Allele-specific PCR detection of sweet cherry self-incompatibility alleles S_3, S_4 and S_9 using consensus and allele-specific primers in the Czech Republic

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Abstract

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Prunus avium species of the Rosaceae family exhibit gametophytic self-incompatibility. Determination of the self-incompatibility genotype of individuals is essential for genetic studies and the development of informed management strategies. The PCR-based detection of *S*-allele helps to promote and speed up traditional breeding activity and hence molecular analysis of the perspective genotypes has become more intensive in all cherry growing countries. The alleles S_3, S_4 and S_9 from 34 accessions of Czech collections were determined using the polymerase chain reaction (PCR) method. Initially, DNA extracts were amplified with consensus primers that amplify across the first, second, or both introns of the *S-ribonuclease* gene which shows a considerable length polymorphism. The new allele specific primers were designed with the goal to overcome some occurring difficulties in the detection of expected alleles by previously published allele specific primers. *S*-alleles fragments of standard cultivars used in this study were PCR amplified, sequenced to validate the designed primers. The study demonstrates the advantage of newly designed primers application in testing of sweet cherry genotypes.

Keywords: *Prunus avium*; *S*-alleles; allele-specific primers; consensus primers

Sweet cherries *Prunus avium* (L.) are usually diploid (2*n* = 2× = 16) trees (Webster 1996; Iezzoni 2008) which are characterized by mighty growth and a pyramidal tree top. Gametophytic self-incompatibility (GSI) in cherry is controlled by the multi-allelic *S*-locus, which prevents self-fertilization (de Nettancourt 2001; Franklin-Tong, Franklin 2003). The *S*-genotype is agronomically important because cultivars with the same *S*-genotype are cross incompatible.

The stylar component of GSI was determined to be a *ribonuclease* (*S-RNase*) gene in the Rosaceae (Bošković, Tobutt 1996; Sassa et al. 1996). In the past, several *P. avium S*-alleles were identified as a result of cross pollination experiments and progeny testing in sweet cherry (Crane, Brown 1937) and later correlated with stylar ribonucleases (Bošković, Tobutt 1996). These *S-RNases* were also characterized at the molecular level (Tao et al. 1999; Sonneveld et al. 2001, 2003; Wiersma

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et al. 2001; Wunsch, Hormaza 2004; De Cuyper et al. 2005).

Several common structural features (C1, C2, C3, C4 and C5) and a region of high variability (RHV) were characterized for rosaceous *S-RNases* which exhibit evidence of positive selection (Ishimizu et al. 1998; Ushijima et al. 1998). These (hypervariable) RHV regions are important for *S*-allele recognition and for initiating the self-incompatibility response. In cherry the allelic variants of *S-RNase* contain two introns and both of them unveiled considerable length polymorphism (Tao et al. 1999; Sonneveld et al. 2003).

To identify the *S-RNase* alleles in sweet and wild cherry, polymerase chain reaction techniques are employed along with primers designed within conserved regions flanking each of the *S-RNase* introns (Tao et al. 1999; Wiersma et al. 2001; Sonneveld et al. 2003; Kato, Mukai 2004; De Cuyper et al. 2005; Schueler et al. 2006). The application of fluorescently labelled primers for the intron associated with the 5' untranslated region (UTR) of the *SFB* gene and for the first intron of the *S-RNase* gene was also demonstrated for *S*-haplotype identification in *P. avium* (Sonneveld et al. 2006; Vaughan et al. 2006).

Sonneveld et al. (2001, 2003) developed allele specific primers from sequenced cDNAs which revealed the considerable polymorphism and lies in the RHV region of the *S-RNase*. These primers detect the self-compatibility allele and seemed to be useful for genotyping of cultivars and seedlings.

