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ORIGINAL PAPER

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Identification of incompatibility alleles in the tetraploid species sour cherry

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Abstract The incompatibility genetics of sour cherry (*Prunus cerasus*), an allotetraploid species thought to be derived from sweet cherry (diploid) and ground cherry (tetraploid), were investigated by test crossing and by analysis of stylar ribonucleases which are known to be the products of incompatibility alleles in sweet cherry. Stylar extracts of 36 accessions of sour cherry were separated electrophoretically and stained for ribonuclease activity. The zymograms of most accessions showed three bands, some two or four. Of the ten bands seen, six co-migrated with bands that in sweet cherry are attributed to the incompatibility alleles S_1, S_3, S_4, S_6, S_9 and S_{13}. 'Čačanski Rubin', 'Erdi Botermo B', 'Koroš' and 'Ujfehertoi Furtoš', which showed bands apparently corresponding to S_1 and S_4, were test pollinated with the sweet cherry 'Merton Late' (S_1 S_4). Monitoring pollen tube growth, and, in one case, fruit set, showed that these crosses were incompatible and that the four sour cherries indeed have the alleles S_1 and S_4. Likewise, test pollination of 'Marasca Piemonte', 'Marasca Savena' and 'Morello, Dutch' with 'Noble' (S_6 S_{13}) showed that these three sour cherries have the alleles S_6 and S_{13}. S_{13} was very frequent in sour cherry cultivars, but is rare in sweet cherry cultivars, whereas with S_3 the situation is reversed. It was suggested that the other four bands are derived from ground cherry and one of these, provisionally attributed to S_B, occurred frequently in a small set of ground cherry accessions surveyed. Analysing some progenies from sour by sweet crosses by *S* allele-specific PCR and monitoring the success of some sweet by sour crosses were informative. They indicated mostly disomic inheritance, with sweet cherry *S* alleles belonging to one locus and, presumably, the ground cherry alleles to the other, and helped clarify the genomic arrangement of the alleles and the interactions in heteroallelic pollen.

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Introduction

Sour cherry (*Prunus cerasus* L.) includes self-compatible as well as fully or partially self-incompatible cultivars (Crane and Lawrence 1929; Hruby 1963; Montalti and Selli 1984; Redalen 1984b) and there are reports of cross-incompatibility, reciprocal or unilateral (Hruby 1963; Redalen 1984a; Apostol 1996). It is a tetraploid species (Darlington 1927; Kobel 1927), apparently an allotetraploid derived from hybridisation of sweet cherry, *P. avium* L. ($2n=2x=16$), and ground cherry, *P. fruticosa* Pall. ($2n=4x=32$) (Olden and Nybom 1968).

In the diploid sweet cherry, nearly all cultivars are self-incompatible and many pairs of cultivars are reciprocally cross-incompatible. This incompatibility was ascribed to a multi-allelic *S* locus with gametophytic expression (Crane and Lawrence 1929). In due course, about 160 cultivars were allocated to 13 incompatibility groups, to ten of which pairs of alleles from S_1 to S_6 were assigned (Matthews and Dow 1969). Seven more *S* alleles, S_7, S_9, S_{10}, S_{12}, S_{13}, S_{14} and S_{16}, have recently been reported and 14 more incompatibility groups have been genotyped (Bošković et al. 1997; Bošković and Tobutt 2001; Sonneveld et al. 2001, 2003; Tobutt et al. 2001). The *S* alleles in cherry code for stylar ribonucleases (*S*-RNases). These can be distinguished by isoelectric focusing of stylar proteins and staining for activity (Bošković and Tobutt, 1996, 2001; Bošković et al. 1997) or by allele-specific PCR (Sonneveld et al. 2001, 2003). Little is known about incompatibility in ground cherry.

If inheritance is disomic in sour cherry, as indicated by the isoenzyme studies of Beaver and Iezzoni (1993), the diploid pollen grains of 'regular' sour cherries will each contain one *S* allele from sweet cherry and one from ground cherry, if the latter indeed has *S* alleles. Of course, this may not be the case in the pollen grains of 'sour cherries' that are the result of backcrossing to either parental species. Some of the complications that might be expected in the genetic control of incompatibility in tetraploids were pointed out by Crane and Lawrence (1929) and Lawrence (1930), and later Crane and Lawrence (1938) amplified these observations to offer an explanation for unilateral incompatibility between certain tetraploids. A significant advance in the understanding of the subject was the proposal by Lewis and Modlibowska (1942) that the self-compatibility of a tetraploid sport of pear could be attributed to the compatibility of the heteroallelic diploid pollen in the selfed style. Lewis discussed evidence from further species, especially *Oenothera organensis*, that in heteroallelic diploid pollen in some species, the two *S* alleles may interact competitively, so that such pollen is not rejected in styles having one or both of the same *S* alleles (Lewis 1943, 1947, 1954), or else one allele can dominate the other, so that the pollen is rejected in styles having the dominant allele (Lewis 1947, 1954). In sour cherry, no detailed explanations of the genetics of (in)compatibility have been offered. Yenikeyev (1973) looked at inheritance of self-incompatibility and self-compatibility in some progenies. Lansari and Iezzoni (1990) found some self-incompatible seedlings in progenies from crosses between self-compatible cultivars, suggesting those self-compatible parents carry self-incompatibility alleles. Recently, Yamane et al. (2001) identified and characterised several *S* ribonucleases in sour cherry by PCR amplification of genomic DNA, using consensus primers or by Southern blot analysis.

We decided to extend our work on sweet cherry to sour cherry with the aim of contributing to the elucidation of (in)compatibility in this tetraploid species. First, we surveyed stylar ribonucleases in a range of cultivars, to see if any alleles from sweet cherry appeared in sour cherry cultivars, whether self-compatible or self-incompatible; and we examined a few accessions of ground cherry to see if other sour cherry alleles were shared with this species. Then, noting that some sour cherry cultivars appeared to have two sweet cherry *S* alleles, we pollinated some of those with sweet cherries having the same two *S* alleles and assessed the results by monitoring pollen tube growth with fluorescence microscopy and/or by recording fruit set. Our use of pollen from diploids to clarify incompatibility genotypes in tetraploids was prompted in part by the work of Lewis (1943, 1947) in *O. organensis*. From some of those crosses, we analysed progenies to determine the inheritance of the ribonucleases using specific PCR primers that have recently become available for various alleles. Finally, to clarify the differences in self-(in)compatibility status for two cultivars with the same stylar ribonuclease pattern and gain insight into allelic interaction in heteroallelic diploid pollen, appropriate sweet cherry by sour cherry crosses were carried out, again prompted by the work of Lewis (1943).

