Wild and Rare Self-Incompatibility Allele S_{17} Found in 24 Sweet Cherry (*Prunus avium* L.) Cultivars

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Abstract

The pollination of self-incompatible diploid sweet cherry is determined by the S-locus alleles. We resolved the S-alleles of 50 sweet cherry cultivars grown in Estonia and determined their incompatibility groups, which were previously unknown for most of the tested cultivars. We used consensus primers SI-19/20, SI-31/32, PaConsI, and PaConsII followed by allele-specific primers and sequencing to identify sweet cherry S-genotypes. Surprisingly, 48% (24/50) of the tested cultivars, including 17 Estonian cultivars, carry the rare S-allele S_{17} , which had initially been described in wild sweet cherries in Belgium and Germany. The S_{17} -allele in Estonian cultivars could originate from ‘Leningradskaya tchernaya’ ($S_6|S_{17}$), which has been extensively used in Estonian sweet cherry breeding. Four studied cultivars carrying S_{17} are partly self-compatible, whereas the other 20 cultivars with S_{17} have not been reported to be self-compatible. The recommended pollinator of seven self-incompatible sweet cherries is of the same S-genotype, including four with S_{17} -allele, suggesting heritable reduced effectiveness of self-infertility. We classified the newly genotyped sweet cherry cultivars into 15 known incompatibility groups, and we proposed four new incompatibility groups, 64–67, for S-locus genotypes $S_3|S_{17}$, $S_4|S_{17}$, $S_5|S_{17}$, and $S_6|S_{17}$, respectively, which makes them excellent pollinators all across Europe. Alternatively, the frequency of S_{17} might be underestimated in Eastern European populations and some currently unidentified sweet cherry S-alleles might potentially be S_{17} .

Keywords Sweet cherry · Self-incompatibility · S-locus · S-alleles · S-RNase · SFB

Introduction

Sweet cherry (*Prunus avium* L.) is a fruit crop with growing popularity in Estonian home gardens. A wide range of local and foreign sweet cherry cultivars are grown, including 30 sweet cherry cultivars that have been bred in Estonia during

the last half a century, primarily to improve their midwinter cold hardiness and increase the size and taste of the fruit (Jänes et al. 2010). Sweet cherry yield depends on planting appropriate pollinators nearby. Additionally, the right weather conditions, sufficient amount of pollen and corresponding blooming times are crucial factors for fertilization.

Most sweet cherry cultivars are self-incompatible, which is controlled by a single gametophytically expressed multiallelic S-locus (Crane and Lawrence 1928). In sweet cherry, the S-locus encodes two genes, the pollen-expressed F-box protein (SFB) and stylar ribonuclease (*S-RNase*) (Matsumoto and Tao 2016). Self-incompatibility occurs if both the pollen and the pistil carry the same S-allele (de Nettancourt 1977). Pollen is haploid and expresses SFB of one S-allele. Pistil is diploid and expresses S-RNases from both S-locus alleles. Additionally, S-locus F-box like proteins (SFLs) and S-haplotype-specific F-box proteins (SFBL), which are encoded by genes outside the S-locus, act as general inhibitors by recognising and mediating the polyubiquitination of S-RNases, thus keeping the environment in the pistil detoxified (Matsumoto and Tao 2016, 2019). When the pollen reaches pistil and its SFB-allele does not match

Key Message

- Self-incompatibility allele S_{17} has previously been found in wild sweet cherries and only about a dozen cultivars. We report S_{17} allele in 24 previously unresolved sweet cherry cultivars. Four of the cultivars carrying S_{17} are partly self-fertile. New allele S_{17} -specific primers were designed. We propose four new incompatibility groups.

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either of the S-RNase alleles in the pistil, then S-RNases stay inactivated, and the pollen is able to fertilize the sweet cherry flower. However, when the SFB-allele in pollen matches either of the S-RNase alleles in the pistil, then pollen SFB inhibits polyubiquitination of S-RNases of the matching allele. Active S-RNase in the pistil causes a cytotoxic environment and disrupts pollen tube growth (Matsumoto and Tao 2016, 2019). Mutations, which cause absence of SFB in the pollen or truncated version of SFB, such as in alleles S_3' , S_4' , and S_5' , result in self-fertility (Ushijima et al. 2004; Sonneveld et al. 2005; Marchese et al. 2007). Additionally, downregulation of *MGST* (*M LOCUS-ENCODED GST*) gene has also been shown to result in self-compatibility (Ono et al. 2018).