The primer pairs for S_3, S_4 and S_9 alleles often gave weak amplification or did not amplify frequently in cultivars grown in the Czech Republic in congruence to the analysis of intron 1 and 2. The length variation of the introns and the conservation of various regions of S_3, S_4, and S_9 exons (Sonneveld et al. 2001, 2003) prompted us to design primers which would unambiguously amplify specific alleles. These newly designed allele specific primers would be primarily useful for determining the genotypes of still untested cultivars and seedlings. The cDNAs sequences of these genes were used to determine which DNA regions are highly conserved. This is important for designing of new specific primers and used with allele specific primers to investigate the anomalies in several cultivars which were described as positive for these specific alleles. Furthermore, to investigate their general applicability in cherry, the consensus and allele specific primers were tested on a range of cherry varieties collected from the Research and Breeding Institute of Pomology in Holovousy (RBIPH), Czech Republic. In this article, the application background and suitability of newly designed primers for cherry genotyping which significantly reduced the problem of false negatives are described.

MATERIAL AND METHODS

Plant material. Leaf samples for DNA isolation were collected from cultivars of *P. avium* (Table 1) in plantations of the Research and Breeding Institute of Pomology in Holovousy, Czech Republic (RBIPH). Six cultivars were used as standard for alleles S_3 (Kordia, Granát), S_4 (Sam, Merton late) and S_9 (Burlat, Sweetearly) and other cultivars were used to check the consistency of the PCR banding patterns and ruling out of both false positives and negatives. Absolute majority of selected cultivars has never been tested for *S* locus allelic constitution by any molecular technique in the Czech Republic. Information of cultivars origin is mentioned in Table 1. Majority of them originate in foreign countries and are used as genetic pool for Czech cherry breeding. Czech cultivars are representatives of national breeding using selection from hybridization programs.

Consensus primers designing. Full-length cDNA sequences of S_3, S_4 and S_9 (European Molecular Biology Laboratory (EMBL) accession numbers: AJ298312, AJ298313, AB028154 and AJ635271, respectively) (Tao et al. 1999; Sonneveld et al. 2001, 2003) were aligned using the ClustalW method of the European Molecular Biology Laboratory (EMBL) program. Using coding and intron sequence information as appropriate, allele-specific primers for S_3, S_4, and S_9 were designed (Table 2) from three regions i.e. hypervariable region, conserved regions C1 and C3 to C5 that were highly conserved among these alleles.

Allele-specific PCR amplification and sequencing. Genomic DNA from leaves of cherry cultivars was extracted using miniprep version of the acetyl trimethylammonium bromide (CTAB) extraction method (Saghai-Maroof et al. 1984) with slight modification. Modifications included the changes in incubation time at 65°C (increased from 1 to 2 h), spinning at 5,000 rpm, precipitation of DNA with equal volume of isopropanol, removal of polyphenolic compounds with equal volume of chloroform isoamylalcohol (24:1) instead of chloroform: octanol and final precipitation of DNA pellet using centrifugation at 5,000 rpm. PCR amplification of cultivars was performed using designed allele-specific primers for S_3, S_4, and S_9. For PCR ampli-

Table 1. Presence (+) or absence of *S-RNase* allele using genomic DNA PCR amplification in RBIPH cherry cultivars with consensus primers intron 1st, 2nd and designed S_3, S_4, S_9 allele specific primers