Materials and methods

Stylar ribonuclease analyses

To determine the stylar ribonuclease patterns of a range of sour cherries, 36 accessions were analysed, some self-incompatible and some self-compatible or partly so, principally from collections at Horticulture Research International (HRI) East Malling or the National Fruit Collections, Brogdale, UK (Table 1). There are several accessions of 'Morello' type—'Morello' is considered to represent a population rather than a single cultivar. Although 'Koroš' and 'Crisana' are regarded as synonyms, the two accessions are listed separately, as 'Koroš'/'Crisana' is also considered a population rather than a single cultivar (Iezzoni et al. 1991). To see if any sour cherry ribonucleases were shared with ground cherry, we also analysed eight accessions of ground cherry collected from a wild population in northwest Yugoslavia, at Koševac on the south bank of the Danube, and numbered EM5590 to EM5597.

Styles were collected and the proteins extracted following the procedures of Bošković and Tobutt (1996), with the modification that just ten styles were used with 1 ml of extraction solution. The extracts were loaded on acrylamide gels, prepared in accord with Bošković and Tobutt (1996), and, with a view to testing of apparent homologies and/or detecting otherwise cryptic variation, were run under two sets of conditions. Isoelectric focusing (IEF) comprised 1 h at 150 V, 2 h at 300 V and 3 h at 450 V, and non-equilibrium pH gradient electrofocusing (NEPHGE) 1 h at 150 V, 2 h at 300 V and 1 h 45 min at 450 V. The gels were stained following the procedure described by Bošković and Tobutt (1996). A range of sweet cherry cultivars of known incompatibility genotype were included on preliminary gels for comparison. For the photographs, just a set of relevant sweet cherry cultivars or ladders representing the alleles S_1 to S_6, S_9 and S_{13} from sweet cherry were included.

Test pollinations

To check possible identity of ribonucleases in sweet and sour cherry, seven sour cherry cultivars were test pollinated with sweet cherry cultivars in 1998 so that pollen tube growth could be observed, after the viability of the pollen had been confirmed by germination on 10% sucrose and 250 ppm boric acid in agar. 'Čačanski Rubin', 'Erdi Botermo B', 'Koroš', and 'Ujfehertoi Furtos', which showed bands apparently identical with the bands for the sweet cherry alleles S_1 and S_4, were pollinated with 'Merton Late' (S_1 S_4), 'Van' (S_1 S_3) and 'Governor Wood' (S_3 S_6). 'Marasca Piemonte', 'Marasca Savena' and 'Morello, Dutch', which showed bands apparently corresponding to S_6 and S_{13}, were each pollinated with 'Noble' (S_6 S_{13}) and 'Merton Late' (S_1 S_4). The pollinations of 'Čačanski Rubin' were made at Čačak in the field following Cerović et al. (1992). The other pollinations were made at East Malling on detached shoots brought to the laboratory and stood in water-soaked florists' foam (Oasis); flowers were emasculated at the balloon stage and pollinated 1 day later. Seventy-two hours after pollination, about 20 pistils per cross of 'Čačanski Rubin' and about ten pistils per cross of the other cultivars were fixed in FPA (40% formaldehyde: propionic acid: 70% ethanol, 5:5:90) and stored at 4°C until required. They were then squashed and stained and examined by fluorescence microscopy following the procedures of Preil (1970).

Two of the sour cherry cultivars, 'Erdi Botermo B' and 'Marasca Piemonte', were hand pollinated in 1998 with the sets of cultivars just described to observe fruit set. In addition, to elucidate how two sour cherry cultivars could have the same ribonuclease phenotype but different self-(in)compatibility status, pollen of 'Amarena di Verona P.C.' and 'Montmorency', which showed the

same three bands including one apparently corresponding to S_6, but were, respectively, self-incompatible (Albertini et al. 1988) and self-compatible (Montalti and Selli 1984; Redalen 1984b; Albertini et al. 1988; Lansari and Iezzoni 1990), was used to pollinate 'Merton Late' (S_1 S_4) and 'Sasha' (S_3 S_6) in 2000. The crosses were made on emasculated flowers on potted trees in an insect-proof glasshouse, generally 100 or more flowers per cross. Fruit set was recorded after 8 weeks.

Progeny analysis by *S* allele-specific PCR

To test the reported disomic inheritance in sour cherry (Beaver and Iezzoni 1993), and clarify the allelic constitution of diploid gametes in two sour cherries, three small progenies were raised, from sour cherry by sweet cherry crosses. These were 'Erdi Botermo B', which showed four bands including those apparently corresponding to S_1 and S_4, crossed with 'Van' (S_1 S_3) and with 'Governor Wood' (S_3 S_6), and 'Marasca Luxardo', which showed three bands including those apparently for S_6 and S_9, with 'Victor' (S_2 S_3). (The last two crosses were between cultivars having no stylar ribonuclease alleles in common.) The resulting seedlings, and parents, were analysed by PCR, using *S* allele-specific primers for S_1, S_3, S_4, S_6 and S_9, in accordance with Sonneveld et al. (2001, 2003).

Results

Stylar ribonuclease analyses of sour cherry cultivars and ground cherry accessions

Separation of stylar extracts of the sour cherries under IEF and NEPHGE conditions and staining for ribonuclease activity revealed two to four principal bands per cultivar, seen as pale bands on a blue background. Running extracts of various sweet cherries alongside showed that some of the bands in sour cherry co-migrated with sweet cherry alleles.

Figure 1a, b shows the zymograms of 15 sour cherry cultivars, run under IEF and NEPHGE respectively, encompassing the complete range of phenotypes seen, together with reference 'ladders' for the sweet cherry alleles S_1 to S_6, S_9 and S_{13}. Each cultivar reveals at least one band apparently identical, under both sets of conditions, with a band corresponding to a sweet cherry allele. These bands we will refer to temporarily as '1', '3', '4', '6', '9' and '13', the number indicating the corresponding sweet cherry *S* allele. In addition, four more bands were

