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Identification of new self-incompatibility alleles in sweet cherry (*Prunus avium* L.) and clarification of incompatibility groups by PCR and sequencing analysis

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Abstract Correct assignment of sweet cherry cultivars to cross-compatibility groups is important for the efficient production of cherry fruit. Despite considerable confusion in the literature, these groups continue to be an effective tool for predicting pollination effectiveness for breeders and growers. PCR fragments generated from cherry S-RNase sequences coincided with specific S-allele phenotypes. Twenty five genomic DNA fragments, representing the six most common alleles, were cloned and sequenced. In addition, fragments were characterized from four new S-alleles. These genomic and cDNA sequences were invariant among cultivars with the same S-allele. Using the sequence data, PCR and restriction enzyme-based methodology was developed for rapid analysis of S-genotypes. Analysis and description of fragmentation patterns for S-allele determination are discussed. The method was utilized to characterize the S-allele composition of 70 sweet cherry cultivars obtained from collections in North America, including many of the named releases from the Canadian breeding programs at Agriculture and Agri-Food Canada in Summerland, B.C., and Vineland, Ontario. A number of differences between published S-allele assignments and PCR data were discovered and a new listing of cultivar S-allele assignments is presented.

Keywords *Prunus avium* L., · Incompatibility groups · S-RNase genes · PCR fragment pattern · DNA sequence

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Introduction

Sweet cherry flowers express an effective self-incompatibility system to reduce self-fertilization. Fruit set is often close to 0% unless cross-pollination is provided. The agronomic importance of this trait has been the basis for many studies world-wide on the cross-compatibility of cherry cultivars (Crane and Brown 1937; Kobel et al. 1938; De Vries 1968; Matthews and Dow 1969; Tehrani and Brown 1992). The trait has been mapped to a single locus for which multiple alleles have been demonstrated (the S-alleles; Crane and Lawrence 1931). Many important sweet cherry cultivars have been arranged into a set of 13 incompatibility groups by using data from observed fruit-set after controlled pollinations. Six S-alleles are sufficient to define most of these groups although additional alleles are predicted to explain some of the groups containing only a few cultivars. The designation of "O" has been given for cultivars which are self-incompatible but for which no cross-incompatibilities have been determined. It has been uncertain whether such cultivars contain new alleles or rare combinations of known alleles. In addition, there are self-fertile cultivars such as those derived from the John Innes lines (Matthews 1970; represented by an apostrophe following the allele designation, e.g., S4′). It is uncertain what changes have occurred to produce self-fertility but the mutations are closely linked to the S-locus.

For sweet cherry, as for the other Rosaceae examined to-date, incompatibility is expressed gametophytically as an allele-specific ribonuclease expressed in stylar tissue and an unknown pollen component. The RNases are highly conserved in structure (Sassa et al. 1996). S-RNase proteins and/or genes have been characterized from the related fruit species, apple (Broothaerts et al. 1995; Janssens et al. 1995; Sassa et al. 1996), Japanese pear (Sassa et al. 1992, 1996; Ishimizu et al. 1996; Norioka et al. 1996), European and Chinese pear (Tomimoto et al. 1996), almond (Tao et al. 1997), apricot (Burgos et al. 1998) and sweet cherry (Mau et al. 1982; Williams et al. 1982; Bŏsković and Tobutt 1996; Bŏsković et al. 1997; and Tao et al. 1999).

Molecular detection methods have been developed to speed-up the analysis of allele type and circumvent some of the problems associated with determining alleles from conventional controlled crosses. These new methods include zymogram analysis of stylar RNases which were found to segregate with varietal S-type (Bŏsković and Tobutt 1996; Bŏsković et al. 1997) and PCR-based analysis with fragment size differentiation of S-allele types (Janssens et al. 1995; Tao et al. 1999). In this study we have further developed and utilized PCR methodology for the characterization of the S-allele genotypes of 70 cherry cultivars. The S-allele identifications were used to clarify groupings, to group new cultivars and to distinguish and identify four new S-alleles.

Materials and methods

Plant materials

Young leaf tissue (spring) or winter buds of 70 sweet cherry cultivars were collected from a number of research centres across North America. Among them, 17 cultivars were kindly provided by William Lay, University of Guelph, Vineland Station, Ontario, Canada: namely, Vic, Angela, Early Lyons, Noble, Mona, Schmidt, Noir de Guben, Bada, Hedelfingen, Merton Glory, Republican, Ramon Oliva, Early Amber, Merton Heart, Elton Heart, Early Burlat and Gold. Fifteen cultivars were obtained from Bill Howell, NRSP5, Washington State University, Prosser, Wash., USA: namely, Chinook, Venus, Schneiders Späte Knorpelkirsche, Lyons, Vic, Vogue, Emperor Francis, Black Tartarian, Valera, Black Republican, Hedelfingen, Windsor, Deacon, Velvet and Victor. The rest of the cultivars were collected at the Pacific Agri-Food Research Centre, Summerland, B.C., Canada.