S-genotypes have been used to divide sweet cherry accessions into 63 incompatibility groups, one group of universal pollen donors, and one group of self-compatible cultivars (Schuster 2012, 2020; Marchese et al. 2017). Determination and assignment of the cultivars to the correct cross-compatibility groups are essential for sweet cherry breeding and crop production. Cross-compatibility groups and S-alleles have traditionally been identified by controlled pollination tests over several years and counting the number of formed fruits, which is very time and resource consuming and difficult to test. Molecular methods were developed to overcome these obstacles by being rapid, cheap and applicable regardless of the plants' age and time of the year (Wiersma et al. 2001; Sonneveld et al. 2001, 2003). S-locus can also be used as a genetic marker for cultivar identification or pollen donor differentiation (Sebolt and Iezzoni 2009; Cachi and Wünsch 2014).

S-allele genotyping method is based on the knowledge that *S-RNase* gene alleles of sweet cherry have several conserved domains and two introns of varying size (Tao et al. 1999). Consensus primers for the polymerase chain reaction (PCR) have been designed to bind to the conserved regions of sweet cherry *S-RNase* so that they span either the first and/or the second intron (Tao et al. 1999; Wiersma et al. 2001; Sonneveld et al. 2001). Therefore, S-alleles are differentiated according to the size of the PCR-fragment. Consensus primer set SI-19 + SI-20 and SI-31 + SI-32 (Wiersma et al. 2001) can identify seven S-alleles (S_1 – S_6 and S_9). Another set of consensus primers, PaConsI-F + PaConsI-R and PaConsII-F + PaConsII-R (Sonneveld et al. 2003), can distinguish between 13 S-alleles (S_1 – S_{16}). After *S-RNase* gene alleles were cloned, allele-specific PCR primers were developed (Sonneveld et al. 2001, 2003; Schuster et al. 2007; Szikriszt et al. 2012).

More than 30 S-alleles have been characterized both in cultivated and wild sweet cherries: S_1 – S_{16} (Sonneveld et al. 2001, 2003), S_{17} – S_{22} (De Cuyper et al. 2005), S_{23} – S_{25} (Wünsch and Hormaza 2004) and S_{27} – S_{32} (Vaughan et al. 2008). However, the total number of unique S-alleles is currently 22 as several S-alleles have been found to be indistinct from each other, e.g. S_8 , S_{11} and S_{15} are not sufficiently

different from S_3 , S_7 and S_5 , respectively (Sonneveld et al. 2001, 2003; Schuster 2020). While some alleles are rarely found in cultivated sweet cherries (Schuster 2012), nearly all alleles are thought to originate from wild populations, with the possible exception of S_5 , which is very common in cultivated sweet cherries but seldom found in wild sweet cherries (Schuster 2012). Some S-alleles, such as S_{10} , S_{13} , S_{16} , S_{17} , S_{19} , S_{21} – S_{24} , and S_{30} , have been previously found mostly in wild sweet cherries and are rare in European cultivated sweet cherries (De Cuyper et al. 2005; Vaughan et al. 2008; Schuster 2012, 2020). Wild sweet cherry alleles S_{27} – S_{29} and S_{31} – S_{32} have not been found in cultivated sweet cherries thus far (Vaughan et al. 2008; Schuster 2020).

S-allele frequency varies widely in different regions (Lacis et al. 2008; Ipek et al. 2011; Schuster 2012; Cachi and Wünsch 2014; Ivanovych 2016). In Europe, S_3 , S_6 and S_1 are the most common S-alleles of sweet cherry cultivars (Schuster 2012). In the Baltic region, S_3 , S_6 and S_9 were the most frequent S-alleles found in Lithuanian sweet cherry cultivars (Stanys et al. 2008). A recent study of Russian sweet cherry cultivars detected S_3 , S_5 and S_6 as the most frequent S-alleles, but also suggested occurrence of rare S-alleles such as S_{17} and S_{30} or even yet undescribed S-alleles (Bezlepina et al. 2020). However, in Latvian and Swedish sweet cherry collections only S-alleles S_1 – S_6 were genotyped (Lacis et al. 2008). Estonia shares mainland border with Latvia and Russia and has partly similar climatic conditions; thus, sweet cherry S-allele frequency in Estonian sweet cherry cultivars could be similar to neighbouring countries. However, S-alleles of Estonian sweet cherries have not been verified thus far.

