount'	BC	Summerland, BC, Canada	S_4S_5	S_1S_4	IX
Second representatives of group VIII, XXI and XXII					
'Merpet'		Brogdale, UK	S_4S_9	S_4S_9	XXI
'Princess'		Summerland, BC, Canada	S_3S_{12}	S_3S_{12}	XXII
'Vista'		BAZ, Dresden, Germany	S_2S_5	S_2S_5	VIII

[a] EM = Bošković and Tobutt (2001), NY = Choi et al. (2000), AH = Schmidt et al. (1999), BC = Wiersma et al. (2001), MI = Hauck et al. (2001)
[b] Unexpectedly three bands for Ramon Oliva BC; subsequently found to be tetraploid and therefore not *P. avium*

Table 6 'New' cultivars from the Cherry Club planting at East Malling for which the *S* genotype was not given in the Harmonization Table (Tobutt et al. 2001), and their parentage, analysed by PCR using consensus primers for the first and the second intron. The presence of the S_1 and the S_{13} allele was determined/confirmed by allele-specific PCR

Cultivar	Parentage/Origin	Ref.[a]	*S* alleles[b]	Incompatibility group
Reportedly self-incompatible				
'Aida'	'Moldvai Fekete' (?) × H236 (?)	A	S_6S_{12}	XXIII (new)
'Canada Giant'	may be 'Summit' (S_1S_2)	B, J	S_1S_2	I
'Penny' (East Malling C73-5)	'Colney' (S_5S_6) × 'Inge' (S_4S_9)	C	S_6S_9	X
'Late Maria'	seedling of 'Lambert' (S_3S_4) or 'Bing' (S_3S_4) (?)	D	S_3S_4	III
'Vera'	'Ljana' (?) × 'Van' (S_1S_3)	A	S_1S_3	II
Incompatibility status not reported				
'New Moon'	may be a sport of 'Newstar' (2S-28-28) (S_3S_4')	B, J	S_2S_3	IV
'Staccato'	'Sweetheart' (S_3S_4') open pollinated	B	S_3S_4'	SC
'Summer Sun' (JI 14039)	'Merton Glory' (S_4S_6) open pollinated	E	S_4S_9	XXI
Reportedly self-compatible				
'Alex'	'Van' (S_1S_3) × Cherry SF 46 (S_1S_3' or $S_1'S_3$) [c]	A	S_3S_3'	SC
'Blaze Star'	'Lapins' (S_1S_4') × 'Durone Compatto di Vignola' (?)	F	$S_4'S_6$	SC
'Columbia'	'Stella' (S_3S_4') × 'Beaulieu' (?)	G	$S_4'S_9$	SC
'Early Star'	'Burlat' (S_3S_9) × 'Stella Compact' (S_3S_4')	F	$S_4'S_9$	SC
'Glacier'	'Stella' (S_3S_4') × 'Early Burlat' (S_3S_9)	H	$S_4'S_9$	SC
'Index'	'Stella' (S_3S_4') open pollinated	H	S_3S_4'	SC
'Peter'	'Burlat' (S_3S_9) × 'Stella' (S_3S_4')	A	S_3S_4'	SC
'Sir Don'	'Black Douglas' (?) × 'Stella' (S_3S_4')	I	$S_4'S_{13}$	SC
'Sir Tom'	'Black Douglas' (?) × 'Stella' (S_3S_4')	I	S_3S_{13}	XIX
'Sumesi'	'Van' (S_1S_3) × 2S-22-05 ['Stella' (S_3S_4') o.p.]	J	S_3S_4'	SC

[a] A. Brózik and Apostol (2000); B. Kappel (personal communication); C. Tobutt (2002); D. Sheehan (personal communication); E. Matthews (personal communication); F. Sansavini and Lugli 1997; G. Lang (personal communication); H. Olmstead et al. 2000; I. Granger (personal communication); J. Edin et al. 1997
[b] The distinction between S_4 and S_4' and between S_3 and S_3' is based on the consideration of pedigree
[c] Cherry SF 46 is an accession received in Hungary from the John Innes Institute, possibly as 1411/46 (Apostol, personal communication); 1411/46 = JI 2538 (Matthews and Lapins 1967); JI 2538 was genotyped by Schmidt (1999)

Genotyping of previously untested cherry cultivars

Genotypes were assigned to 18 'new' cultivars with the use of both pairs of consensus primers and appropriate allele-specific primers. Banding patterns from amplification with consensus primers for the second intron and specific amplification for S_1, S_{13} and S_3 are given in Fig. 4 and scores in Table 6. For several of them some further comment is needed.