Isolation of Genomic DNA

Fresh young leaves or buds of each cultivar were used for extraction and purification of genomic DNA with a FastDNA kit (BIO101, La Jolla, Calif., USA) according to the manufacturer's recommendation and using the supplied reagents (CLS-VF, PPS, SEWS-M, lysing matrix) with minor modifications. In brief, approximately 200 mg of leaf tissue were introduced into a tube containing 800 µl of CLS-VF, 200 µl of PPS and lysing matrix. Concentrated 2-mercaptoethanol (20 µl) was added to make a final concentration of 2%. The samples were homogenized twice in a FastPrep Instrument (BIO101, La Jolla, Calif., USA) for 20 s at a speed of 4.0. The homogenate was centrifuged at 14000 g for 5 min and the supernatant was transferred into a clean microcentrifuge tube. One volume of binding matrix was added, the mixture was incubated at room temperature for 5 min and centrifuged at 14000 g for 1 min. The pellet was gently washed with 500 µl of SEWS-M solution. Finally, genomic DNA was eluted from the binding matrix in 200–400 µl of water and stored at –20°C.

PCR amplification

Degenerate PCR primer SI-11 (YCARTTYGTNCARCARTGGCC; where R=A or G; S=A or C; Y=C or T; and N=A or C or G or T) was designed based on the N-terminal amino-acid sequences (FQFVQQWP) of almond S-RNases (Tao et al. 1997). First-strand cDNA was synthesized using a tagged oligo(dT) primer [ASBdT18; CCTGGCCAGGGCCCGTCGACGGATCC$(T)_{18}$] and a total RNA sample isolated from the styles of the cultivar Star. Primers SI-11 and the cDNA synthesis tag [i.e., ASB; same as above without the (dT) tail] were able to amplify the 3′end of the S-RNase genes. The PCR product was cloned, sequenced and identified as S4 based on its homology to apple and pear S-RNases and its distribution among cherry cultivars (Wu and Wiersma, unpublished). Genomic clones for alleles S1 and S4 were also identified by PCR and sequencing. Based on the conserved nucleotide sequence information for these genes, along with sequences from the GenBank database for alleles S2 (AB010304), S3 (AB010306), and S6 (AB010305) (Tao et al. 1999), four PCR primers were selected for the identification of all S-alleles. They were SI-19 (CCACCGACCAACTGCAGAGT), SI-20 (TGGTACGATTGAAGCGT), SI-31 (STTSTTGSTTTTGCTTTCTTC) and SI-32 (CATAGGCCATGRATGGTG). PCR reactions of a total of 25 µl or 50 µl were run in a RoboCycler 40 (Stratagene, La Jolla, Calif., USA) with one cycle of 3 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 50°C and 90 s at 72°C; and one cycle of 5 min at 72°C. The PCR products were analyzed in 1% or 2% agarose gels in 1×TAE buffer and visualized by staining with ethidium bromide.

Restriction endonuclease digestions of PCR fragments

All the restriction endonucleases were purchased from Gibco BRL Life Technologies. The unique enzymes were selected for a specific allele based on the sequence information of all cloned alleles (Wu and Wiersma, in preparation). One-tenth of the PCR reaction was digested with a particular enzyme (2–5 U) in 1× enzyme buffer at 37°C for 1 h. The digested PCR fragments were analyzed in 1% or 2% agarose gels. Molecular markers are 100 bp or 1 kb from Gibro BRL (e.g. in Fig. 1A the 100 bp is at left and 1 kb at right).

Results

Degenerate PCR primers were designed for the N-terminal coding sequences of published almond S-RNases (Tao et al. 1997) and from the conserved regions found in alignments of other S-RNases (Sassa et al. 1996). These primers amplified specific fragments by RT-PCR from total RNA extracted from the styles of the cherry cultivar Star (S3S4). These fragments were cloned and sequenced and the accumulation of this sequence data allowed an iterative process of primer re-design and fragment analysis that revealed more S-alleles. By this means, alleles S1–S4 were tentatively identified and cloned from cultivars with S-allele designations consistent in the literature. The end result of this process was the set of primers reported here which were capable of producing specific amplification products with either genomic DNA or cDNA. Sequence data for all six of the best-characterized alleles (S1 to S6) were obtained from genomic DNA of representative cultivars, and all had high homology within the coding region of other S-RNases. Genomic sequence data for the alleles (S1–S6) were obtained from 4, 3, 7, 5, 2 and 4 different cultivars, respectively. The sequence data is presently being prepared for publication (Wu and Wiersma, in preparation). Briefly, the genomic sequences for the S-alleles showed an intron structure which is unique to sweet cherry S-RNases. Each S-RNase coding region was interrupted by two introns at exactly the same position for all six genes. The introns for each allele differed in sequence and size from all other tested alleles. These differences form the basis of distinguishing the alleles by PCR.