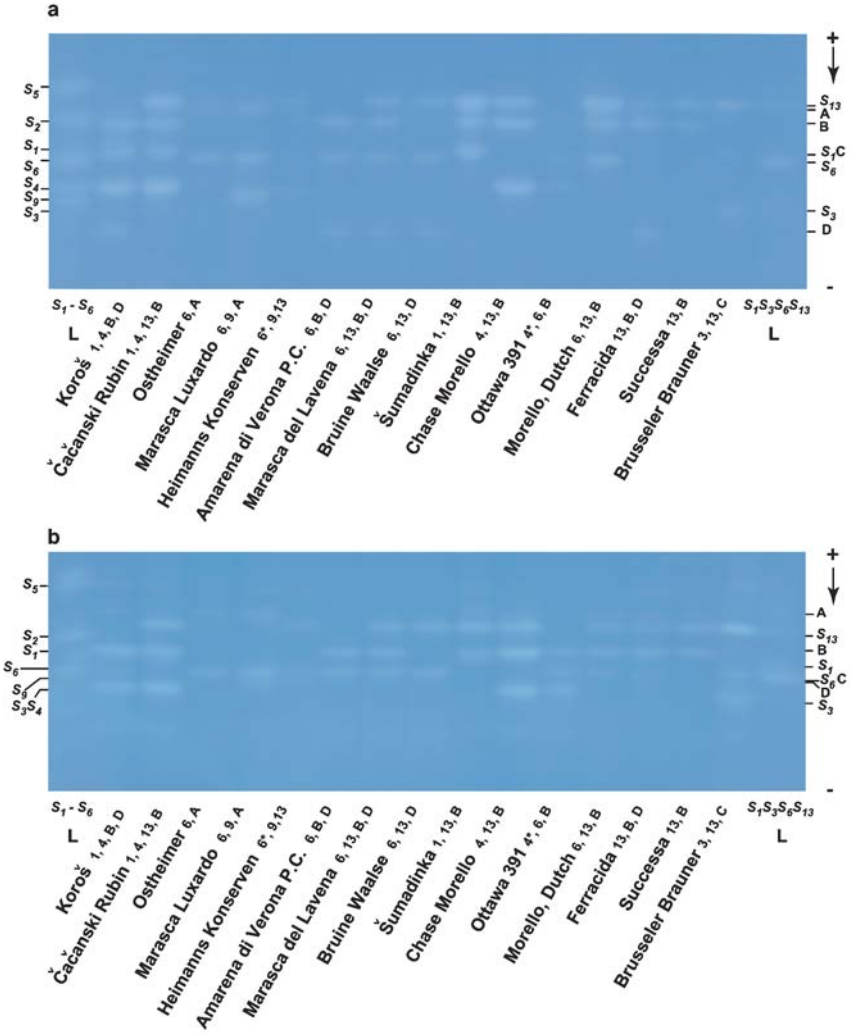

Fig. 1 Stylar ribonucleases of 15 sour cherries representing a range of different phenotypes, together with reference ladders for the sweet cherry alleles S_1 to S_6, S_9, and S_{13}, run under IEF (**a**) and NEPHGE (**b**). The relative positions of S_3 and S_4, of A and S_{13} and of D with respect to S_1 and S_6 are affected by running conditions. (*PCR did not confirm the presence of S_6 in 'Heimanns Konserven' and S_4 in Ottawa 391)

Table 1 Sour cherry cultivars analysed for stylar ribonucleases and bands observed

Cultivar	Source[a]	Bands observed	SI/SC[b]	Reference[c]
Amarena di Verona P.C.	EM	6, B, D	SI	Al
Bruine Waalse	Wi	6, 13, D	SI	D
Brusseler Brauner	Br	3, 13, C	SI	H
Čačanski Rubin	EM	1, 4, 13, B	SC	Ce
Chase Morello	EM	4, 13, B	SI	R
Crisana	Br	1, 4, B, D	SI	L
Diemitzer Amarelle	Br	13, B, D	SC	R
Elmer	EM	13, B	SC	EM
Erdi Botermo B	EM	1, 4, B, D	SC	EM
Favorit	Br	13, B, D	SC	Ap
Ferracida	Br	13, B, D	SC	Al
Flemish	Br	6, 13, B	SC	EM
Gay Maraschino	Br	6, 13, B	SC	EM
Heimanns Konserven	Ca	6[d], 9, 13	SC	Al, R
Kentish Morello	Br	6, 13, B	SC	EM
Kentish Red A	Br	13, B	SC	Cr
Kentish Red C	Br	6, 13, B		
Konigliche Amarelle	EM	13, B, D	SI/SC	Al, R/D
Koroš	EM	1, 4, B, D	SI	Al, H, L, R
Kronio	EM	6, B, D	SC	Ca
Meikers[e]	Wi	6, 13, B	SC	D
Marasca del Lavena	EM	6, 13, B, D		
Marasca Luxardo	EM	6, 9, A	SC	Al
Marascone Nero di Verona	EM	6, B, D	SI?	EM
Marasca Piemonte	EM	6, 13, B	SC	EM
Marasca Savena	EM	6, 13, B, D	SI	Al
Montmorency	Br/Wi	6, B, D	SC/SI	Al, L, M, R/D
Morello EMLA	EM	6, 13, B	SC	L, R
Morello, Dutch	Br	6, 13, B	SC?	
Nabella	Br	13, B	SC	Al, M, R
Ostheimer	EM	6, A	SI	R, Y
Ottawa 391	Ul	4[d], 6, B	SI	R
Schattenmorelle	Dr	6, 13, B	SC	M, R
Successa	Br	13, B	SC	EM
Šumadinka	EM	1, 13, B	SC	Ce
Ujfehertoi Furtos	EM	1, 4, B, D	SC	Ap

[a] *Br* Brogdale, UK; *Ca* Čačak, Yugoslavia; *Dr* Dresden, Germany; *EM* East Malling, UK; *Ul* Ullensvang, Norway; *Wi* Wilhelminadorp, The Netherlands
[b] *SI* Self-incompatible; *SC* self-compatible, at least partially
[c] *Al* Albertini et al. (1988), *Ap* Apostol (1996), *Ca* Calabrese et al. (1984), *Cr* Crane (1927), *Ce* Cerović (1992), *D* De Vries (1968), *EM* East Malling data, *H* Hruby (1963), *L* Lansari and Iezzoni (1990), *M* Montalti and Selli (1984), *R* Redalen (1984b), *Y* Yenikeyev (1973)
[d] PCR did not confirm the presence of S_6 in 'Heimanns Konserven' and S_4 in Ottawa 391 (data not shown)
[e] Considered to be Duke cherry (De Vries 1968)

seen that did not match any of the sweet cherry alleles S_1 to S_{14}; all cultivars, except perhaps 'Heimanns Konserven', showed at least one such band. These are provisionally labelled 'A', 'B', 'C' and 'D'. The pI values of these are: A, 8.35; B, 8.75; C, 8.90; and D, 9.70. The resolution of the bands depended to some extent on the separating conditions. Generally, the order of the bands was the same under IEF and NEPHGE. However, the bands for S_3 and S_4, though clearly separate under IEF, more or less co-migrated under NEPHGE. The A band co-migrated with the band for S_{13} under IEF, but was clearly distinct with NEPHGE. The C band, seen only in 'Brusseler Brauner', co-migrated with the S_1 band under IEF but with the S_6 band under NEPHGE. The most cathodal band in Fig. 1b seen in all cultivars represents a ribonuclease not involved in incompatibility. The phenotypes of these 15 cultivars are given in Table 1. This also lists 21 more accessions which showed a set of the same phenotypes. Most cultivars (24) showed three bands; five showed two and seven showed four. The phenotype 6, 13, B was the most frequent with nine accessions, including four forms of 'Morello'. Other relatively common phenotypes, with four accessions each, were: 13, B; 6, B, D; 13, B, D; and 1, 4, B, D.