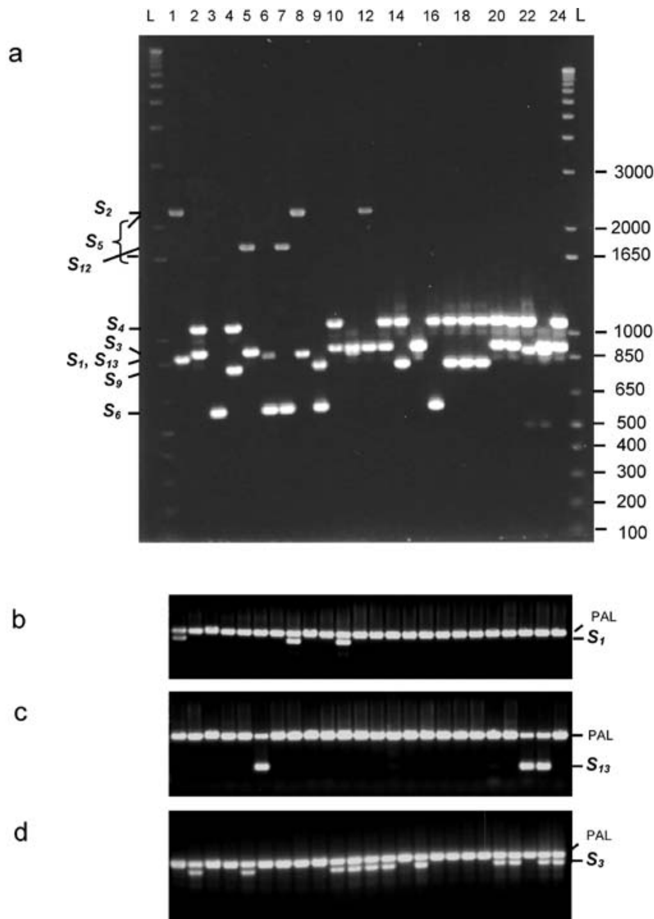

Fig. 4 PCR amplification of new cultivars listed in Table 6 and standards with **a** consensus primers for the second intron of cherry *S*-RNases, **b** specific primers for S_1, **c** specific primers for S_{13}, and **d** specific primers for S_3. Samples on the gel are (from the left): 1-kb + ladder, *1* 'Early Rivers' (S_1S_2), *2* 'Napoleon' (S_3S_4), *3* 'Colney' (S_5S_6), *4* 'Inge' (S_4S_9), *5* 'Schneiders Späte Knorpelkirsche' (S_3S_{12}), *6* 'Noble' (S_6S_{13}), *7* 'Aida', *8* 'Canada Giant', *9* 'Penny', *10* 'Late Maria', *11* 'Vera', *12* 'New Moon', *13* 'Staccato', *14* 'Summer Sun', *15* 'Alex', *16* 'Blaze Star', *17* 'Columbia', *18* 'Early Star', *19* 'Glacier', *20* 'Index', *21* 'Peter', *22* 'Sir Don', *23* 'Sir Tom', *24* 'Sumesi', 1-kb + ladder

All self-incompatible cultivars could be assigned to existing incompatibility groups (Tobutt et al. 2001), except for 'Aida' (S_6S_{12}), which should form a new incompatibility group, XXIV, with 'Flamentiner' (S_6S_{12}), which had previously been placed in Group O.

'Canada Giant' was scored as S_1S_2, which is the same genotype as 'Summit', to which it is said to be very similar and perhaps identical (see Table 6). The genotype of S_2S_3 for 'New Moon' is not consistent with 'New Moon' being a sport of 'New Star' (S_3S_4').

Whether the cultivars of unknown incompatibility status are self-compatible can be deduced from their scores and pedigrees. The consensus primers also detect the self-compatibility allele S_4', but can not distinguish it from the S_4 allele. 'Staccato', a seedling of 'Sweetheart' (S_3S_4'), showing bands for S_3 and S_4, should be self-compatible; S_3 must have originated from 'Sweetheart' and the other band must represent the self-compatibility allele S_4' to have succeeded on the 'Sweetheart' style. The parentage could be 'Sweetheart' selfed. 'New Moon' does not show the S_4/S_4' band and should be self-incompatible. 'Summer Sun' should be self-incompatible, as it has inherited S_4 from 'Merton Glory' and S_9 from the unknown pollen parent.

'Alex' gave a single band in the S_3 position with both first and second intron consensus primers. On the basis of its reported self-compatibility and parentage we deduce that this pattern indicates S_3S_3'. Its parent Cherry SF 46 is likely to be the self-compatible John Innes selection JI 2538 (footnote Table 6), reported by Schmidt (1999) as S_1S_3' or $S_1'S_3$ ($'$ = pollen-part mutation). If 'Alex' is indeed a seedling of JI 2538 and has the genotype S_3S_3' then JI 2538 must be S_1S_3', unless the mutation is not 'linked' with one particular allele.