Several sets of primers, designed to hybridize to the conserved coding region flanking the two introns, am-

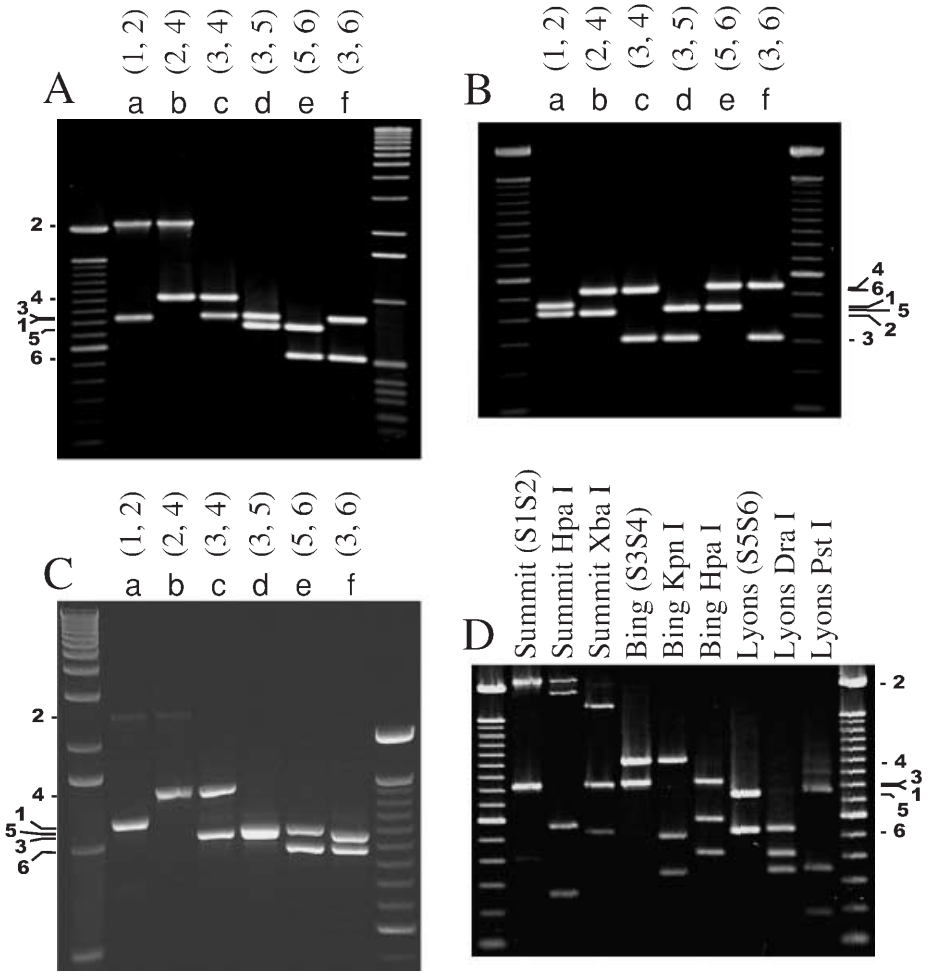

Fig. 1A–D PCR analysis of S-alleles from sweet cherry cultivars demonstrating the six predefined alleles. For **A**, **B** and **C** the cultivars used were: *a* Early Rivers (1, 2); *b* Schmidt (2, 4); *c* Star (3, 4); *d* Burlat (3, 5); *e* Lyons (5, 6) and *f* Merton Heart (3, 6). Fragments associated with specific alleles are indicated next to the figure. **A** Primer pair 19/20; **B** primer pair 31/32; **C** primer pair 31/20; **D** primer pair 19/20 with the restriction enzyme digest for the three cultivars as indicated.

plified all six of the common sweet cherry S-alleles. Primer pair SI-19+SI-20 spanned the sequence of the second intron and produced fragments from 530 to 2500 bp (Fig. 1A). The consistent pattern of fragments from each of the alleles allowed the dependable discrimination of alleles S2, S4, S5 and S6 but not S1 and S3, both of which migrate at approximately 820 bp. A tradeoff was necessary when running this reaction to allow ample time for the 2.2-kb S2 fragment to elongate while keeping times short enough not to produce non-specific fragments. (Optimum reaction conditions in our laboratory were as reported in the Materials and methods section but slight variations of annealing temperature and elongation time were required for some reactions.) A more-consistent pattern was obtained with primer pair SI-31+SI-32 which spanned the sequence of the first intron and produced fragments from 310 to 530 bp (Fig. 1B). Band intensities were usually comparable due to the similar size of the fragments. On standard 2.0% agarose gels, S1 was differentiated from S3 and thereby complemented the information from primer pair SI-19+SI-20 as well as confirming assignments for other alleles. With this second primer pair, allele pairs S4 and S6 as well as S1 and S5 were not well-resolved.

It was also possible to amplify allele-specific fragments using the primer pair SI-31+SI-20 which spanned both introns. As seen in Fig. 1C this produced fragments of larger size and it was often difficult to obtain these fragments consistently (note particularly the large 2.5-kb S2 fragment). The banding pattern was similar to Fig. 1A as the length of intron 2 contributes most of the length to both fragments.