Figure 2a and b shows, after IEF and NEPHGE, the zymograms of seven sour cherry cultivars that appeared to possess at least two sweet cherry alleles in combinations already found in sweet cherry together with four sweet cherry cultivars that were used for various test pollinations. 'Čačanski Rubin', with the phenotype 1, 4, 13, B, and 'Erdi Botermo B', 'Koroš' and 'Ujfehertoi Furtos', all 1, 4, B, D, clearly have the two bands seen in 'Merton Late', which is known to be S_1 S_4. 'Marasca Piemonte', with the phenotype 6, 13, B, and 'Marasca Savena', 6, 13, B, D, clearly have the two bands seen in 'Noble', which is known to be S_6 S_{13} (Bošković and Tobutt 2001). Again,

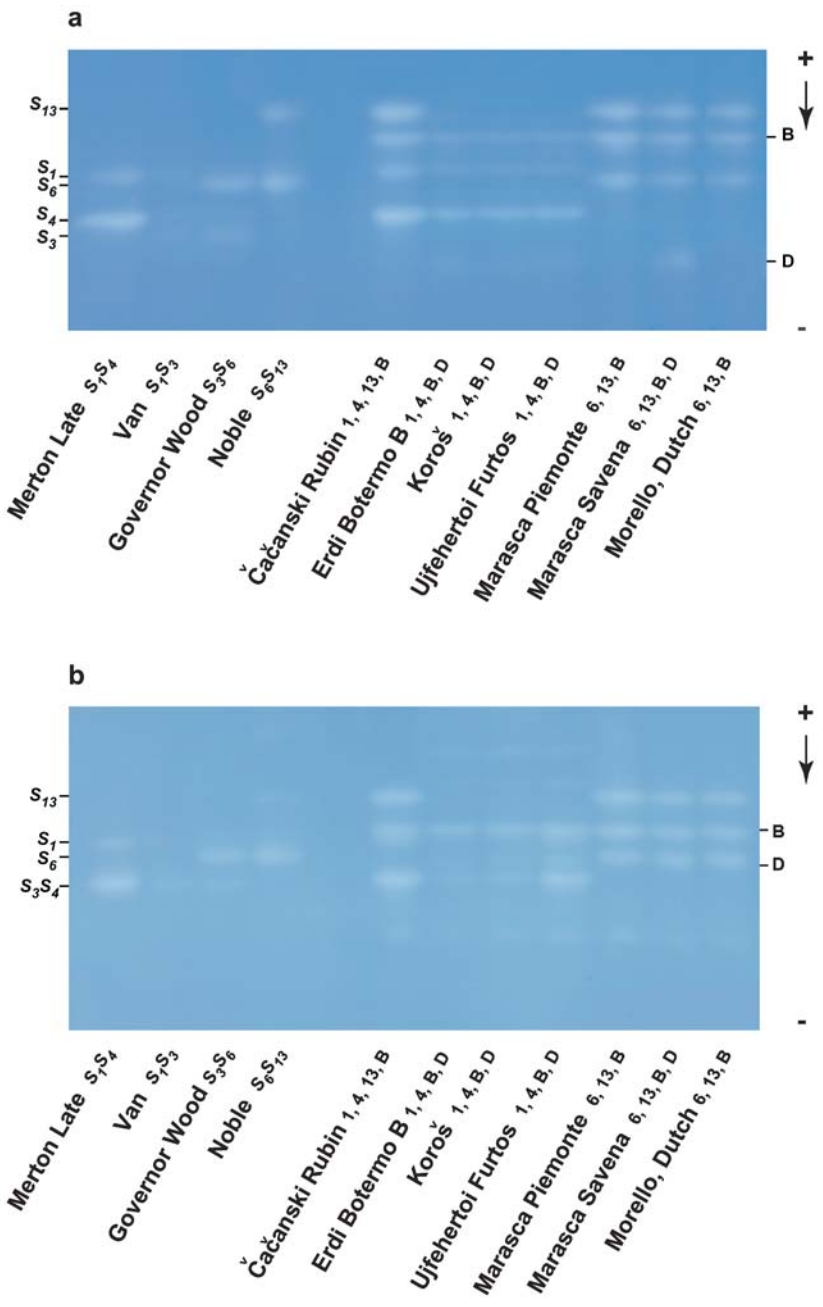

Fig. 2 Stylar ribonucleases of seven sour cherries, *right*, and of four sweet cherries used in test pollinations, *left*, run under IEF (**a**) and NEPHGE (**b**)

the S_3 and S_4 bands, though distinct under IEF conditions, co-migrate under NEPHGE conditions, and the relative position of band C changes markedly from IEF to NEPHGE.

Figure 3 shows stylar ribonuclease zymograms of eight accessions of ground cherry run under NEPHGE conditions alongside four sour cherry cultivars with the range of stylar ribonuclease alleles seen in sour cherry. All showed the B band, which is frequently seen in sour cherry. Four accessions also showed a 'new' band, anodal to S_{13}, that we designate as 'E'; three showed a different 'new' band, slightly cathodal to B, that we designate as 'F'; and one accession showed only the B band.

Test pollinations of sour × sweet cherry

Table 2 reports the pollen tube growth as monitored by fluorescence microscopy after the sour × sweet pollinations that were made to check the functional identity of certain sweet and sour cherry ribonucleases. 'Čačanski Rubin', 'Erdi Botermo B', 'Koroš' and 'Ujfehertoi Furtos' clearly rejected 'Merton Late' (S_1 S_4) pollen, with no tubes reaching the ovary. However with 'Van' (S_1 S_3) and 'Governor Wood' (S_3 S_6), the pollen tubes penetrated to the ovary in at least 50% of the styles; in the case of pollination by 'Van', which has one band in common with the sour cherries, a few incompatible pollen

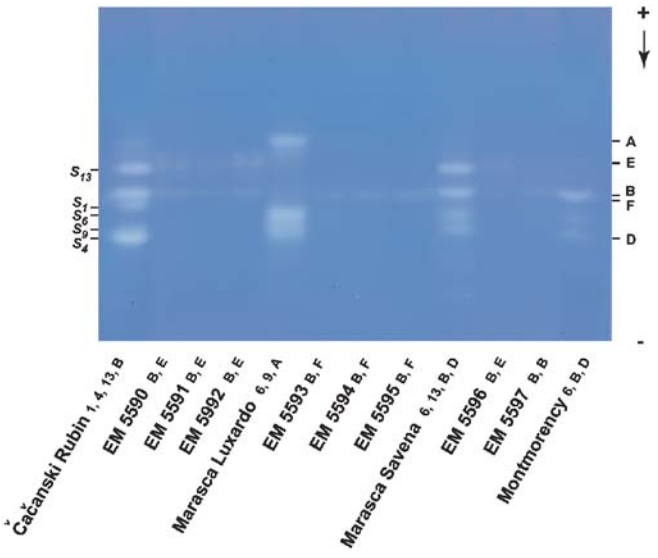

Fig. 3 Stylar ribonucleases of eight ground cherries and of four sour cherries, used as the standards, run under NEPHGE

tubes were observed. This indicates that the bands apparently the same as S_1 and S_4 in these four sour cherry cultivars do indeed represent these alleles. Figure 4a and b shows the incompatible reaction in 'Čačanski Rubin' × 'Merton Late' and Fig. 4c, the compatible reaction in 'Čačanski Rubin' × 'Governor Wood'. Likewise, 'Marasca Piemonte', 'Marasca Savena' and 'Morello, Dutch' were clearly incompatible with 'Noble' (S_6 S_{13}), but not with 'Merton Late' (S_1 S_4). Thus, the bands apparently the same as S_6 and S_{13} in these three cultivars represent these alleles. Figure 5a shows the incompatible reaction in 'Marasca Piemonte' × 'Noble' and Fig. 5b and c, the compatible reaction in 'Marasca Savena' × 'Merton Late'.