'Sir Tom', reported as self-compatible, unexpectedly appears to have inherited the S_3 allele from 'Stella' (S_3S_4') rather than the self-compatibility allele S_4', raising the possibility that the accession received by East Malling is actually self-incompatible.

Finally, on the basis of the genotypes of the cultivars derived from them, it can be concluded that 'Beaulieu', 'Black Douglas' and 'Durone Compatto di Vignola', of unknown genotype, must have the alleles S_9, S_{13} and S_6, respectively.

Testing consensus primers in cherry species

When the consensus primers for the first and the second intron were tested in the diploid, presumably self-incompatible, cherry species, they amplified one or two bands in each accession (Fig. 5). This indicates that the sweet cherry consensus primers recognise *S*-RNase sequences in other cherry species. A single band on the gel could mean either that the two alleles have introns of the same size, which is especially likely in the case of a thick band, or that one allele is not amplified because the primers do not match the sequence of that allele. It is not known whether weak bands represent *S* alleles or secondary bands, as examples of both are found in sweet cherry. We have confirmed by sequencing that the two second intron products of *P. nipponica* F1292 represent *S*-RNase sequences (data not shown). More work is needed to confirm that the bands represent *S* alleles in the other accessions, and to find out why some give a single band on the gel with one or both consensus primer pairs.

Discussion

We have developed consensus primers for PCR amplification of the first and the second intron of cherry *S*-RNases which can detect the known cherry *S* alleles, S_1 to S_{16}, and, in most cases, distinguish them because of length variation of the intron. In addition, we have extended the range of allele-specific primers, so that specific primers

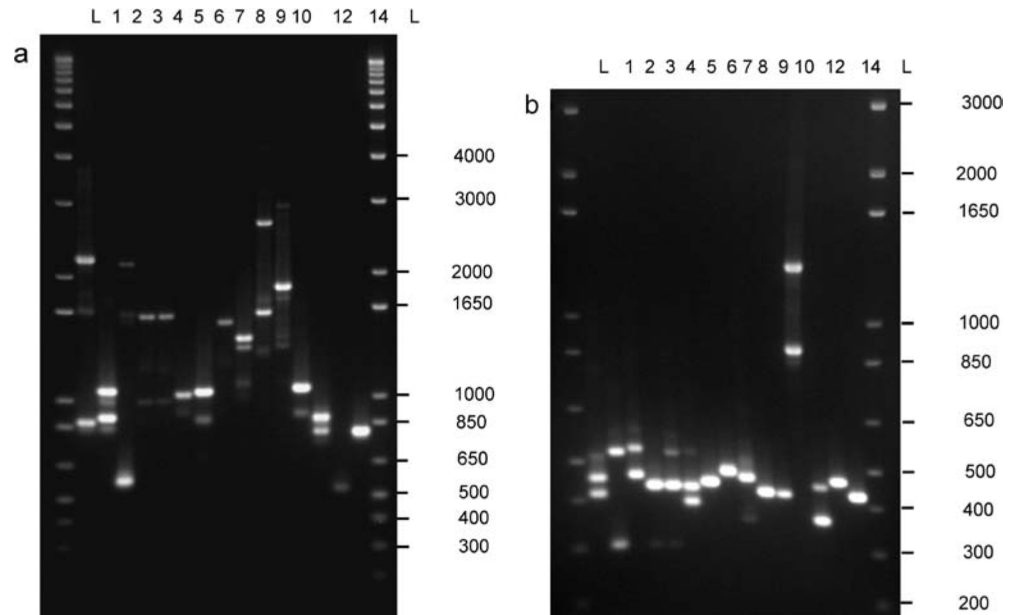

Fig. 5 PCR amplification of cherry species with consensus primers for **a** the second intron, and **b** the first intron of cherry *S*-RNases. Samples on both gels are (from the left): 1-kb + ladder, *1 P. avium* 'Early Rivers', *2 P. avium* 'Napoleon', *3 P. avium* 'Colney', *4 P. canescens* G254, *5 P. canescens* GM79 0065, *6 P. incisa* E621, *7 P. incisa* F283, *8 P. mahaleb* 'Magyar', *9 P. mahaleb* SL64, *10 P. nipponica* F1292, *11 P. nipponica* var. *kurilensis* F1301, *12 P. sargentii* clone 34, *13 P. sargentii* D5774, *14 P. subhirtella* 'Ascendens' 1878, *15 P. subhirtella* 'Pendula' F1131, 1-kb + ladder

are now available for all the reported *S* alleles of cherry. We have demonstrated their utility in genotyping new cultivars of interest to growers and resolving anomalies in previous work. We have also indicated their likely use in other cherry species. The genotyping method presented here is a rapid and convenient technique that can be used on vegetative material, e.g. leaves or buds.