The objective of this study was to determine the S-locus alleles of sweet cherry cultivars grown in Estonia and to find the most suitable method for genotyping sweet cherry S-alleles for advancement of sweet cherry breeding in Estonia as well as to verify the list of sweet cherry recommended pollinators based on the S-locus genotyping.

Materials and Methods

Plant Materials and Genomic DNA Isolation

A total of 50 sweet cherry accessions widely grown in Estonia were analysed in this study, including 32 Estonian sweet cherry cultivars ($n = 25$) and breeding lines ($n = 7$). Samples were collected from Polli Horticultural Research Centre, Päidre Tree Nursery and from tissue culture collection in Estonian Crop Research Institute. Young sweet cherry branches were collected between March 2018 and December 2020. Branches were placed in water and forced to sprout. Genomic DNA was extracted from young leaves using a modified CTAB protocol. Plant tissue was ground in

CTAB extraction buffer (2% cetyl trimethylammonium bromide w/v, 100 mM Tris–HCl, 1.4 M NaCl, 20 mM EDTA, 2% PVP-40 in water) and incubated for 25 min at 55 °C. The sample was centrifuged and supernatant pipetted into a new tube. Five hundred microliters of isopropanol was added and mixed by inverting several times. The mixture was incubated at –20 °C for 15 min, spun down at maximum speed, supernatant discarded and pellet was allowed to dry at room temperature. The pellet was dissolved in 50 µl of water. ‘Leningradskaya tchernaya’ is known and registered as ‘Leningradi must’ in Estonia.

S-Allele Genotyping

Estonian sweet cherries were S-genotyped using two methods: (1) consensus primers SI-19 + SI-20 and SI-31 + SI-32 (Wiersma et al. 2001), which can distinguish S_1 – S_6 and S_9 and (2) consensus primers PaConsI-F + PaConsI-R and PaConsII-F + PaConsII-R, which can distinguish S_1 – S_{16} (Sonneveld et al. 2003) (Table S1). Additionally, allele-specific primer pairs of sweet cherry S-alleles S_1 to S_{17} were used for confirming genotypes (Sonneveld et al. 2001, 2003; Szikriszt et al. 2012) (Table S1). New S_{17} -specific primers were also designed: PaS17_F3 (5′-GGGTCGCAATTTAAGAGATTGG-3′) and PaS17_R3 (5′-GCTGAGACATCCAAGCACATAG-3′) (Table S1).

PCR with SI-19 + SI-20 and SI-31 + SI-32 was optimized for FIREPol® Master Mix (Solis BioDyne, Estonia) with final concentrations: 1× FIREPol® Master Mix with 2.5 mM $MgCl_2$, 0.2 µM of each primer and 50 ng of genomic DNA. The reaction was performed as described by Wiersma et al. (2001), except annealing steps were performed at 54 °C for 90 s. Gel electrophoresis of SI-19 + SI-20 and SI-31 + SI-32 PCR products was run approximately for 2 h at 120 V using 1.5% agarose gel with ethidium bromide.

PCR with PaConsI-F + PaConsI-R and PaConsII-F + PaConsII-R primers was done as described by Sonneveld et al. (2003). FIREPol® DNA Polymerase (Solis BioDyne, Estonia) was used in all these PCR reactions. Overnight gel electrophoresis was performed at 50 V for 16 h using 2% agarose gel with ethidium bromide for PaConsI and 1.5% agarose gel for PaConsII PCR products.

Allele-specific PCR reactions were done as described by Sonneveld et al. (2001, 2003) and Szikriszt et al. (2012). Either FIREPol® DNA Polymerase (Solis BioDyne, Estonia) or DreamTaq DNA Polymerase (Thermo Scientific) (1× DreamTaq Green buffer with 0.2 mM dNTP mix, 2 mM $MgCl_2$, 0.2 µM of each primer and 50 ng of genomic DNA) was used in all these PCR reactions. PCR conditions for previously published S_{17} -specific primers (EM-PC2consFD + PavS17R2) were optimized as following: 5 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, followed by 5 min at 72 °C. PCR conditions for new

S_{17} -specific primers were the following: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 62 °C and 30 s at 72 °C, followed by 5 min at 72 °C. Gel electrophoresis was run at 90 V using 1.5% agarose gel with ethidium bromide.