Confirmation was provided by the crosses made to determine fruit set (Table 3). 'Erdi Botermo B' failed to set fruit when pollinated with 'Merton Late'. 'Marasca Piemonte' failed to set fruit when pollinated with 'Noble'. Both sour cherry cultivars set fruit, from 5% to 38%, after pollination by sweet cherry cultivars with one or no stylar ribonuclease bands in common.

Progeny analysis using *S* allele-specific PCR

PCR amplification with specific primers of the sour cherry parents of the progenies analysed to clarify the genomic arrangement of the alleles, 'Erdi Botermo B' (1, 4, B, D) and 'Marasca Luxardo' (6, 9, A), was in accord with the phenotypes deduced from the NEPHGE gels.

Table 2 Test pollinations of sour × sweet cherries for determining compatibility by examination of pollen tube growth

Sour cherry cultivar	Sweet cherry cultivar	Mean number of pollen tubes near stigma	Pistils with pollen tubes reaching ovary (%)
Čačanski Rubin (1, 4, 13, B)	× Merton Late (S_1 S_4)	40.2	0
	× Van (S_1 S_3)	77.7	100
	× Governor Wood (S_3 S_6)	36.3	66.7
Erdi Botermo B (1, 4, B, D)	× Merton Late (S_1 S_4)	30.0	0
	× Van (S_1 S_3)	18.2	100
	× Governor Wood (S_3 S_6)	26.0	100
Koroš (1, 4, B, D)	× Merton Late (S_1 S_4)	26.6	0
	× Van (S_1 S_3)	17.6	57.1
	× Governor Wood (S_3 S_6)	24.7	100
Ujfehertoi Furtos (1, 4, B, D)	× Merton Late (S_1 S_4)	30.0	0
	× Van (S_1 S_3)	15.5	100
	× Governor Wood (S_3 S_6)	12.0	100
Marasca Piemonte (6, 13, B)	× Merton Late (S_1 S_4)	15.2	50
	× Noble (S_6 S_{13})	36.5	0
Marasca Savena (6, 13, B, D)	× Merton Late (S_1 S_4)	67.4	100
	× Noble (S_6 S_{13})	20.9	0
Morello, Dutch (6, 13, B)	× Merton Late (S_1 S_4)	17.3	100
	× Noble (S_6 S_{13})	14.9	0

Table 3 Sour cherries test pollinated with sweet cherries for determining compatibility by noting fruit set

Sour cherry cultivar	Sweet cherry cultivar	Number of flowers pollinated	Number of fruit sets
Erdi Botermo B (1, 4, B, D)	× Merton Late (S_1 S_4)	127	0
	× Van (S_1 S_3)	79	21
	× Governor Wood (S_3 S_6)	52	11
Marasca Piemonte (6, 13, B)	× Noble (S_6 S_{13})	475	0
	× Merton Late (S_1 S_4)	264	14
	× Governor Wood (S_3 S_6)	120	8

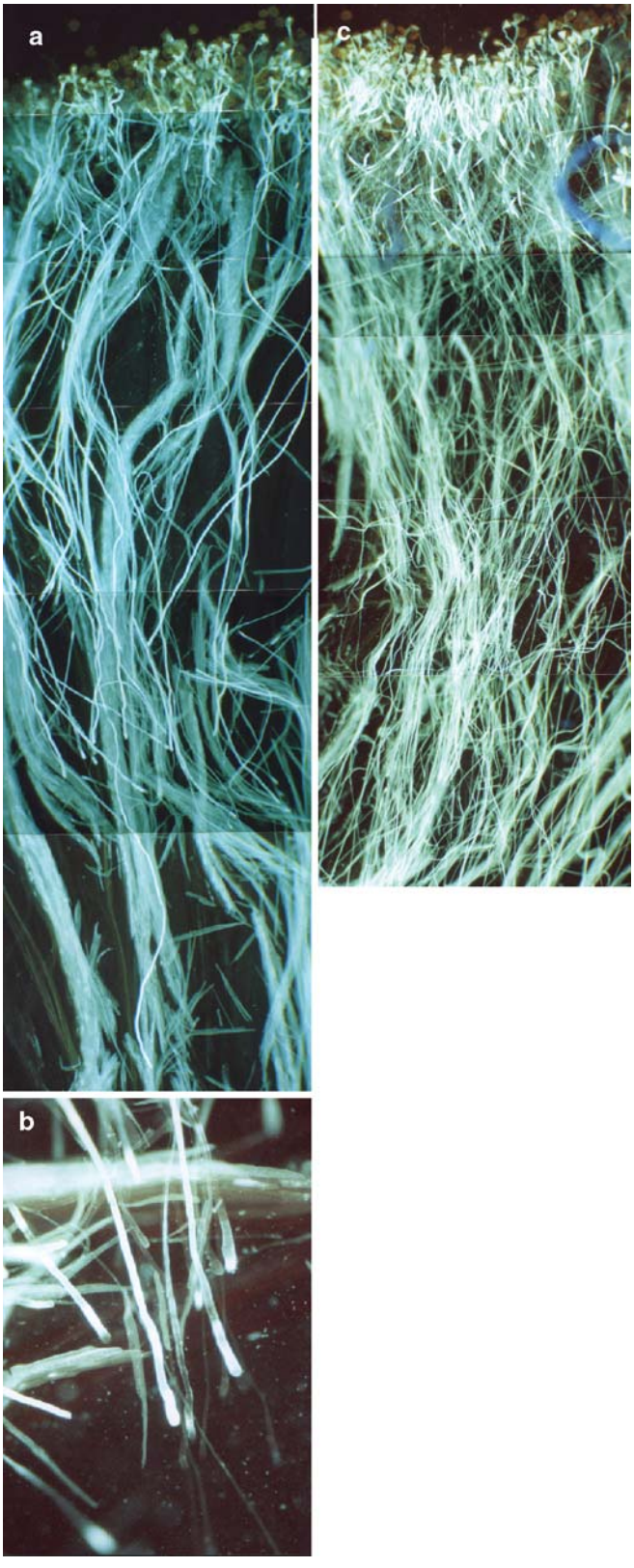

Fig. 4 Pollen tube growth 72 h after crossing 'Čačanski Rubin' (1, 4, 13, B) × 'Merton Late' (S_1 S_4) **(a, b)** and × 'Governor Wood' (S_3 S_6) **(c)**. Typical incompatible reaction is seen in **a**, tubes stopping in the upper third of the style, and **b** shows a close up of the swollen tips. Compatible growth is seen in **c**