Identity of alleles

Sequencing established that the wild cherry allele previously published as S_{11}, on the basis of the iso-electric point of the *S*-RNase on IEF gels (Bošković et al. 1997; Bošković and Tobutt 2001), has the same partial DNA sequence as the S_7 allele found in various sweet cherries. These two alleles also appear to be functionally identical on the basis of the 'semi-compatible' segregation pattern in the progeny of 'Charger' (S_1S_7) × Orleans 171 ($S_{10}S_{11}$). Minor variation of the iso-electric point, but identity of function, has previously been reported for S_8, which is functionally the same as S_3 (Sonneveld et al. 2001).

Interestingly, from the RFLP profiles for cherry *S*-RNases recently reported by Hauck et al. (2001), it appears that S_7 and S_{11} do not have bands of the same size for all four restriction enzymes used. However, these authors also found such variation for S_9 from two different cultivars, indicating that restriction sites in regions flanking the *S*-RNases are not always conserved. Surprisingly, their PCR data also suggested that the DNA sequence of S_{11} is different from S_7, as they amplified S_7 but not S_{11} with the consensus primers of Wiersma et al. (2001) for both the first and the second intron. Presumably this was the result of failure of the PCRs for Orleans 171 ($S_{10}S_{11}$). In our experience, S_{11} of Orleans 171 is amplified with these primers and gives a band of the same size as S_7 (data not shown). Comparing full-length sequences of S_7 with S_{11} and S_3 with S_8 would show whether any variation exists at the amino-acid level. Alternatively, the slight electrophoretic difference may be the result of variation in glycosylation of the *S*-RNases.

The allele previously published as S_x (Bošković and Tobutt 2001) we have named S_{16}, as its genomic PCR products have a unique sequence. Although the S_{15} of Wiersma et al. (2001) has now been identified as S_5 (Tobutt et al. 2001), we have not re-used the S_{15} label, as it was agreed by groups working on cherry self-incompatibility (at the Cherry Symposium in Oregon, USA, June 2001) that S_{15} (and also S_8) should be 'retired' rather than 'recycled' to avoid possible confusion. (For the same reason, we will not re-use S_{11}).

Characteristics of S_5 and S_{13}

We could not determine why the second intron consensus primers produce an extra band for S_5, as this lower band could not be cloned or sequenced. The lower band may be an artefact resulting from secondary structure of the DNA, and represent shorter products after the *Taq* DNA polymerase has missed out a 'hairpin' during PCR. Or perhaps some of the top band products are able to run faster on an agarose gel as a result of having a different conformation. Interestingly, the S_5 allele has a long microsatellite $[(TA)_{\sim 20}]$ in the second intron that is not found in introns of the other alleles, except S_{13}, which often shows two extra lower bands on the gel.

Failure of the first intron consensus primers for S_{13} suggests that the primer positions are not conserved in S_{13}. Alternatively, the structure of the intron may prevent amplification, as suggested by Tamura et al. (2000) for the second intron of some almond alleles.

Principles of consensus and allele-specific primer approach

The intron length variation of cherry *S*-RNases and conserved regions in the exons are the basis for the consensus primers approach. The first intron is unique to *Prunus S*-RNases. Considerable variation in length of the second intron has also been reported for other rosaceous species, particularly those of other *Prunus*, and consensus primers for *S*-RNases to detect this length variation have been used not only in cherry (Tao et al. 1999; Wiersma et al. 2001), but also in Japanese pear (*Pyrus serotina*) (Ishimizu et al. 1999), apple (*Malus pumila*) (Matsumoto and Kitahara 2000), almond (*Prunus dulcis*) (Tamura et al. 2000; Channuntapipat et al. 2001) and Japanese apricot (*Prunus mume*) (Tao et al. 2000; Yaegaki et al. 2001). In contrast, solanaceous *S*-RNase introns are relatively short (85–120 bp) and show very little length polymorphism (e.g. Coleman and Kao 1992; Saba-El-Leil et al. 1994; Matton et al. 1995). It would be interesting to investigate why rosaceous *S*-RNase introns are so variable in length and why *Prunus* species have an additional intron. For example, mobile genetic elements could have contributed to this.