To verify the presence or absence of S_3 ′ self-compatible allele, primers by Sonneveld et al. (2005) were used to amplify and sequence *SFB3* gene (Table S1). To verify the presence or absence of S_4 ′ self-compatible allele, new primers to amplify 279 bp long fragment were designed for *SFB4*: SFB-4_F2 and SFB-4_R2 (Table S1). PCR conditions were 5 min at 95 °C, 40 cycles of 20 s at 95 °C, 20 s at 58 °C and 20 s at 72 °C, followed by 5 min at 72 °C. Gel electrophoresis was run at 90 V using 1.5% agarose gel with ethidium bromide. *SFB4* PCR products were also sequenced.

Selected PCR products were Sanger sequenced at the University of Tartu, Institute of Genomics Core Facility with Applied Biosystems capillary electrophoresis analyser. Sequence alignment was conducted with Clustal Omega MSA tool by EMBL-EBI (Madeira et al. 2019). A selection of 32 sequences was also uploaded to the NCBI Nucleotide database and annotated with respective accession numbers (Table S2).

Assessment of Sweet Cherry Flowering Time

Sweet cherry flowers bloom before or during leaf budding. In Estonia, this usually occurs in the beginning or the first half of May. The beginning of flowering time was registered when 5% of flowers had bloomed. Full flowering (25% of flowers) starts 2–3 days later and lasted 6–10 days (25–75% of flowers). Flowering duration depends on the cultivar and the weather. On 2 years, also end of flowering was registered (when majority of petals have fallen).

Data Availability

Genomic DNA sequences have been submitted to NCBI Nucleotide database; accession numbers of the submitted sweet cherry genomic sequences are listed in Table S2.

Results

S-allele composition of 32 Estonian sweet cherry cultivars and 18 foreign cultivars grown in Estonia were analysed using two sets of consensus primers (Fig. 1 and Table 1). The first set of degenerate PCR primers (Wiersma et al. 2001) successfully identified the S-allele genotypes for 15 out of 50 studied cultivars. The second set of consensus primers PaConsI and PaConsII, which amplify introns I and II, respectively (Sonneveld et al. 2003), could determine S-allele genotypes for 23 out of 50 studied cultivars. S-allele composition was confirmed with allele-specific

Fig. 1 Agarose gel images of two sets of consensus primers for S-genotyping sweet cherry cultivars grown in Estonia. S-allele consensus primers SI-19 + SI-20 and SI-31 + SI-32 determine S-alleles S_1 – S_6 and S_9 . S-allele consensus primers PaConsI-F + PaConsI-R and PaConsII-F + PaConsII-R determine S_1 – S_{16} . S_{17} can be detected and is distinct from all other alleles with each consensus primer set. Agarose gel images of PCR products amplified by **a** SI-19 and SI-20 primers, **b** SI-31 and SI-32 primers, **c** PaConsI-F and PaConsI-R primers, and **d** PaConsII-F and PaConsII-R primers. Cultivars ‘Lapins’ $S_1|S_4$, ‘Kompaktnaja’ $S_2|S_4$, ‘Anne’ $S_3|S_6$, ‘Krupnoplodnaya’ $S_5|S_9$, ‘Madissoni roosa’ $S_4|S_{13}$ and ‘Irma’ $S_3|S_{17}$ were selected to represent all S-alleles detected in this study

primer pairs (Sonneveld et al. 2001, 2003). Majority of the initially unidentified sweet cherry cultivars carried S-allele S_{17} .

High S_{17} Allele Frequency in Estonian Cultivars

S-genotyping of 32 Estonian sweet cherry cultivars (Table 1) resulted in detection of six different S-alleles (S_3 , S_4 , S_5 , S_6 , S_{13} , S_{17}), with S_{17} and S_6 being the most frequent (Fig. 2). Among the 18 foreign and other cultivars grown in Estonia, we detected nine different S-alleles (S_1 , S_2 , S_3 , S_4 , S_5 , S_6 , S_9 , S_{13} , S_{17}), also with S_{17} being the most frequent. Altogether, 53% (17/32) of Estonian cultivars (e.g. ‘Piret’, ‘Polli rubiin’, ‘Tiia’) and 39% (7/18) of foreign cultivars (‘Aleksandrs’, ‘Dönisseni kollane’, ‘Elton’, ‘Gronkavaja’, ‘Leningradskaya tchernaya’, ‘Stella (False)’, ‘Zorka’) grown in Estonia carried the S-allele S_{17} (Table 1).