To discriminate better between alleles without going to the effort of sequencing, an additional test of allele-type was developed. Based on the sequences for the cloned alleles, a set of restriction enzyme sites was found that cleave the S-alleles differentially. Each of the six alleles was amplified by PCR with primer pair SI-19+SI-20 and then digested with selected restriction enzymes (Fig. 1D). Alleles S1 and S2 were amplified from the cultivar Summit producing DNA fragments which were both cut by *Hpa*I while only S2 was cut by *Xba*I. Since PCR reaction samples were used directly for restriction digestion it was common for partial digestions to occur, such as for the Summit digested with *Hpa*I. It was clearly shown, however, that the enzymes produced the expected cuts for the alleles. Similarly, the S3 fragment from Bing DNA was cut only by *Kpn*I while S4 was cut by *Hpa*I. S5 and S6 from Lyons were cut differ-

Table 1 Fragment lengths generated by PCR of specific S-alleles

Allele	DNA fragment length in base pairs for specified PCR primer pairs			
	31/20	31/32	19/20	19/20; restriction fragments
1	1195	458	820	*Hpa*I (558, 262); *Eco*RV (469, 284, 67)
2	2493	422	2154	*Xba*I (512, 1642); *Hpa*I (256, 1898)
3	1057	306	828	*Kpn*I (490, 338)
4	1459	526	1013	*Hpa*I (588, 425); *Sca*I (678, 335)
5	1096	431	745	*Dra*I (408,337)
6	967	521	529	*Alu*I (187, 342)
12	1650	1000	727	*Nde*I (529, 198)
13	2051	423	1711	*Xho*I (625, 1086)
15		465		

entially by *Dra*I and *Pst*I. Additional restriction sites can be determined from sequences deposited in GenBank to give more detailed analysis. A summary of the results from the methods demonstrated in Fig. 1 is given in Table 1. Predicted fragment sizes for specific sets of primers were calculated for eight S-alleles. Restriction enzyme sites giving useful digestion patterns with one of these primer pairs (SI-19+SI-20) were also identified from sequence data.

The S allele composition of 70 sweet cherry cultivars was determined by the PCR method, in most cases requiring only the two reactions with primer pairs SI-31+SI-32 and SI-19+SI-20. To confirm unexpected results the fragments from the SI-19+SI-20 reaction were also digested with diagnostic restriction enzymes. In Table 2, cultivars are arranged first by allele type and then by the level of correspondence of the PCR data to the original assignment of that allele type (i.e., by Note). For example, of the four cultivars containing alleles S1 and S2 the PCR determination of the first three corresponded exactly to previous allele assignments (indicated by an "A" in the "Notes" column), while the remaining one did not.

Table 2–Note "A." Twenty four cultivars possessed the S-alleles assigned to them previously through cross-pollination studies (Matthews and Dow 1969; Tehrani and Brown 1992; Schmidt and Schulze 1998). Cultivars from which sequence data were obtained are indicated by the allele number in the "Seq." column of Table 2. Each of the six most-common S-alleles, except S5, was represented multiple times in those cultivars showing PCR patterns consistent with published S-allele data. For S5, the assignment of allele type from published data was more difficult. Of the cultivars listed as containing allele S5 by Mathews and Dow (1969) we examined three from Group VII (S4S5; Early Burlat, Black Republican and Hedelfingen) and Noir de Schmidt from Group VIII (S2S5). In our initial experiments, only Early Burlat contained a sequence distinct from the other five standard alleles. Black Republican was S1S4, Hedelfingen was originally thought to be S1S3 (see below) and Noir de Schmidt was S2S4. Therefore, the sequenced fragment found in Early Burlat was used as the standard sequence for allele S5.

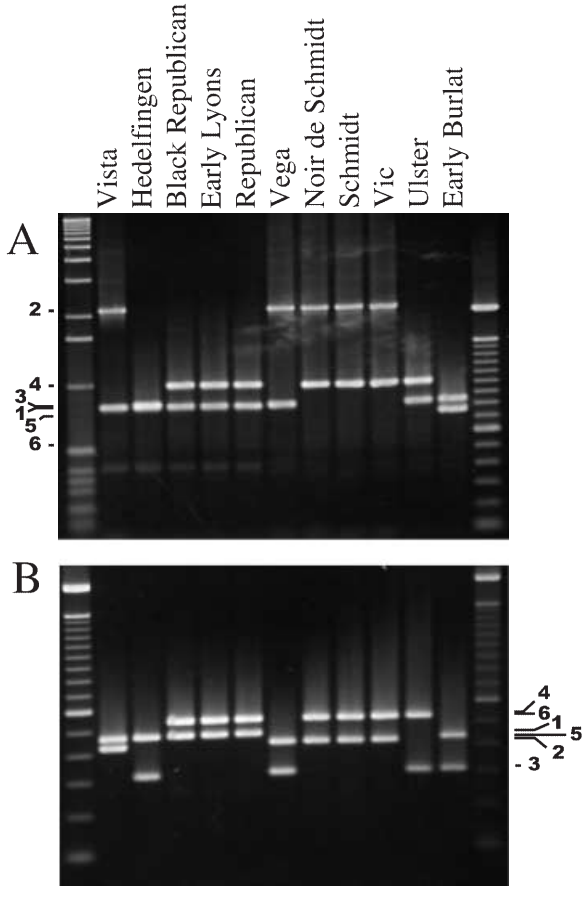

Fig. 2A, B PCR patterns for S-alleles from cultivars where the analysis differed from the published alleles. **A** Primer pair 19/20; **B** primer pair 31/32. The variety is indicated above each lane and specific fragments are also indicated