A rare example of intra-allelic variation in intron length that has recently been found in almond (Channuntapipat et al. 2001; Ma and Oliveira 2001) indicates the need for caution in characterizing potential new alleles on the basis of intron length alone, without confirmation by sequencing and/or test crossing.

Sequence variation of the *S*-RNases is the basis for allele-specific primers. The exon sequences we obtained were not always sufficiently variable for the design of good specific primers, and the use of intron sequences was necessary in some cases. Allele-specific primers have been used in cherry (Sonneveld et al. 2001), apple (Broothaert et al. 1995; Janssens et al. 1995, 1996; Verdoodt et al. 1998; Matsumoto and Kitahara 2000; Sakurai et al. 2000; Van Nerum et al. 2001) and almond (Tamura et al. 2000; Channuntapipat et al. 2001; Ma and Oliveira 2001), sometimes in combination with restriction enzyme digestions of PCR products, to confirm the genotype score or to distinguish between two alleles amplified with the same 'specific' primer pair. The report of intra-allelic point mutations in the coding sequence (not leading to amino-acid changes) in apple (Van Nerum et al. 2001) suggests that confirmation by allele-specific restriction enzyme digestions or primers may not always be 100% reliable.

Consensus primers will be especially useful for genotyping accessions of unknown parentage. For cultivar surveys we recommend the following strategy: an initial screen with consensus primers for intron 1 and 2, a deduction of provisional genotypes and a confirmation with the appropriate allele-specific primers. The allele-specific primers for S_1 and S_{13}, S_2 and S_7, S_{10} and S_{14} are particularly useful as these pairs of alleles cannot be distinguished easily with the consensus primers, even when results for introns 1 and 2 are combined. The new S_2-specific forward primer (PaS2-Fnew) is recommended instead of the forward primer (PaS2-F) published earlier (Sonneveld et al. 2001), as it is more reliable and gives stronger amplification. It is recommended that standards of known alleles be included. New alleles can also be detected with the consensus primers.

Comparison of PCR-based genotyping methods

Our consensus primers for the second intron amplify all the known alleles from cherry (S_1 to S_{16}), unlike those of Tao et al. (1999) and Wiersma et al. (2001), neither of which can amplify S_5. Perhaps the C5 region from which our reverse primer was designed, is more conserved at the DNA level than the C4 region used by these authors.

We have not encountered the problem of preferential amplification of particular alleles that was reported for consensus primers in almond (Tamura et al. 2000; Channuntapipat et al. 2001), so long as good quality DNA is used, although some alleles usually give stronger bands than others. As pointed out in the Materials and methods, the particular PCR cycling conditions we use are essential for amplification of certain alleles.

The first intron primers, although revealing less polymorphism and not amplifying S_{13}, can be useful for distinguishing between some alleles that have second introns of similar size [e.g. S_1/S_3/S_{13} and S_2/S_5 (top band)]. Consensus primers to detect length variation for the first intron have been reported in cherry previously (Tao et al. 1999; Wiersma et al. 2001), but have not been tested on all alleles now known.

The allele-specific primers to distinguish between certain alleles are an alternative to the allele-specific restriction enzyme digestion of PCR products amplified by consensus primers used by Yamane et al. (2000b) and Wiersma et al. (2001). Confirmation of the score from consensus primers is advisable, as discussed below. When used on representatives of S_1 to S_{16}, our primers are indeed specific. Limitations and advantages of consensus primers, specific primers and the use of restriction enzymes have been discussed by Sonneveld et al. (2001). Here we have shown that the main drawback of allele-specific primers, i.e. the problem of detecting false negatives, can be overcome by using an internal control.

Application of consensus and allele-specific primers to cultivars

We were able to attribute the anomalies indicated in the Harmonization Table (Tobutt et al. 2001) to mis-scoring or to the existence of different clones having the same cultivar name.

We found that both accessions of each of 'Noble', 'Merchant' and 'Viscount' that we analysed had the same genotype, S_6S_{13}, S_4S_9 and S_1S_4 respectively, indicating mis-scoring by one of the institutes who had genotyped these previously. For 'Noble' the score of S_1S_6 by Choi et

al. (2000) could have been caused by the second intron of S_{13} being the same size as that of S_1. Similarly, 'Viscount' might have been scored as S_4S_5 by Wiersma et al. (2001) because the first intron PCR product of S_1 cannot easily be distinguished from S_5. The possibility that 'Noble' and 'Viscount' were mis-scored because certain alleles have introns of similar size, highlights the need for confirmation of genotypes obtained from consensus primers, either by allele-specific restriction enzyme digestion of PCR products or use of allele-specific primers.