Cultivar	Origin	Allele based on intron 1			Allele based on intron 2			Allele specific PCR			Confirmed alleles
		S_3	S_4	S_9	S_3	S_4	S_9	S_3	S_4	S_9	
Adriana	HU										
Amazonka	UA										
Amid	CZ	+	+		+	+		+	+		S_3S_4
Black star	FR	+						+			S_3
Burlat*	FR			+			+			+	S_9
Carmen	HU		+			+			+		S_4
Ferprime	FR				+					+	S_9
Fertard	FR	+			+			+			S_3
Granát*	CZ	+			+			+			S_3
Greema	?										
Halka	CZ		+			+			+		S_4
Hedelfingenská	DE	+			+			+			S_3
Helga	CZ	+			+			+			S_3
Hertford	GB										
Hong Deng	CN	+	+					+		+	S_3S_9
Irena	CZ		+			+			+		S_4
Jacinta	CZ	+	+		+	+		+	+		S_3S_4
Kordia*	CZ	+			+			+			S_3
Krupnoplodnaja	UA										
Lambert	US	+	+		+	+		+	+		S_3S_4
Lapins	US		+			+			+		S_4
Merton late*	GB		+			+			+		S_4
Navon	DE		+	+		+	+		+	+	S_4S_9
New York 200	US										
Nočka	?				+						
Regina	DE							+			S_3
Rivan	SE										
Sam*	CA		+			+			+		S_4
Sandra Rose	US		+		+	+		+	+		S_3S_4
Satin Sumele	BE										
SIMCOE Probla	US	+			+			+			S_3
Staccato	US	+	+		+	+		+	+		S_3S_4
Sumgita Canada Giant	CA										
Sweetearly*	IT			+			+			+	S_9
Symphony	US		+			+			+		S_4
Van	US	+			+			+			S_3
Vanda	CZ						+				
Vilma	CZ										

*cultivars used as standard; RBIPH – Research and Breeding Institute of Pomology in Holovousy

Table 2. Nucleotide sequences of allele-specific primers for S_3, S_4, and S_9, cDNA position, optimal annealing temperature for PCR (Tm) and estimated size of genomic amplification product

Primer	Sequence 5'→3'	cDNA position	Tm (°C)	Amplicon size (bp)	Gene ID
PaS3F2	CCG AAC GTT GTA AGT AGC A	356	58–59.4*	325	AJ298312
PaS3R2	TTT GCA TCC TGC TGT TCG A	718			
PaS4F1	CAA TTG CGT TCC GAT CTG	75	61–57.4*	390	AJ298313
PaS4R4	CGTTTATTGAAACGAGATGACTTTC	458			
PaS9F1	TCCTATAATCAGCATTGCGT	59	58–59.4*	300	AJ635271
PaS9R1	AAGGTCTCTTTCGAGCTCTG	363			

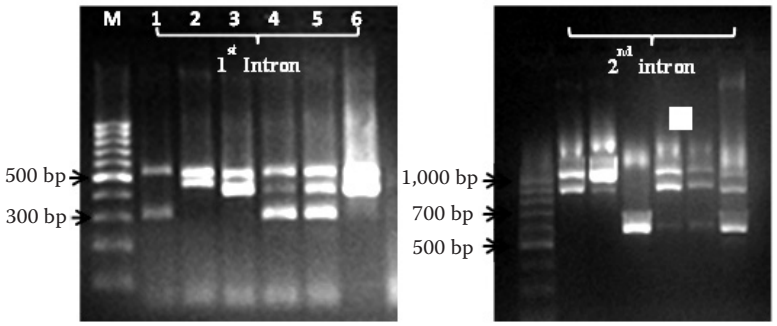
*PCR reaction set up with internal control primers

fication, the reaction volume was 12.5 µl, including KCl PCR buffer without $MgCl_2$, 2.0 mM $MgCl_2$, 0.2 mM of dNTP mixture, 0.1 µM of the PCR primers, 0.625 U of *Taq*DNA polymerase (both Fermentas, Vilnius, Lithuania) and about 30 ng of genomic DNA. The PCR amplification consisted of following steps: initial denaturation at 95°C for 2 min followed by 36 cycles consisting of denaturation at 94°C for 30 s, annealing at 58–61°C depending on primer pair used (Table 2) for 45 s and extension at 72°C for 2 min and followed by a final extension at 72°C for 10 minutes. Amplified products were subjected to 1.2% agarose gel electrophoresis, stained with ethidium bromide (EtBr) and observed and documented using Geldoc XR (BioRad, Hercules, USA) gel imaging system. PCR products were purified using the Qiagen kit and sequenced in both directions using an automated sequencer ABI Prism 310 Genetic Analyzer (Applied Biosystems, Waltham, USA).