Table 2–Note "B." Nine cultivars which had previously been assigned to an incompatibility group gave the PCR pattern expected for another group. Reassignment of S-alleles was based on consistent banding patterns for PCR across both intron 2 (Fig. 2A) and intron 1 (Fig. 2B). Most of the alleles were unambiguously assigned by fragment patterns produced with primer pair SI-19+SI-20 (Fig. 2A) with the the differentiation between S1 and S3 being resolved with SI-31+SI-32 (Fig.

Table 2 Identification of sweet cherry S-alleles by PCR and sequencing

Variety	PCR	Seq.	Group	Assigned by	Notes
Black Tartarian	1, 2		I	MD, TB	A
Early Rivers	1, 2		I	MD, TB	A
Sparkle	1, 2		I	MD, TB	A
Summit	1, 2	1, 2	I		S
Van	1, 3	1, 3	II	MD, TB	A
Venus	1, 3		II	MD, TB	A
Windsor	1, 3		II	MD, TB	A
Cristalina	1, 3		II		S
Samba	1, 3		II		S
Sonnet	1, 3		II		S
Black Giant	1, 4		IX	TB	A
Chinook	1, 4		IX	MD, TB	A
Rainier	1, 4		IX	MD, TB	A
Lapins	1, 4	4	IX, 4′	SS	A
Black Republican	1, 4		IX	MD,TB (VII; 4,5)	B a
Early Lyons	1, 4		IX	TB (X)	B
Republican	1, 4		IX	MD (XII),TB (IX)	B
Celeste	1, 4		IX		S
Salmo	1, 4		IX		S
Santina	1, 4		IX, 4′		S
Skeena	1, 4		IX, 4′		S
Summer Jewel	1, 4		IX		S
Sylvia	1, 4		IX		S
Symphony	1, 4		IX		S
Bada	1, 4		IX		X
Dawson	1, 4		IX		X
	1, 5		?		D
	1, 6		?		D
Sue	2, 3	3	IV	MD, TB	A
Velvet	2, 3		IV	MD, TB	A
Victor	2, 3		IV	MD, TB	A
Viva	2, 3	3	IV	TB	A
Vogue	2, 3		IV	TB	A
Vega	2, 3		IV	TB (O)	B
Sam	2, 4	2, 4	XIII	MD (?), SS	A c
Noir de Schmidt	2, 4	4	XIII	MB (VIII; 2,5)	B b
Schmidt	2, 4		XIII	T (VIII; 2,5)	B b
Vic	2, 4		XIII	MD, TB (O)	B
Deacon	2, 4		XIII	MD (?)	X c
Patricia	2, 4		XIII		X
	2, 5		?		D
	2, 6		?		D
Bing	3, 4	4	III	MD, TB	A
Emperor Francis	3, 4		III	MD, TB	A
Lambert	3, 4		III	MD	A
Napoleon	3, 4		III	MD, TB	A
Star	3, 4	3, 4	III	MD, TB	A
Ulster	3, 4	3	III	MD (XIII; 2,4)	B d
Newstar	3, 4		III, 4′		S
Sandra Rose	3, 4		III, 4′		S
Sonata	3, 4		III, 4′		S
Stella	3, 4		III, 4′		S
Sunburst	3, 4		III, 4′		S
Sweetheart	3, 4		III, 4′		S
Angela	3, 4		III		X
Kristin (NY1599)	3, 4		III		X
Early Burlat	3, 5	3, 5	VII	MD, TB (4, 5)	B e
Early Amber	3, 6	6	VI	MD	A
Merton Heart	3, 6	6	VI	MD	A
Stark's Gold	3, 6		VI	MD, TB	A
Merpet (Mermaid)	4, 5			JI1 (O)	C
Merchant	4, 5			JI2 (O)	C
Elton Heart	4, 6			MD (VI)	C
Lyons	5, 6	5, 6		T (X)	C f
Noir de Guben	1, 15	1		MD (?)	E g
Valera	1, 15	1, 15		TB (?)	E g
Mona	2, 14	2, 14			E h

Table 2 continued

Variety	PCR	Seq.	Group	Assigned by	Notes
Vista	2, 15			TB (O)	E
Schneiders	3, 13	13		MD (III; 3,4)	E
Princess	3, 13				E
Hedelfingen	3, 15	3, 15		MD (VII; 4,5)	E i
Viscount	4, 15			TB (IX)	E
Ramon Oliva	5, 12	12		MD (X)	E
Noble	6, ?	6		MD (XII)	E