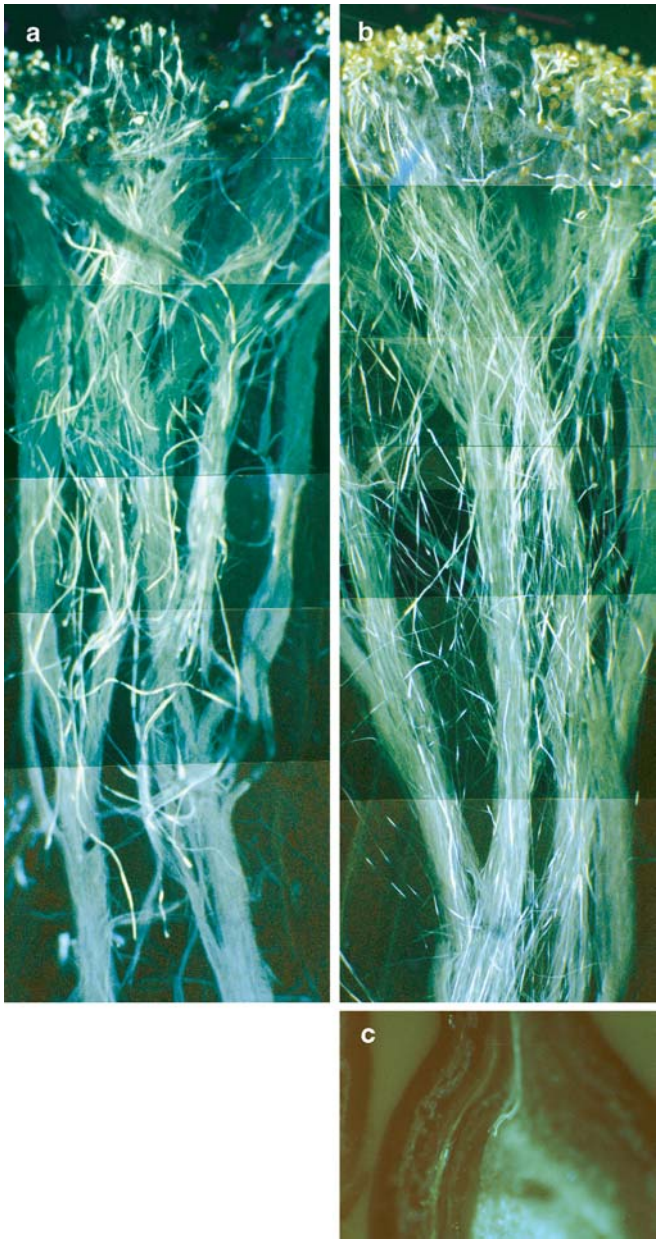

Fig. 5 Pollen tube growth 72 h after crossing 'Marasca Savena' (6, 13, B, D) × 'Noble' (S_6 S_{13}) **(a)**, and × 'Merton Late' (S_1 S_4) **(b, c)**. Typical incompatible reaction is seen in **a**, tubes stopping in the upper third of the style. Compatible growth is seen in **b**, and **c** shows penetration of a tube to the ovary locule

In the progeny from the cross 'Erdi Botermo B' (1, 4, D) × 'Van' (S_1 S_3), two phenotypes were observed, 1, 3 and 4, 3, with three seedlings of each. This indicated that only the S_3 pollen from 'Van' succeeded, the S_1 pollen being rejected by the 1, 4, B, D style and that alleles 1 and 4 in 'Erdi Botermo B' were allelic, as they were not inherited together.

In the progeny from the presumably fully compatible cross 'Erdi Botermo B' (1, 4, B, D) × 'Governor Wood' (S_3 S_6), three phenotypes were observed: five seedlings were 3, 4; one was 4, 6; and one was 1, 3, 4. Thus, both S_3

Table 4 Test pollinations of sweet × sour cherry to understand pairing and interaction of *S* alleles in diploid pollen

Sweet cherry cultivar	Sour cherry cultivar	Number of flowers pollinated	Number of fruit sets
Merton Late (S_1 S_4)	× Amarena de Verona P.C. (6, B, D)	95	60
Merton Late (S_1 S_4)	× Montmorency (6, B, D)	110	57
Sasha (S_3 S_6)	× Amarena de Verona P.C. (6, B, D)	145	0
Sasha (S_3 S_6)	× Montmorency (6, B, D)	269	22

and S_6 pollen from 'Governor Wood' could succeed. Bands 1 and 4 in 'Erdi Botermo B' were generally allelic, though the seedling revealing both these bands indicates occasional tetrasomic inheritance. The absence of seedlings inheriting just allele 1 from 'Erdi Botermo B' is not unexpected in such a small progeny.

The progeny from another fully compatible cross, 'Marasca Luxardo' (6, 9, A) × 'Victor' (S_2 S_3), segregated into four phenotypes 2, 6; 2, 9; 3, 6 and 3, 9 with three, two, two and one seedling(s), respectively. Thus, both S_2 and S_3 pollen from 'Victor' succeeded and bands 6 and 9 in 'Marasca Luxardo' were allelic, as they were not inherited together.

Test pollinations of sweet × sour cherry

Table 4 shows the different effects of pollen of two sour cherries, 'Amarena di Verona P.C.' and 'Montmorency', which have the same stylar ribonuclease phenotype (6, B, D), but are self-incompatible and self-compatible, respectively, when used to pollinate the sweet cherries 'Merton Late' (S_1 S_4) and 'Sasha' (S_3 S_6). On 'Merton Late', both crosses were successful, giving over 50% fruit set and demonstrating the fertility of the pollen. In contrast, on 'Sasha', the pollination with self-incompatible 'Amarena di Verona P.C.' failed, while that with self-compatible 'Montmorency' succeeded, giving 8% fruit set. This suggests that all the 'Amarena di Verona P.C.' pollen grains express S_6, perhaps because this cultivar has two allelic copies of S_6, whereas not all the 'Montmorency' pollen grains do so. A fuller explanation is offered in the Discussion.

Discussion

Ribonuclease phenotypes and genotypes

The ribonuclease analyses showed that the cultivars of tetraploid sour cherry cultivars each had two, three or four stylar ribonucleases in the range pI 8.45–9.70, and that six of the ten variants co-migrated with bands that in sweet cherry are attributed to *S* alleles.

The test pollinations with cultivars of diploid sweet cherry, an approach inspired partly by the work of Lewis (1943, 1947) with *O. organensis*, and the monitoring of pollen tube growth or fruit set established that the four sour cherry variants 1, 4, 6 and 13 do indeed represent *S* alleles functionally identical with sweet cherry S_1, S_4, S_6 and S_{13}, respectively. This bears out the conclusion of Lansari and Iezzoni (1990) that self-compatible cultivars of sour cherry such as 'Morello' and 'Erdi Botermo' carry self-incompatibility alleles. The availability of sweet cherry cultivars of appropriate genotype was crucial to the success of this approach. The recent report of sweet cherries of genotype S_3 S_{13} (group XIX) and S_6 S_9 (group X) (Bošković and Tobutt 2001) provides cultivars that could be used to test for S_3 in sour cherries of phenotype 3, 13, C and for S_9 in phenotypes 6, 9, 13 and 6, 9, A.