In addition, PCR amplification was performed using *S-RNase* intron 1 and intron 2 consensus primers (Sonneveld et al. 2001) to confirm allelic variation in evaluated collection.

RESULTS AND DISCUSSION

The preliminary goal of the work was to evaluate a large number of sweet cherry cultivars grown and used in breeding programs in the Czech Republic with the aim to identify the cultivar to the one of existing pollination groups according to *S*-locus self-incompatibility. Previously published research results gave very good theoretical and methodological base for the research, but alleles S_3, S_4 and S_9 were identified using consensus primer pairs amplifying intron 1 and 2 but it was not possible to confirm them reliably by allele specific amplification. It is not exactly



S-allele	1st intorn PCR product (PaCons I F/R)	2nd intron PCR products (PaCons II F/R)
S_3	303	898 (+ ~825 + ~950)
S_4	523	1,064 (+ ~950 + ~1,200)
S_9	428	798

Fig. 1. PCR amplification of cultivars with consensus primers for the first and second intron of cherry *S-RNases*
samples on the gel M: 1-kb ladder for 1st intron and 1-kb + ladder for 2nd intron; 1. Amid, 2. Halka, 3. Vilma, 4. Sandra Rose, 5. Staccato, 6. Symphony

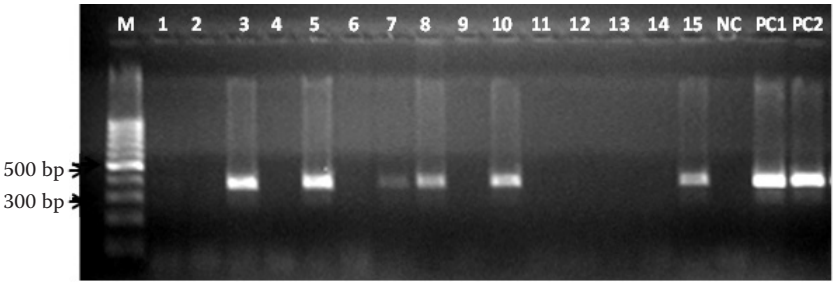


Fig. 2. PCR amplification of genomic DNA with S_3 allele specific primers of cultivars including positive and negative control
1. Vilma, 2. Symphony, 3. Amid, 4. Satin Sumele, 5. Sandra Rose, 6. Greema, 7. Probla, 8. Kordia, 9. Samor, 10. Hong Deng, 11. Amazonka, 12. Nočka, 13. Adriana, 14. Vanda, 15. Black star, NC. negative control, PC1. Kordia, PC2. Granát

known where the origin of these troubles is. All used varieties should have common genetic background and for this high reproducibility of previously used methodology was presumed. When potential problem with DNA (consensus markers for both introns amplified well) was excluded new primers specific for S_3, S_4 and S_9 alleles had to be designed and used instead of the Sonneveld's primer sets (Sonneveld et al. 2001, 2003). Conserved sequences of the *S-RNases*, specifically exon sequences, are not always variable and so they are suitable for designing of new primers (Sonneveld et al. 2003). The *S*-allele specific primers described by Sonneveld et al. (2001, 2003) for S_3, S_4 and S_9 amplified fragments of length 960, 820 and 495 bp, respectively, but amplicons were often very weak and/or unreproducible.

The primer sets PaConsI-F-PaConsI-R and PaConsII-F-PaConsII-R (Sonneveld et al. 2003) were used to amplify the first and second intron in order to identify S_3, S_4 and S_9 alleles in collection of cultivars (Fig. 1). The newly designed primers for S_3, S_4 and S_9 alleles yielded PCR product of 325 bp (Figs 2 and 3), 390 bp (Fig. 4) and 300 bp (Fig. 5), respectively. The length of S_3 marker amplified with the primer pair PaS3F2-PaS3R2 had similarity to the length of partial hypervariable region to conserved regions C5 of genomic sequence of *S-RNase* from *P. avium*. The primer pairs PaS4F1 and PaS4R4 amplified part of gene had similarity to partial C3 to C5 conserved region. Primers PaS9F1-PaS9R1 amplified part of intron 1 and C1 conserved region.