MD=Matthews and Dow 1969; JI1=Matthews and Dow 1977
JI2 = Matthews and Bullen 1979; TB=Tehrani and Brown 1992
T=Thompson 1996; SS=Schmidt and Schulze 1998
A Same as published
B Reassignment to defined group
C Assignment with new combination of 1–6
D Unassigned combinations of 1–6
E Assignment with new uncharacterized allele
S Assignment of Summerland cultivars
X Assignment of other cultivars
a Same as Republican (Hedrick, 1915)
b Noir de Schmidt and Schmidt possibly same
c, g These cultivars in same group but not assigned by MD
d Parentage is Schmidt (2,4) ×Lambert (3,4)
e Reassigned by Boskovic et al. (1997) and Tao et al. (1999)
f Same as Bigarreau Jaboulay (Thompson, 1996)
h Second intron not amplified by primer pair 19/20 for S14
i Two clones gave same results

2B). These cultivars included a number of releases from the Vineland (Ontario, Canada) breeding program for which the original assignments were based on parent cultivars Hedelfingen and Schmidt having the allele types of S4S5 and S2S5, respectively (Tehrani and Lay 1991). For example, the cultivar Vista was previously assigned an "O" grouping but was shown by PCR to belong to the previously defined S1S2 group (the conflict indicated by the "B" in the note column). At this resolution of detection the alleles for the cultivar Hedelfingen (lane 3) were indistinguishable from S1S3 although further analysis demonstrated it to be S3S15. The clones Black Republican and Republican gave results consistent with each being the same cultivar as suggested by Hedrick (1915). It is uncertain from the literature whether Schmidt and Noir de Schmidt, which showed the same PCR pattern here, are distinct cultivars. Early Lyons, Ulster and Early Burlat also showed different than expected patterns.

Table 2–Note "C." Fourteen cultivars produced 11 allele combinations which had not previously been designated in sweet cherry. The designation "Groupless" or "O" has been given when a variety was self-incompatible but was able to pollinate all defined groups (Crane and Brown 1955). These could be cultivars with unique combinations of the six common alleles or those with new alleles. Merchant, Merpet, Elton Heart and Lyons showed new combinations of the six S-alleles (Fig. 3) and were designated "C" in the notes of Table 2. One of the fragments from Lyons gave a sequence identical to S5 from Early Burlat.

Table 2–Note "E." Ten cultivars with new allele combinations include one allele from the most-common six allele types and one of four different new genes (designated here S12 through S15). These were first detected as PCR fragment sizes different from the previously characterized six alleles (Fig. 4A). A single primer pair determination was not always sufficient to differentiate the new alleles. The sequence data for allele S13 predicted a fragment only 1 bp different from that for S2 when

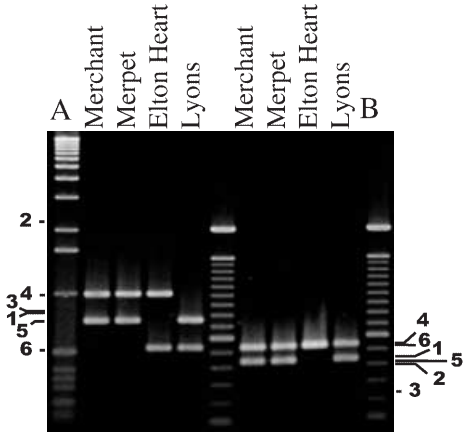

Fig. 3 Sweet cherry cultivars with new combinations of alleles 1 through 6. DNA from the indicated cultivars was amplified with *A*, primer pair 19/20; or *B*, primer pair 31/32

primer pair SI-31+SI-32 was used (Table 1), which could not be resolved on agarose gels. Similarly, the cultivars Mona and Hedelfingen were thought to contain S1 until sequence data from the fragments which migrated with S1 showed them to be new alleles (S14 and S15, respectively). Fragments for S1 and S15 from the cultivar Hedelfingen were most easily distinguished by restriction digestion (Fig. 4B) which was important for the analysis of the Vineland cultivars discussed below. Fragments for the cultivars Schneiders and Princess were very similar in migration but PCR and restriction analysis (Fig. 4C) showed that S13 (identified by sequencing from Schneiders) was contained in both cultivars. This tentatively differentiates four new alleles which can be identified by their PCR patterns in the same fashion as the previous six alleles. In addition to the standard usage of alleles S1 through S6 as designated by the John Innes Institute group (i.e., Matthews and Dow 1969), assignments for five different alleles (S7 through S11) were proposed according to the isozyme patterns of Bŏsković et al. (1997). To avoid conflicting designations, the new alleles ob-