Neither of the consensus primer sets was initially able to clearly identify allele S_{17} , although unidentified PCR products were detected. The unidentified PCR products were most similar to S_5 with PaConsI primers and to S_9 with PaConsII primers; however, neither was confirmed to be correct with allele-specific primers. The initially unidentified PCR product sizes using PaConsI and PaConsII were also similar to those previously reported for S_{17} (De Cuyper et al. 2005; Vaughan et al. 2008). Previously published

allele-specific primers for S_{17} (Sutherland et al. 2004; Szikriszt et al. 2012) as well as newly designed more specific primers for S_{17} (PaS17_F3 + PaS17_R3) were used to confirm that indeed, S_{17} allele was the most frequent S-allele in Estonian sweet cherry cultivars (Table 1; Figs. 3 and S1). Thus, we confirmed that S_{17} can be detected with PaConsI and PaConsII. Moreover, allele S_{17} can be also detected, although less reliably, with SI-19 and SI-20 primers, where allele S_{17} sometimes has a weak band of about 750 bp and with SI-31 and SI-32 primers, where S_{17} had a band approximately 480 bp, which was indistinguishable from S_1 and S_5 allele (Fig. 1).

The S-alleles of selected cultivars were sequenced in order to reverify the correct alleles. Sequencing confirmed that our S-genotyping results had been correct for alleles S_3 , S_4 , S_6 and S_{17} . Additionally, the presence of allele S_{16} was screened with allele-specific primers and was not detected.

S-allele S_{13} was found in five Estonian and two foreign cultivars. S_{13} can be detected using both consensus primer sets. Using SI-19 and SI-20 primers, alleles S_9 and S_{13} may appear as S_1 and S_3 producing a band around 850 bp. For allele S_{13} primer pair SI-31 and SI-32 had a band of similar size as S_4 and S_6 .

All samples of ‘Stella’ collected in Estonia were S-genotyped as $S_6|S_{17}$, although ‘Stella’ was previously reported to

Table 1 S-alleles of 32 Estonian sweet cherry cultivars and breeding lines and 18 foreign cultivars grown in Estonia. Genotyping was the result of using two different sets of consensus primer pairs (all potential alleles amplified by the consensus primers are shown). Allele-specific primers were used to verify consensus primer results ('+' indicates presence of allele, '-' indicates absence of allele)