Primers were designed to specific polymorphic sites conservative for the subjected allele. Sequencing of amplicons and next matching of obtained sequences confirmed identity to alleles.

These primers enabled us to identify clearly S_3, S_4 and S_9 alleles, because testing showed that all three pairs gave specific results at the optimized conditions. Primer sequences and their positions for each of these *S*-alleles together with the optimal annealing temperature and the expected size of amplicons are presented in Table 2.

Sequences amplified using designed primers for allele S_3 (cv. Kordia), S_4 (cv. Sam) and S_9 (cv. Burlat) were submitted in NCBI under accession numbers KJ590777, KJ590778 and KJ590779, respectively. The sequences were similar to those described by (Sonneveld et al. 2001, 2003). The length of amplification products with consensus primer for analysis of introns 1 and 2 varied from

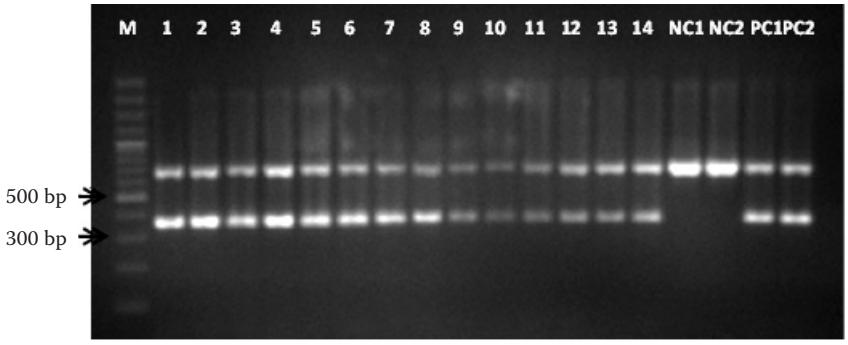


Fig. 3. PCR amplification of genomic DNA with S_3 allele specific primers and ITS internal control of cultivars including positive and negative control
1. Amid, 2. Sandra Rose, 3. Staccato, 4. Kordia, 5. Probla, 6. Hong Deng, 7. Black star, 8. Regina, 9. Jacinta, 10. Lambert, 11. Hedelfingenská, 12. Van, 13. Helga, 14. Fertard, NCI. Hertford, NC2. Rivan, PC1. Kordia, PC2. Granát

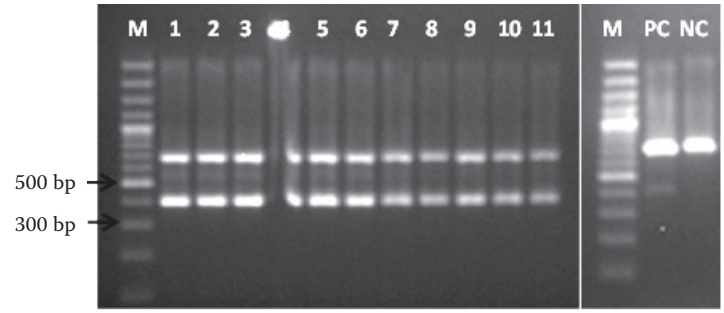


Fig. 4. PCR amplification of genomic DNA with S_4 allele specific primers and ITS internal control of cultivars including positive and negative control

1. Amid, 2. Halka, 3. Sandra Rose, 4. Staccato, 5. Symphony, 6. Jacinta, 7. Navon, 8. Lapins, 9. Fertard, 10. Carmen, 11. Irena, PC. Sam, NC. Rivan