We found evidence of different clones for 'Mona' and 'Ramon Oliva'. For 'Mona', until the authenticity of the material has been established, we propose recording the accessions as 'Mona' BC (S_2S_5) and 'Mona' MI (S_3S_9). Analysis of 'Mona' from another source (Genbank Obst, Dresden) did not help to determine which clone was likely to be true to name, as that score (S_3S_6) was different from either (data not shown). The 'Ramon Oliva' clone used by Wiersma et al. (2001) was scored here as $S_2S_9S_{new}$, indicating that it was not diploid and therefore not a sweet cherry. Cytometric analysis for determination of ploidy level showed it to be a tetraploid, so it cannot be true to name. Perhaps it was mistakenly propagated from a rootstock. Rootstocks used for sweet cherry are often of hybrid origin, with a higher ploidy level. As there is no evidence that S_{new} is a sweet cherry allele, it would be inappropriate to number it S_{17}.

For each incompatibility group (Tobutt et al. 2001) at least two cultivars from a known source have now been scored at HRI East Malling.

The genotyping of new cultivars proved to be straightforward and provides useful information for nurserymen and growers concerned with allowing for effective pollination, and indeed for breeders using these in crossing programmes.

'Alex' appears to be the first self-compatible cultivar having S_3' (from JI 2538), rather than S_4'. The S_4' allele, derived from JI 2420, is the source of self-compatibility of all other self-compatible cultivars. The S_3' allele from JI 2538, which was raised at the John Innes Institute as a spontaneous self-compatible mutant (Lewis and Crowe 1954; Matthews and Lapins 1967), should be distinct from the S_3' allele reported previously for JI 2434 (EM and AH clone), which is an X-ray induced mutation (Lewis and Crowe 1954; Bošković et al. 2000). Both are pollen-part mutations with a normal stylar function and our S_3 specific primers amplify both (data not shown).

The identity and incompatibility status of the 'Sir Tom' trees at East Malling is now in doubt. 'Sir Tom', from the cross 'Black Douglas' (?) × 'Stella' (S_3S_4'), has been reported as self-compatible, but was scored here as S_3S_{13}. If it is self-compatible, it should have inherited the S_4' allele from 'Stella'. As discussed by Bošković et al. (2000), and confirmed by our unpublished data, the pollen-part mutation of JI 2420, from which 'Stella' derives, is linked in coupling with the S_4 allele; so the S_3 allele of 'Stella' should not confer self-compatibility.

We have identified five cultivars that are the first to have S_4' in combination with alleles other than S_1 or S_3: 'Blaze Star' ($S_4'S_6$), 'Columbia', 'Early Star' and 'Glacier' ($S_4'S_9$) and 'Sir Don' ($S_4'S_{13}$). This increases the range of semi-compatible crosses that can be made to give fully self-compatible progenies, e.g. $S_6S_x \times S_4'S_6$ gives $S_4'S_6$ and $S_4'S_x$.

Forward look

The consensus and allele-specific primers could be applied to wild *P. avium* for studies of population genetics and gene flow. Consensus primers could detect new alleles, perhaps in cultivars from Eastern Europe and Western Asia which have been less studied, and also in wild material. As shown, our consensus primers designed from sweet cherry *S*-RNase sequences also give PCR amplification in a range of cherry species, and they could therefore be useful for studies of self-incompatibility in these species. They may also prove to be useful in other *Prunus* species, as trans-specific evolution has been observed for *Prunus* *S*-RNases, i.e. the sequences do not form species-specific clusters in a phylogenetic tree (Ushijima et al. 1998; Igic and Kohn 2001). However, the sequence information that has recently become available for many *Prunus* *S*-RNases could be used to design *Prunus* consensus primers that are more universal.

The relatively short length of the first introns of sweet cherry *S*-RNases (145 to 368 bp) opens up the possibility of sizing fluorescently labelled PCR products on an automated sequencer, especially if the primers used are close to the intron so that the products are <500 bp. Microsatellite markers are now scored routinely in this way. This would enable more-accurate scoring of the first intron products and could be a convenient alternative to running products on an agarose gel.

The partial sequences of an additional seven *S*-RNases of cherry could contribute to phylogenetic analyses of rosaceous *S*-RNases, to the investigation of the basis of intron length variation and the presence of the extra intron of *Prunus* *S*-RNases and to studies of allele-specificity.

Acknowledgements We are grateful to Emma-Jane Lamont (National Fruit Collections, Brogdale, UK), Dr. M. Fischer (BAZ, Dresden, Germany), Dr. R. Andersen (Cornell University, New York, USA), Dr. P. Wiersma and Dr. F. Kappel (Agriculture and Agri-Food Canada, Pacific AgriFood Research Centre, Summerland, Canada), Dr. D. Thompson (Saanich, Canada) and Dr. C. Weeks (USDA/ARS Germplasm Repository Davis, California, USA) for supply of material. Tineke Sonneveld acknowledges receipt of a studentship from the University of Nottingham and Horticulture Research International.