Cultivar	S-genotype	Origin	Consensus primers			Allele-specific primers										
			SI19 SI20	SI31 SI32	PaConsI-F PaConsI-R	PaConsII-F PaConsII-R	S ₁	S ₂	S ₃	S ₄	S ₅	S ₆	S ₉	S ₁₃	S ₁₆	S ₁₇
Adelina	S ₃ S ₅	RU	S ₇ S ₃	S ₇ S ₃ S ₃	S ₃ S ₁ , S ₅ , S ₁₆	S ₃ S ₁ , S ₃ , S ₁₃	-	+	+		+					
Aelita	S ₃ S ₉		S ₉	S ₇ S ₅	S ₇ , S ₅ , S ₁₆ S ₂ , S ₇ , S ₁₂ , S ₉ , S ₁₀	S ₃ S ₉			+		+	+				
Aleksanders	S ₃ S ₁₇	LV	S ₉	S ₇ S ₅	S ₇ , S ₅ , S ₁₆	S ₃ S ₉	-				+		-			+
Anne	S ₃ S ₆	EE	S ₇ , S ₃ S ₆	S ₃ S ₄ , S ₆	S ₃ S ₄ , S ₆	S ₇ , S ₃ , S ₁₃ S ₆		+			+	+				
Anu	S ₃ S ₁₇	EE	S ₉	S ₇ S ₅	S ₇ , S ₅ , S ₁₆	S ₃ S ₉	-				+		-			+
Arthur	S ₃ S ₆	EE	S ₇ , S ₃ S ₆	S ₃ S ₄ , S ₆	S ₃ S ₄ , S ₆	S ₇ , S ₃ , S ₁₃ S ₆		+			+	+				
Dõnisseni kollane ^a	S ₆ S ₁₇	DE	S ₆	S ₇ , S ₃ S ₄ , S ₆	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₆ S ₉	-				-	+	-			+
Elle	S ₃ S ₁₃	EE	S ₇ , S ₃	S ₇ , S ₃ S ₄ , S ₆	S ₇ , S ₅ , S ₁₆	S ₃ S ₁ , S ₃ , S ₁₃	-				+	-		+		+
Elo	S ₃ S ₁₇	EE (bl)	S ₆	S ₇ , S ₅	S ₇ , S ₅ , S ₁₆	S ₃ S ₉	-				+	-	-			+
Elton ^a	S ₆ S ₁₇	GB	S ₆	S ₇ , S ₃ S ₄ , S ₆	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₆ S ₉	-				-	+	-			+
Ene	S ₁₃ S ₁₇	EE	S ₄	S ₇ , S ₃ S ₄ , S ₆	S ₇ , S ₅ , S ₁₆	S ₇ , S ₃ , S ₁₃ S ₉	-				-	+	-			+
Gronkavaja	S ₄ S ₁₇	BY	S ₄	S ₇ , S ₃ S ₄ , S ₆	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₄ S ₉	-		+		-					+
Helgi	S ₃ S ₆	EE	S ₇ , S ₃ S ₆	S ₃ S ₄ , S ₆	S ₃ S ₄ , S ₆	S ₇ , S ₃ , S ₁₃ S ₆		+			+	+		+		+
Iput	S ₃ S ₁₃	RU	S ₇ , S ₃	S ₃ S ₄ , S ₆	S ₃	S ₇ , S ₃ , S ₁₃		+			-	-				
Irma	S ₃ S ₁₇	EE	S ₇ , S ₃	S ₃ S ₁ , S ₅	S ₃ S ₁ , S ₅ , S ₁₆	S ₇ , S ₃ , S ₁₃ S ₉	-		+		-	-	-			+
Jaago	S ₄ S ₆	EE (bl)	S ₄ S ₆	S ₄ , S ₆	S ₄ , S ₆	S ₄ S ₆			+		+	+				
Jaama maguskirss	S ₄ S ₆	EE	S ₄ S ₆	S ₄ , S ₆	S ₄ , S ₆	S ₄ S ₆			+		+	+				
Johan	S ₃ S ₆	EE	S ₆	S ₄ , S ₆ S ₁ , S ₅	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₃ S ₆	-				+	+				
Juku	S ₆ S ₁₃	EE	S ₇ , S ₃ S ₆	S ₄ , S ₆	S ₄ , S ₆	S ₃ S ₆	-				-	+	-	+		
Jurgita	S ₆ S ₉	LT	S ₆ S ₉	S ₂ S ₄ , S ₆	S ₄ , S ₆ S ₂ , S ₇ , S ₁₂ , S ₉ , S ₁₀	S ₆ S ₉		-				+	+			
Kadrin	S ₃ S ₁₇	EE (bl)	S ₉	S ₇ , S ₅	S ₇ , S ₅ , S ₁₆	S ₃ S ₉	-				+	-				+
Kaie	S ₃ S ₆	EE	S ₇ , S ₃ S ₆	S ₃ S ₄ , S ₆	S ₃ S ₄ , S ₆	S ₇ , S ₃ , S ₁₃ S ₆			+		+	+				
Karmel	S ₄ S ₆	EE	S ₄ S ₆	S ₄ , S ₆	S ₄ , S ₆	S ₄ S ₆			+		+	+				
Kaspar	S ₆ S ₁₇	EE	S ₆	S ₇ , S ₃ S ₄ , S ₆	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₆ S ₉	-				-	+	-			+
Kõhrkirss 12	S ₃ S ₁₇	EE (bl)	S ₇ , S ₃ S ₉	S ₃ S ₁ , S ₅	S ₃ S ₁ , S ₅ , S ₁₆	S ₇ , S ₃ , S ₁₃ S ₉	-		+		-	-	-			+
Kompaktnaja	S ₂ S ₄	RU	S ₂ S ₄	S ₂ S ₄ , S ₆	S ₄ , S ₆ S ₂ , S ₇ , S ₁₂ , S ₉ , S ₁₀	S ₄		+			+	+	+			
Krupnoplodnaya	S ₃ S ₉	UA	S ₉	S ₇ , S ₅	S ₇ , S ₅ , S ₁₆ S ₂ , S ₇ , S ₁₂ , S ₉ , S ₁₀	S ₃ S ₉	+									
Lapins	S ₁ S ₄	CA	S ₇ , S ₃ S ₄	S ₄ , S ₆ S ₁ , S ₅	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₁ S ₄			+		+		+			
Leningradskaya tchernaya	S ₆ S ₁₇	RU	S ₆	S ₇ , S ₃ S ₄ , S ₆	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₆ S ₉					-	+	-			+
Madissoni roosa	S ₄ S ₁₃	EE	S ₄	S ₄ , S ₆	S ₄ , S ₆	S ₄ S ₉			+		-	-	-	+		
Meelika	S ₄ S ₁₇	EE	S ₄ S ₉	S ₇ , S ₃ S ₄ , S ₆	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₄ S ₉	-		+		-	-	-			+
Mupi	S ₄ S ₁₇	EE	S ₄	S ₇ , S ₃ S ₄ , S ₆	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₄ S ₉	-		+		-	-	-			+
Norri	S ₄ S ₁₇	EE	S ₄	S ₇ , S ₃ S ₄ , S ₆	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₄ S ₉	-		+		-	-	-			+
Paula	S ₃ S ₉	LV	S ₇ , S ₃ S ₉	S ₂ S ₃	S ₃ S ₂ , S ₇ , S ₁₂ , S ₉ , S ₁₀	S ₃ S ₉		-	+			+	+			