303 to 523 bp (intron 1) and from 577 to 2,383 bp (intron 2). Sonneveld et al. (2003) described allele specific amplicons of alleles S_3, S_4 and S_9 based on intron 1 primers in length of 303, 523 and 428 bp and based on intron 2 primers approximately 898, 1064 and 798 bp. Here evaluated cultivars were at first amplified with both first and second intron primers and validated with recently designed allele specific primers. Results are reported in Table 1. At least, consensus primers and then newly designed allelic specific primers together confirmed the presence or absence of S_3, S_4 and S_9 alleles. The S_3 allele has been identified in 14 genotypes, S_4 allele in 11 and S_9 allele in 3 genotypes (Table 1). Genotyping brought first time information about allelic constitution of *S* locus in cvs Amid, Sandra Rose, Staccato and Jacinta (S_3S_4), Hong Deng (S_3S_9) and Navon (S_4S_9) (Table 1). In remaining genotypes none of the examined alleles was identified since these were occupied with other *S*-alleles. For final allele specific amplification and *S*-locus evaluation of standard cultivars collection having S_3 (cvs Kordia, Granát), S_4 (cvs Sam, Merton late) and S_9 (cvs Burlat, Sweetearly) were used with the evaluated cultivars. To be able to identify the false negative in PCR reactions with allele specific primers, the primers ActF1 (5'-ATGGTGAGGATATTCAACCC-3') and ActR1 (5'-CTTCCTGTGGACAATGGATGG-3') described by Ushijima et al. (2003) for the S_9 allele and ITS-1 and ITS-4 (White et al. 1990) for the alleles S_3 and S_4 were included in the PCR as an internal control of amplification. The *S* allele specific band tended to be weaker when the internal control was included; to optimize it, it is recommended to set higher annealing temperature. Very important finding of this research is that the genotyping of *S*-locus is very sensitive and needs high quality genomic DNA and strictly optimized PCR conditions. Apart from specific primers, these are strong limiting factors for preferential amplification of specific allele. Finally, no problem of detecting false negatives with new primers was observed, however internal controls as used in this study are recommended to prevent this problem. The newly designed allele specific primers will be applied to the larger collection of *P. avium* cultivars and new breeds potentially grown in the Czech Republic as an alternative to primers previously used. The allele specific primers are reliable when used alone or combined with internal control primers (Figs 1–5) under described standardized conditions. However, the possibility to optimize new annealing temperature when the designed primer would be used in different labs cannot be denied, as was similarly admitted by Sonneveld et al. (2001).

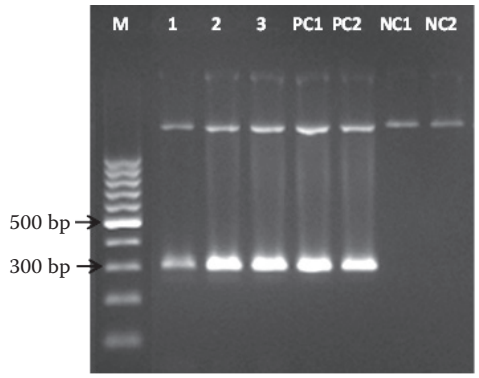


Fig. 5. PCR amplification of genomic DNA with S_9 allele specific primers and actin internal control of cultivars including positive and negative control

1. Hong Deng, 2. Navon, 3. Ferprime, PC1. Burlat, PC2. Sweetearly, NC1. Amid, NC2. Halka.

References

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

Crane M.B., Brown A.G., 1937. Incompatibility and sterility in the sweet cherry, *Prunus avium* L. Journal of Pomology and Horticultural Science, *15*: 86–116.

De Cuyper B., Sonneveld T., Tobutt K.R., 2005. Determining self-incompatibility genotypes in Belgian wild cherries. Molecular Ecology, *14*: 945–955.

de Nettancourt D., 2001. Incompatibility and Incongruity in Wild and Cultivated Plants. Berlin, Springer.

Franklin-Tong N., Franklin F.C.H., 2003. Gametophytic self-incompatibility inhibits pollen tube growth using different mechanisms. Trends in Plant Science, *8*: 598–605.