References

Brózik S, Apostol J (2000) In: Brózik S, Kállay Tné (eds) Csonthéjas gyümölcsfajták. Mezögazda Kiadó, Budapest

Bošković R, Tobutt KR (1996) Correlation of stylar ribonuclease zymograms with incompatibility alleles in sweet cherry. Euphytica 90:245–250

Bošković R, Tobutt KR (2001) Genotyping cherry cultivars assigned to incompatibility groups, by analysing stylar ribonucleases. Theor Appl Genet 103:475–485

Bošković R, Russell K, Tobutt KR (1997) Inheritance of stylar ribonucleases in cherry progenies, and reassignment of incompatibility alleles to two incompatibility groups. Euphytica 95:221–228

Bošković R, Tobutt KR, Schmidt H, Sonneveld T (2000) Re-examination of (in)compatibility genotypes of two John Innes self-compatible sweet cherry selections. Theor Appl Genet 101:234–240

Broothaerts W, Janssens GA, Proost P, Broekaert WF (1995) cDNA cloning and molecular analysis of two self-incompatibility alleles from apple. Plant Mol Biol 27:499–511

Channuntapipat C, Sedgley M, Collins G (2001) Sequences of the cDNAs and genomic DNAs encoding the S_1, S_7, S_8 and S_f alleles from almond, *Prunus dulcis*. Theor Appl Genet 103:1115–1122

Choi C, Livermore K, Andersen RL (2000) Sweet cherry pollination: recommendation based on compatibility groups and bloom time. J Am Pomological Soc 54:148–152

Coleman CE, Kao T-h (1992) The flanking regions of two *Petunia inflate S* alleles are heterogeneous and contain repetitive sequences. Plant Mol Biol 18:725–735

Crane MB, Brown AG (1937) Incompatibility and sterility in the sweet cherry, *Prunus avium* L. J Pomol Hort Sci 15:86–116

Crane MB, Lawrence WJC (1929) Genetical and cytological aspects of incompatibility and sterility in cultivated fruits. J Pomol Hort Sci 7:276–301

Edin M, Lichou J, Saunier R (1997) Les variétés. In: Edin M, Lichou J, Saunier R (eds) Cerise, les variétés et leur conduite. Ctifl, Paris, pp 135–229

Hauck NR, Iezzoni AF, Yamane H, Tao R (2001) Revisiting the *S*-allele nomenclature in sweet cherry (*Prunus avium*) using RFLP profiles. J Am Soc Hort Sci 126:654–660

Igic B, Kohn JR (2001) Evolutionary relationships among self-incompatibility RNases. Proc Natl Acad Sci USA 98:13,167–13,171

Ishimizu T, Inoue K, Shimonaka M, Saito T, Terai O, Norioka S (1999) PCR-based method for identifying the *S*-genotypes of Japanese pear cultivars. Theor Appl Genet 98:961–967

Janssens GA, Goderis IJ, Broekaert WF, Broothaerts W (1995) A molecular method for *S*-allele identification in apple based on allele-specific PCR. Theor Appl Genet 91:691–698

Janssens GA, Van Haute AM, Keulemans J, Broothaerts W, Broekaert FW (1996) PCR analysis of self-incompatibility alleles in apple applied to leaves, seed embryos and in vitro shoots. Acta Hort 484:403–407

Lewis D, Crowe LK (1954) Structure of the incompatibility gene. IV. Types of mutations in *Prunus avium* L. Heredity 8:357–363

Ma R-C, Oliveira MM (2001) Molecular cloning of the self-incompatibility genes S_1 and S_3 from almond (*Prunus dulcis* cv Ferragnès). Sex Plant Reprod 14:163–167

Matsumoto S, Kitahara K (2000) Discovery of a new self-incompatibility allele in apple. HortScience 35:1329–1332

Matthews P, Dow KP (1969) Incompatibility groups: sweet cherry (*Prunus avium*). In: Knight RL (ed), Abstract bibliography of fruit breeding and genetics to 1965, *Prunus*. Commonwealth Agricultural Bureaux, Farnham Royal, pp 540–544

Matthews P, Lapins K (1967) Self-fertile sweet cherries. Fruit Var Hortic Digest 21:36–37

Matton DP, Mau S-L, Okamoto S, Clarke AE, Newbigin E (1995) The *S*-locus of *Nicotiana alata*: genomic organization and sequence analysis of two *S*-RNase alleles. Plant Mol Biol 28:847–858