Table 1 (continued)

Cultivar	S-genotype	Origin	Consensus primers			Allele-specific primers															
			SI19 SI20	SI31 SI32	PaConsI-F PaConsI-R	PaConsII-F PaConsII-R	S ₁	S ₂	S ₃	S ₄	S ₅	S ₆	S ₉	S ₁₃	S ₁₆	S ₁₇					
Piret	S ₅ S ₁₇	EE			S ₁ , S ₅	S ₁ , S ₅ , S ₁₆	S ₅ S ₉	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Poesia	S ₃ S ₆	RU	S ₁ , S ₃ S ₆		S ₃ S ₄ , S ₆	S ₃ S ₄ , S ₆	S ₁ , S ₃ , S ₁₃ S ₆			+	+	+	+	+	+	+	+	+	+	+	+
Polli murel	S ₃ S ₆	EE			S ₃ S ₄ , S ₆	S ₃ S ₄ , S ₆	S ₁ , S ₃ , S ₁₃ S ₆			+	+	+	+	+	+	+	+	+	+	+	–
Polli rubiin	S ₃ S ₁₇	EE	S ₁ , S ₃ S ₉		S ₃ S ₁ , S ₅	S ₃ S ₁ , S ₅ , S ₁₆	S ₁ , S ₃ , S ₁₃ S ₉	–		+	+	–	–	–	–	–	–	–	–	–	+
Raimu	S ₆ S ₁₇	EE (bl)	S ₆ S ₉		S ₁ , S ₃ S ₄ , S ₆	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₆ S ₉	–		–	–	–	–	–	–	–	–	–	–	–	+
Rannaya rozowinka	S ₃ S ₅	UA	S ₁ , S ₃		S ₁ , S ₃ S ₃	S ₃ S ₁ , S ₅ , S ₁₆	S ₃ S ₁ , S ₃ , S ₁₃	–		+	+	+	+	+	+	+	+	+	+	+	+
Reelu	S ₄ S ₆	EE (bl)	S ₄ S ₆		S ₄ , S ₆	S ₄ , S ₆	S ₄ S ₆				+	+	+	+	+	+	+	+	+	+	+
Sunburst	S ₃ S ₄	FR	S ₁ , S ₃ S ₄		S ₃ S ₄ , S ₆	S ₃ S ₄ , S ₆	S ₄ S ₁ , S ₃ , S ₁₃			+	+	+	+	+	+	+	+	+	+	+	+
Stella (false) ^b	S ₆ S ₁₇		S ₆		S ₁ , S ₃ S ₄ , S ₆	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₆ S ₉			–	–	–	–	–	–	–	–	–	–	–	+
Taki	S ₅ S ₁₇	EE			S ₁ , S ₅	S ₁ , S ₅ , S ₁₆	S ₅ S ₉	–				+	+	+	+	+	+	+	+	+	+
Tiia	S ₆ S ₁₇	EE (bl)	S ₆		S ₁ , S ₃ S ₄ , S ₆	S ₄ , S ₆ S ₁ , S ₅ , S ₁₆	S ₆ S ₉	–				–	–	–	–	–	–	–	–	–	+
Tiki	S ₄ S ₁₃	EE	S ₁ , S ₃ S ₄		S ₄ , S ₆	S ₄ , S ₆	S ₄ S ₁ , S ₃ , S ₁₃	–		–	–	–	–	–	–	–	–	–	–	–	+
Tõmmu	S ₃ S ₁₇	EE	S ₁ , S ₃ S ₉		S ₃ S ₁ , S ₅	S ₃ S ₁ , S ₅ , S ₁₆	S ₁ , S ₃ , S ₁₃ S ₉	–		–	–	–	–	–	–	–	–	–	–	–	+
Tontu	S ₄ S ₆	EE	S ₄ S ₆		S ₄ , S ₆	S ₄ , S ₆	S ₄ S ₆				+	+	+	+	+	+	+	+	+	+	+
Veidenbergi maguskirss	S ₃ S ₁₇	EE (bl)	S ₁ , S ₃		S ₃ S ₁ , S ₅	S ₃ S ₁ , S ₅ , S ₁₆	S ₁ , S ₃ , S ₁₃ S ₉	–		+	+	–	–	–	–	–	–	–	–	–	+
Zorka ^a	S ₁₃ S ₁₇	RU			S ₁ , S ₃ S ₄ , S ₆	S ₁ , S ₅ , S ₁₆	S ₁ , S ₃ , S ₁₃ S ₉	–		–	–	–	–	–	–	–	–	–	–	–	+