Hodgkin, T., Roviglioni R., De Vicente M.C., Dudnik N., 2001. Molecular methods in the conservation and use of plant genetic resources. Acta Horticulturae (ISHS), 546: 107–118.

Iezzoni A.F., 2008. Cherries. In: Hancock J.F. (ed.), Temperate Fruit Crop Breeding: Germplasm to Genomics. Berlin, Springer: 151–175.

Ishimizu T., Endo T., Yamaguchi-Kabata Y., Nakamura K.T., Sakiyama F., Norioka S., 1998. Identification of regions in which positive selection may operate in *S-RNase* of Rosaceae. Implication for *S*-allele specific recognition sites in *S-RNase*. FEBS Letters, *440*: 337–342.

Kato S., Mukai Y., 2004. Allelic diversity of *S-RNase* at the self-incompatibility locus in natural flowering cherry populations (*Prunus lannesiana* var. *speciosa*). Heredity, *92*: 249–256.

Saghai-Maroof M.A., Soliman K.M., Jorgensen R.A., Allard R.W., 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proceedings of the National Academy of Sciences of the USA, *81*: 8014–8018.

Sassa H., Nishio T., Kowyama Y., Hirano H., Koba T., Ikehashi H., 1996. Self- incompatibility (*S*) alleles of the Rosaceae encode members of a distinct class of the T-2/*S ribonuclease* superfamily. Molecular & General Genetics, *250*: 547–557.

Schueler S., Tusch A., Scholz F., 2006. Comparative analysis of the within-population genetic structure in wild cherry (*Prunus avium* L.) at the self-incompatibility locus and nuclear microsatellites. Molecular Ecology, *15*: 3231–3243.

Sonneveld T., Robbins T.P., Boskovic R., Tobutt K.R., 2001. Cloning of six cherry self-incompatibility alleles and development of allele specific PCR detection. Theoretical and Applied Genetics, *102*: 1046–1055.

Sonneveld T., Robbins T.P., Tobutt K.R., 2006. Improved discrimination of self-incompatibility *S-RNase* alleles in cherry and high throughput genotyping by automated sizing of first intron PCR products. Plant Breeding, *125*: 305–307.

Sonneveld T., Tobutt K.R., Robbins T.P., 2003. Allele-specific PCR detection of sweet cherry self-incompatibility (*S*) alleles S1 to S16 using consensus and allele-specific primers. Theoretical and Applied Genetics, *107*: 1059–1070.

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. Journal of the American Society for Horticultural Science, *124*: 224–233.

Ushijima K., Sassa H., Tao R., Yamane H., Dandekar A.M., Gradziel T.M., 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. Molecular & General Genetics, *260*: 261–268.

Ushijima K., Sassa H., Dandekar A.M., Gradziel T.M., Tao R., Hirano H., 2003. Structural and transcriptional analysis of the self-incompatibility locus of almond. Plant Cell, *15*: 771–781.

Vaughan S.P., Russell K., Sargent D.J., Tobutt K.R., 2006. Isolation of *S*-locus *F-box* alleles in *Prunus avium* and their application in a novel method to determine self-incompatibility genotype. Theoretical and Applied Genetics, *112*: 856–866.

Webster A.D., 1996. The taxonomic classification of sweet and sour cherries and a brief history of their cultivation. In: Webster A.D., Looney N.E. (eds), Cherries: Crop Physiology, Production and Uses. Wallingford, CAB International: 3–24.

White T.J., Bruns T., Lee S., Taylor J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis et al. (eds), PCR Protocols. London, Academic Press: 315–322.

Wiersma P.A., 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. Theoretical and Applied Genetics, *102*: 700–708.

Wunsch A, Hormaza J.I., 2004. Cloning and characterization of genomic DNA sequences of four self-incompatibility alleles in sweet cherry (*Prunus avium* L.). Theoretical and Applied Genetics, *108*: 299–305.

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