The bands A, B, C and D represent stylar ribonucleases. It is unclear if they are *S* ribonucleases, as their function in incompatibility has not been demonstrated. (Indeed, the occurrence of a ground cherry with only the B band creates some doubt that it functions in the incompatibility reaction.) For the rest of this discussion, we will assign them provisionally to the alleles S_A, S_B, S_C and S_D. Without evidence that they occur in sweet cherry, we would not be justified in labelling them in continuation of the S_1 to S_{16} series.

As mentioned earlier, sour cherry is considered to be an allotetraploid derived from diploid sweet cherry and tetraploid ground cherry. It might be that the alleles S_1, S_3, S_4, S_6, S_9 and S_{13} are derived from the former, and S_A, S_B, S_C and S_D from the latter. A more extensive survey, especially of ground cherry, would be needed to confirm this. Of the sour cherry alleles, so far we have detected only S_B in ground cherry. It will be interesting if certain alleles are found both in sweet cherry and ground cherry.

Of the sour cherry cultivars analysed for stylar ribonucleases in this work, four were among those recently analysed by Yamane et al. (2001) by PCR and RFLP: 'Crisana', 'Montmorency', 'Rheinische Schattenmorelle' (if same as 'Schattenmorelle') and 'Ujfehertoi Furtoš'. For these, the sweet cherry alleles we found correspond to PCR bands of the expected size, though the other alleles and bands cannot be reconciled consistently. The RFLP data of Yamane et al. (2001) are neither readily reconcilable with our IEF data, nor indeed with their own PCR data, perhaps because of polymorphism outside the *S*-RNase coding region.

In 29 of the 36 cultivars, we have detected only two or three bands. In general, we do not know if a phenotype such as 6, B, D, for example, corresponds to the genotypes S_6 S_6 S_B S_D, S_6 S_B S_B S_D or S_6 S_B S_D S_D, or perhaps to S_6 S_B S_D S_N, where S_N represents a null allele that maybe does not function in incompatibility. If we expect sour cherries to have two alleles from sweet

cherry, then maybe the first option is most likely, or perhaps the last if S_N comes from sweet cherry. In the absence of other information the genotype can be written as S_6 S_B S_D–, where '–' represents a duplicate or null allele.

An insight into the presence of duplicate alleles or null alleles could be gained by crossing cultivars that have two-banded or three-banded phenotypes with sour or sweet cherry cultivars that have no bands in common and determining the phenotypes of the seedlings. The phenotypes 6, 13, B and 6, 13, D have at least one allele in common with all the other sour cherry phenotypes, and so in these cases the appropriate crosses would have to be with sweet cherry cultivars.

'Čačanski Rubin' (S_1 S_4 S_{13} S_B) and, maybe, 'Heimanns Konserven' (S_6 S_9 S_{13}–) show three bands we provisionally derive from sweet cherry. Occasional tetrasomic inheritance, demonstrated in this work, or backcrossing to diploid sweet cherry via unreduced gametes (cf. Duke cherries, which are considered to be hybrids between sour and sweet) might explain the occurrence of such genotypes. Alternatively, it may be that some alleles are common to sweet cherry and ground cherry, but we have no evidence of this.

Leaving aside the possibilities that cultivars with two-banded or three-banded phenotypes might have two copies of some alleles, or might have null alleles, the occurrence of the commonest alleles, out of the total number of 110, is as follows: S_B 31, S_{13} 25, S_6 20 and S_D 15. It is noteworthy that S_{13}, which is very frequent in these sour cherry cultivars, is rare in sweet cherry cultivars (Bošković and Tobutt 2001) and that S_3, which is very frequent in sweet cherry cultivars (Williams and Brown 1956, Bošković and Tobutt 2001), is rare in sour cherry cultivars. This may indicate that the 'pool' of sweet cherries that contributed to the pedigree of sour cherries was different from that giving rise to modern cultivars of sweet cherry. Alternatively, it may indicate subsequent drift in one or both species. Or it may indicate differential selection for S_{13} versus S_3 in tetraploids versus diploids, perhaps associated with the interactions in heteroallelic pollen briefly discussed later. The most frequent of the non-sweet cherry alleles was S_B which was found in all the ground cherries surveyed.

Genomic arrangements of ribonuclease alleles and heteroallelic pollen

The inheritance of ribonuclease alleles in the three small progenies raised from crosses of sour by sweet cherries showed, with the exception of one seedling, disomic inheritance in two sour cherry cultivars, in which the alleles of presumed sweet cherry origin belonged to the same locus and duly segregated. A convenient way to present the genotypes of the two cultivars is S_1 S_4. S_B S_D for 'Erdi Botermo B', where S_1 and S_4 belong to one locus and S_B and S_D to the other, and S_6 S_9. S_A– for 'Marasca Luxardo'. To determine the genomic arrangements of ribonuclease alleles in other cultivars, appropriate progenies could be analysed. Certain progenies, such as those from crosses between sour cherries having ribonuclease alleles in common, would be best avoided.

The classic work of Lewis (1943, 1947, 1954) on incompatibility in tetraploids focused on autotetraploids of *O. organensis* and so paid no attention to allelic arrangements. Previously, Lawrence (1930) touched on incompatibility in allotetraploids and indicated genotype and arrangement as, for example, S_1 S_2 Z_2 Z_3, where S and Z represent the two homoeologous loci.

A priori, allotetraploid phenotypes with four bands can have three different genomic arrangements if e.g. S_1 S_2. S_3 S_4 is not distinguished from S_3 S_4. S_1 S_2 (Table 5). For phenotypes with three, two and one band(s), there are nine, ten and five arrangements possible, respectively, if the possibility of null alleles is introduced.

As mentioned earlier, Lewis (1943, 1947, 1954) discussed evidence that the alleles in heteroallelic diploid pollen in tetraploids do not necessarily act independently. The *S* alleles may interact competitively so that the pollen grain is not rejected on a style having either or both of the same alleles. If some *S* alleles from sweet cherry and ground cherry interact in this way, this could be one explanation of self-compatibility in various sour cherries, despite the presence of *S* alleles. In some cases, the *S* alleles may show dominance relationships so that the pollen grain expresses one but not the other allele; this would lead to self-incompatibility in certain genotypes as indeed would independent action of the alleles. Although competitive interaction has been taken into account in current models of incompatibility (Thompson and Kirch 1992; Kao and McCubbin 1996; Luu et al. 2000, 2001), whether independent action, reported in *O. organensis* by Lewis (1947), is consistent with them seems not to have been explicitly addressed. The role of the possible null alleles is obscure.

The genotypes of the pollen grains in sour cherries depend not only on the genotype of the cultivar, but also on the genomic arrangement of the alleles. Table 5 shows the possible segregations of *S* alleles in gametes from allotetraploids with one-banded, two-banded, three-banded and four-banded phenotypes. For the one-banded and two-banded phenotypes, certain different genotypes and genomic arrangements give indistinguishable outcomes with respect to pollen phenotypes.