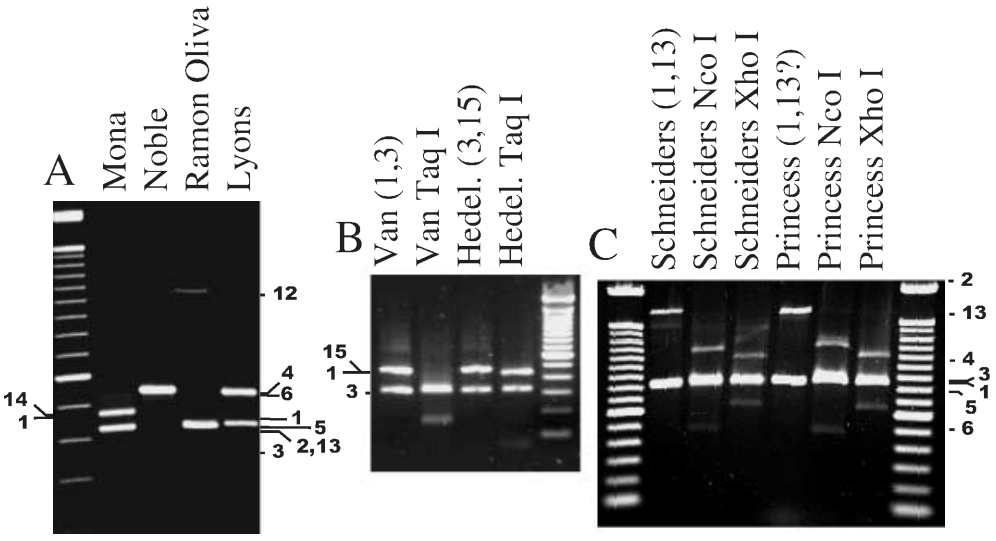

Fig. 4A–C New alleles found in sweet cherry cultivars. **A** Primer pair 31/32 with cultivars showing non-standard patterns except Lyons (5, 6). Allele positions are indicated by the numbers on the sides. **B** Cultivars Hedelfingen and Van compared using primer pair 31/32 and digestion with *Taq*I. **C** Primer pair 19/20 comparing cultivars Schneiders and Princess with and without restriction digestion

Table 3 Evaluation of S-alleles for Agriculture and Agri-Food Canada (Vineland) releases and parent cultivars

Variety	S-type	Parentage
Valera	1, 15	Hedelfingen (3,15) ×Windsor (1,3)
Vega	2, 3	Bing (3,4) ×Victor (2,3)
Velvet	2, 3	Windsor (1,3) ×OP
Venus	1, 3	Hedelfingen (3,15) ×Windsor (1,3)
Vic	2, 4	Bing (3,4) ×Schmidt (2,4)
Victor	2, 3	Windsor (1,3) ×OP
Viscount	4, 15	Hedelfingen (3,15) ×Bing (3,4)
Vista	2, 15	Hedelfingen (3,15) ×Victor (2,3)
Viva	2, 3	Hedelfingen (3,15) ×Victor (2,3)
Vogue	2, 3	Hedelfingen (3,15) ×Victor (2,3)

served in this study by PCR fragment patterns and sequence analysis were numbered starting with S12.

Notes “S”, “X.” Twenty three cultivars which had not been rigorously characterized were analyzed for S-allele composition. Of these, 17 were cultivars from the Summerland breeding program, ten of which were expected to contain the pollen-part self-fertility factor (S4′) inherited from the radiation-induced mutant JI 2420 (Lewis and Crowe 1954; Matthews 1970). Genomic DNA sequences and PCR fragment sizes for the S4-RNases were identical between the standard S4 and the mutant S4′. PCR patterns were consistent with parental types.

As a practical application of this PCR method, the S-allele patterns of a number of Vineland, Ontario, cultivars were compared in Table 3 to the parental cultivars as originally published. Confusion surrounding the assignment of these alleles brought into question the original parent types. The present data shows a good correspondence between the S-alleles of the proposed parents and those inherited by the individual cultivars.

Discussion

In the twenty four cultivars whose S-alleles as found by PCR precisely matched with those previously assigned (Matthews and Dow 1969; Tehrani and Brown 1992; Schmidt and Schulze 1998), the six originally designated alleles (S1-S6) were represented 10-, 9-, 16-, 10-, 0- and 3-times each, respectively. This gives a good representations and confidence in the allele assignment for the first four alleles and reasonable confidence in allele S6. Allele S5 was first characterized from Burlat and is present in only four additional cultivars tested here. Genomic sequence data for these six alleles (S1–S6) was obtained for 4, 3, 7, 5, 2 and 4 different cultivars, respectively. The sequences were invariant in coding region and intron structure for each individual allele, regardless of cultivar source. Genomic DNA was a convenient source of material, available year-round in comparison to stylar tissue which is very small and available for only 1 week out of the year. The highly divergent size of the introns in sweet cherry S-RNase genes provides a convenient basis for distinguishing alleles by PCR-amplification.

Assignment of allele S5 was done in consideration of the conflicting analyses in two lines of work (European: Bŏsković and Tobutt 1996; Bŏsković et al. 1997; Schmidt and Timmann 1997; Schmidt et al. 1999; and Japanese: Tao et al., 1999). In Bŏsković and Tobutt (1996) the S5 allele was not assigned because of uncertain migration of the S-RNase from this allele using the varieties Hedelfingen, Bradbourne Black (both thought to be S4S5) and Late Black Bigarreau (S3S5). Additional varieties from the same groups (Hookers Black, S4S5; Turkey Heart, S3S5) were added in their subsequent work (Bŏsković et al. 1997). Identification of alleles S3 and S4 was conclusively made (data not shown) and it was recommended that the allele assignments for group V (S3S5) and group VII (S4S5) should be switched, with Hedelfingen a representative of the S3S5 type. The assignment of S5 to the