Olmstead JW, Ophardt DR, Lang GA (2000) Sweet cherry breeding at Washington State University. Acta Hort 522:103–109

Saba-El-Leil MK, Rivard S, Morse D, Cappadocia M (1994) The S_{11} and S_{13} self-incompatibility alleles in *Solanum chacoense* Bitt. are remarkably similar. Plant Mol Biol 24:571–583

Sakurai K, Brown SK, Weeden N (2000) Self-incompatibility alleles of apple cultivars and advanced selections. HortScience 35:116–119

Sansavini S, Lugli S (1997) Tre stelle per la cerasicoltura italiana: 'Early Star', 'Blaze Star', 'LaLa Star'. Rivista di Frutticol 10:68–69

Schmidt H (1999) On the genetics of incompatibility in sweet cherries. Acta Hort 484:233–237

Schmidt H, Wolfram B, Bošković B (1999) Befruchtungsverhältnisse bei Süßkirschen (flower biology in sweet cherries). Erwerbsobstbau 41:42–45

Sonneveld T, Robbins TP, Bošković R, Tobutt KR (2001) Cloning of six cherry self-incompatibility alleles and development of allele-specific PCR detection. Theor Appl Genet 102:1046–1055

Tao R, Yamane H, Sugiura A, Murayama H, Sassa H, Mori H (1999) Molecular typing of *S*-alleles through identification, characterization and cDNA cloning for *S*-RNases in sweet cherry. J Am Soc Hort Sci 124:224–233

Tao R, Habu T, Yamane H, Sugiura A, Iwamoto K (2000) Molecular markers for self-compatibility in Japanese apricot (*Prunus mume*). HortScience 35:1121–1123

Tamura M, Ushijima K, Sassa H, Hirano H, Tao R, Gradziel TM, Dandekar AM (2000) Identification of self-incompatibility genotypes of almond by allele-specific PCR analysis. Theor Appl Genet 101:344–349

Tobutt KR (2002) 'Penny' – a new cherry variety from HRI-East Malling. The National Fruit Show [Handbook] 2002:40–41

Tobutt KR, Bošković R, Sonneveld T (2001) Cherry (in)compatibility genotypes – harmonization of recent results from UK, Canada, Germany, Japan and USA. Eucarpia Fruit Breed Sect Newslett 5:41–46

Ushijima K, Sassa H, Tao R, Yamane H, Dandekar AM, Gradziel TM, Hirano H (1998) Cloning and characterization of cDNAs encoding *S*-RNases from almond (*Prunus dulcis*): primary structural features and sequence diversity of the *S*-RNases in Rosaceae. Mol Gen Genet 260:261–268

Van Nerum I, Geerts M, Van Haute A, Keulemans J, Broothaerts W (2001) Re-examination of the self-incompatibility genotype of apple cultivars containing putative 'new' S-alleles. Theor Appl Genet 103:584–591

Verdoodt L, Van Haute A, Goderis IJ, De Witte K, Keulemans J, Broothaerts W (1998) Use of the multi-allelic self-incompatibility gene in apple to assess homozygosity in shoots obtained through haploid induction. Theor Appl Genet 96:294–300

Wiersma PA, Wu Z (1998) A full-length cDNA for phenylalanine ammonia-lyase cloned from ripe sweet cherry fruit (*Prunus avium*; accession no. AF036948) (PGR98-184). Plant Physiol 118:1102

Wiersma PA, Wu Z, Zhou L, Hampson C, Kappel F (2001) Identification of new self-incompatibility alleles in sweet cherry (*Prunus avium* L.) and clarification of incompatibility groups by PCR and sequencing analysis. Theor Appl Genet 102:700–708

Yaegaki H, Shimada T, Moriguchi T, Hayama H, Haji T, Yamaguchi M (2001) Molecular characterization of *S*-RNase genes and *S*-genotypes in the Japanese apricot (*Prunus mume* Sieb. et Zucc.). Sex Plant Reprod 13:251–257

Yamane H, Tao R, Murayama H, Ishiguro M, Abe Y, Soejima J, Sugiura A (2000a) Determining *S*-genotypes of two sweet cherry (*Prunus avium* L.) cultivars, 'Takasago (Rockport Bigarreau)' and 'Hinode (Early Purple)'. J Jap Soc Hort Sci 69:29–34

Yamane H, Tao R, Murayama H, Sugiura A (2000b) Determining the *S*-genotypes of several sweet cherry cultivars based on PCR-RFLP analysis. J Hort Sci Biotechnol 75:562–567