We have already deduced the genotype and arrangement of 'Erdi Botermo B' as S_1 S_4. S_B S_D. Its self-compatibility indicates competitive interaction in at least one of the four types of pollen expected, S_1 S_B, S_1 S_D, S_4 S_B and S_4 S_D. We can now return to the cases of 'Amarena di Verona P.C.', self-incompatible, and 'Montmorency', self-compatible, which share the phenotype 6, B, D, and the test crosses we made following the approach of Lewis (1943) in *O. organensis*. That pollen of 'Amarena di Verona P.C.' failed on sweet cherry 'Sasha' (S_3 S_6) but succeeded on 'Merton Late' (S_1 S_4) indicated that all the pollen behaved as S_6. In contrast, the success of pollen of 'Montmorency' on both sweet cherry cultivars indicated

Table 5 Possible disomic segregations of *S* alleles in gametes of allotetraploid sour cherries

Parent phenotype	Parent genotype and arrangement[a]	Gamete genotypes	Pollen phenotypes
1	$S_1\ S_1.\ S_1\ S_1$	$S_1\ S_1$	1[b]
	$S_1\ S_1.\ S_1\ S_N$	$S_1\ S_1\ S_1\ S_N$	1[b]
	$S_1\ S_1.\ S_N\ S_N$	$S_1\ S_N$	1[b]
	$S_1\ S_N.\ S_1\ S_N$	$S_1\ S_1\ 2\ S_1\ S_N\ S_N\ S_N$	3×1 N
	$S_1\ S_N.\ S_N\ S_N$	$S_1\ S_N\ S_N\ S_N$	1 N
1,2	$S_1\ S_1.\ S_1\ S_2$	$S_1\ S_1\ S_1\ S_2$	1 1,2[c]
	$S_1\ S_1.\ S_2\ S_2$	$S_1\ S_2$	1,2
	$S_1\ S_2.\ S_1\ S_2$	$S_1\ S_1\ 2\ S_1\ S_2\ S_2\ S_2$	1 2×1,2 2
	$S_1\ S_2.\ S_2\ S_2$	$S_1\ S_2\ S_2\ S_2$	1,2 2[d]
	$S_1\ S_1.\ S_2\ S_N$	$S_1\ S_2\ S_1\ S_N$	1 1,2[c]
	$S_1\ S_N.\ S_1\ S_2$	$S_1\ S_1\ S_1\ S_2\ S_1\ S_N\ S_2\ S_N$	2×1 1,2 2
	$S_1\ S_2.\ S_2\ S_N$	$S_1\ S_2\ S_1\ S_N\ S_2\ S_2\ S_2\ S_N$	1 1,2 2×2
	$S_1\ S_N.\ S_2\ S_2$	$S_1\ S_2\ S_2\ S_N$	1,2 2[d]
	$S_1\ S_2.\ S_N\ S_N$	$S_1\ S_N\ S_2\ S_N$	1 2
	$S_1\ S_N.\ S_2\ S_N$	$S_1\ S_2\ S_1\ S_N\ S_2\ S_N\ S_N\ S_N$	1 1,2 2 N
1,2,3	$S_1\ S_2.\ S_3\ S_3$	$S_1\ S_3\ S_2\ S_3$	1,3 2,3
	$S_1\ S_3.\ S_2\ S_3$	$S_1\ S_2\ S_1\ S_3\ S_2\ S_3\ S_3\ S_3$	1,2 1,3 2,3 3
	$S_1\ S_2.\ S_2\ S_3$	$S_1\ S_2\ S_1\ S_3\ S_2\ S_2\ S_2\ S_3$	1,2 1,3 2 2,3
	$S_1\ S_3.\ S_2\ S_2$	$S_1\ S_2\ S_2\ S_3$	1,2 2,3
	$S_1\ S_1.\ S_2\ S_3$	$S_1\ S_2\ S_1\ S_3$	1,2 1,3
	$S_1\ S_2.\ S_1\ S_3$	$S_1\ S_1\ S_1\ S_2\ S_1\ S_3\ S_2\ S_3$	1 1,2 1,3 2,3
	$S_1\ S_2.\ S_3\ S_N$	$S_1\ S_3\ S_1\ S_N\ S_2\ S_3\ S_2\ S_N$	1 1,3 2 2,3
	$S_1\ S_3.\ S_2\ S_N$	$S_1\ S_2\ S_1\ S_N\ S_2\ S_3\ S_3\ S_N$	1 1,2 2,3 3
	$S_1\ S_N.\ S_2\ S_3$	$S_1\ S_2\ S_1\ S_3\ S_2\ S_N\ S_3\ S_N$	1,2 1,3 2 3
1,2,3,4	$S_1\ S_2.\ S_3\ S_4$	$S_1\ S_3\ S_1\ S_4\ S_2\ S_3\ S_2\ S_4$	1,3 1,4 2,3 2,4
	$S_1\ S_3.\ S_2\ S_4$	$S_1\ S_2\ S_1\ S_4\ S_2\ S_3\ S_3\ S_4$	1,2 1,4 2,3 3,4
	$S_1\ S_4.\ S_2\ S_3$	$S_1\ S_2\ S_1\ S_3\ S_2\ S_4\ S_3\ S_4$	1,2 1,3 2,4 3,4

[a] Distinguishing the two homoeologous loci but not specifying which is which
[b, c, d] Outcomes indistinguishable phenotypically

that some of the pollen did not behave as S_6. This is consistent with 'Amarena di Verona P.C.' having the genotype and arrangement $S_6\ S_6.\ S_B\ S_D$; the pollen produced would be $S_6\ S_B$ and $S_6\ S_D$ and, in the absence of competitive interaction, both types would express S_6. 'Montmorency' may have one of the eight other genotypes and arrangements possible, none of which gives rise to S_6 in all pollen grains. This also explains why 'Amarena di Verona P.C.' is self-incompatible, whereas 'Montmorency' is self-compatible.

This approach could contribute to an understanding of the allelic interactions in heteroallelic pollen of other sour cherries. Whether knowledge of the genomic arrangements and the interactions of the various combinations of alleles will be sufficient to explain self-compatibility versus self-incompatibility in all cases remains to be seen.

Conclusion

The ribonuclease approach is useful for detecting alleles coding for functional ribonucleases in styles. PCR and RFLP analyses, which can be applied to young seedlings, may detect possible null alleles lacking RNase activity if these result from minor mutations of the *S*-RNase gene. Whether the PCR primers currently available can detect all expressed alleles is not yet clear.

For a fuller understanding of the genetics of incompatibility relationships in sour cherry, more work would be needed on the following topics: the genotypes, rather than phenotypes, of the cultivars; the function of the ground cherry alleles; the existence or otherwise of null alleles lacking stylar or pollen activity and their functional significance; the arrangement of alleles in the tetraploid genome, which determines the genotypes of the diploid pollen grains; and the interactions in heteroallelic pollen, which determine whether or not the pollen is rejected on a style having one or both of the same alleles. Our work with stylar ribonuclease analysis, test pollinations by and on diploid sweet cherries and progeny analysis by *S* allele-specific PCR, has already provided some insight and a framework for further research.

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