RNase band Sy was also made at this time. The question of Hedelfingen's role in the Vineland varieties was used as evidence of the need to change the assignments. The cultivars containing S5 listed by Schmidt (Schmidt and Timmann 1997; Schmidt et al. 1999) are also based on these data with the exception of cultivar Valera and cross-incompatible cultivars Alma, Annabella and Bianca. Valera was assigned allele S1S5 in a personal communication by P. Matthews as reported by Way (1968) with no additional confirmation shown. Considering the inconsistencies within the original groups V, VII and VIII (which were thought to contain S5) shown by these reports, as well as in the present work, the identification of this allele must be re-examined. Tao et al. (1999) examined only two representative cultivars expected to contain S5 [Burlat and Moreau, S3S5 by the suggestion of Bŏsković et al. (1997) in reassigning group V]. Both cultivars had the same two amplified fragments, one of which was identical to other S3-containing cultivars while the second was assigned to allele S5. The present work also found this fragment in the cultivar Early Burlat but not in the other cultivars assigned this allele (i.e., Schmidt, Hedelfingen, Black Republican). Without a firm context within the original data, the assignment of this allele is arbitrary for those cultivars within S5-containing groups. Until a uniform convention is determined we have chosen to use the assignment based on the molecular data from Tao et al. (1999). Therefore, based on the sequence of the allele in Early Burlat and the consistent fragment pattern found in Merpet, Merchant, Lyons and Ramon Oliva we recommend assignment of S5 to Early Burlat and suggest this cultivar as a standard for further analysis. It will be interesting to see if the S5 and S15 (as used in this work) products share some biochemical characteristics that explain their association with equivalent incompatibility groups.

New alleles in this study were first detected as PCR fragments migrating at undefined positions compared to the standard alleles. These fragments were then cloned and sequenced. To-date, all sweet cherry S-RNases have introns with divergent sizes and few similar restriction sites in the second intron. The sequence data has allowed accurate determination of fragments produced by PCR with and without enzyme digestion. DNA sequencing is the preferred method for the identification of new alleles. PCR patterns and enzyme restriction patterns can be predicted from these sequences. At this point in their characterization the allele sequences are equally distant in identity so there is no confusion with regard to their identification. With fragment lengths, especially with only one set of PCR primers, it is less likely to be certain of the allele type identity. In addition, with the sequence data, each nucleotide acts as an independent data point giving further confidence to the assignment. PCR data and restriction-site analysis each add only one data points per sample. It would be useful if this sequence data were also capable of predicting the migration pattern of S-RNases from stylar extracts as used by Bŏsković and Tobutt (1996). The isoelectric points (pIs) estimated by these authors for alleles S1-S4 and S6 were 8.9, 8.7, 9.3, 9.2 and 9.0, respectively, whereas, the calculated pIs for the putative mature proteins from the sequenced genes were 9.3, 9.7, 9.4, 9.3 and 9.5, respectively. The gene codes were not able to correctly predict the migration pattern of the resulting proteins on isoelectric focusing gels. Perhaps with better information on folding and post-synthesis modifications this prediction might be improved.

The new allele combinations have not yet been retested for pollination consistency, but from the bulk of previously defined types it is likely that this methodology will predict well the incompatibility relationships in sweet cherry cultivars. Much of the confusion associated with allele assignments has been in knowing the genotype of the test pollen donors and acceptors. Standard test cultivars which have been a source of error in the past are not required for the PCR analysis. Once the DNA sequences are deposited in databases any allele will be directly detectable by PCR and/or sequence comparison, and will no longer be dependent on the test cultivars in a collection. The S-allele type has been used as a means of cultivar identification. This continues to be a valid method that is enhanced by the speed and specificity of the PCR methodology.

The PCR and sequencing analysis was unable to distinguish between the S4 and mutant S4′ alleles. This was consistent with the S4′ pollen component being separate from, but closely linked to, the S4-RNase gene. The S-allele pattern was only valid for the stylar component of incompatibility and can, therefore, only be used as a linkage marker for the important self-incompatibility trait.

The confusion surrounding the assignment of specific incompatibility groups for the Vineland selections (Vic, Venus, Vogue, Viva and Viscount) has been the topic of several reports (Way 1968; Tehrani and Dickson 1974; Tehrani 1984). Tehrani and Lay (1991) suggested that the problem was the incorrect assignment of S-alleles to some of the parental cultivars. Cultivars with the names of Hedelfingen and Schmidt, believed to have S-alleles S4S5 and S2S5, respectively (Matthews and Dow 1969), were found in the present study to be S3S15 and S2S4. (Tree and fruit characteristics from the sampled trees were consistent with these variety types. A second clone of Hedelfingen, obtained from the Prosser, Wash., USA, repository gave the same S3S15 patterns by PCR.) As shown in Table 3 this clarified the pattern of inheritance of those cultivars, as each set of parents was able to contribute the observed alleles. The combination of sequencing and PCR analysis was also able to clarify the debated allele assignments.

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