EE (bl) indicates Estonian breeding lines, which have not been registered as cultivars

^aIdentity of the cultivar is inconclusive. For more information, see “Discussion”

^bAdditional sample of ‘Stella’ was obtained from Latvia and genotyped as S₃S₄

Fig. 2 S-allele frequencies of 32 Estonian sweet cherry cultivars (blue bars) and 18 foreign sweet cherry cultivars (green bars) grown in Estonia in comparison to 545 European cultivars shown with gray bars in the background (Schuster 2012). The most common S-allele among both groups was S₁₇

be S₃|S₄. An additional sample was requested from Latvia and S-genotyped as S₃|S₄'. Hence, we renamed the Estonian samples as 'Stella (False)' (Table 1).

New Incompatibility Groups Assigned

The results of S-allele genotyping of sweet cherry cultivars allowed the 50 cultivars to be classified into 19 known incompatibility groups including the proposed four new incompatibility groups, 64–67: LXIV, LXV, LXVI, and LXVII for S₃S₁₇, S₄S₁₇, S₅S₁₇ and S₆S₁₇, respectively (Tables S3 and S4).

Flowering Time Does Not Restrict Compatibility Between Cultivars

Pollination is only possible between cultivars flowering at the same time; hence, we analysed the empirical flowering time of sweet cherry cultivars grown in Estonia. Flowering time data from four different years (Fig. S2) showed that the flowering times of sweet cherry cultivars vary from year to year, but usually, the flowering times of different cultivars have an overlap. Some cultivars tend to flower earlier (e.g. 'Ene', 'Taki', 'Tõmmu') and some later (e.g. 'Iput', 'Madissoni roosa', 'Zorka', etc.); however, the early flowering cultivars are generally still in bloom when the late cultivars start to flower. Hence, the differences in flowering times are unlikely to cause incompatibility in pollination.

Known Self-Compatible Mutations Were Not Present in Estonian Cultivars

It has been reported that some cultivars grown in Estonia might be partly self-fertile, namely 'Dönisseni kollane' (S₆|S₁₇), 'Elton' (S₆|S₁₇), 'Madissoni roosa' (S₄|S₁₃), 'Meelika' (S₄|S₁₇) and 'Veidenbergi maguskirss' (S₃|S₁₇) (Table 2). Cultivar 'Norri' (S₄|S₁₇) is a recommended pollinator both for 'Meelika' (S₄|S₁₇) and 'Mupi' (S₄|S₁₇); however, all belong to the same incompatibility group (Table 2). Hence, sequencing analysis was used to detect possible clues for self-compatibility. Based on the sequence analysis, we showed that neither 'Madissoni roosa', 'Meelika', 'Mupi' nor 'Norri' have the S₄' deletion in the *SFB4* gene (Fig. S3).

Another self-compatible mutation has been reported as S₃' haplotype, where complete deletion of *SFB* gene was detected (Sonneveld et al. 2005). Among sweet cherry cultivars grown in Estonia 'Veidenbergi maguskirss' (S₃|S₁₇) is the only partly self-fertile cultivar with S₃ allele. Cultivar 'Arthur' (S₃|S₆) is a recommended pollinator both for 'Polli murel' (S₃|S₆) and 'Anne' (S₃|S₆); however, all belong to the same incompatibility group (Table 2). Additionally, cultivar 'Tõmmu' (S₃|S₁₇) is a recommended pollinator for 'Polli rubiin' (S₃|S₁₇) despite carrying identical pair of S alleles (Table 2). Hence, we analysed the presence or absence of *SFB3* in the abovementioned cultivars grown in Estonia. We showed that all abovementioned cultivars carry *SFB3* gene detected with primers PaSFB3-F and PaSFB3-R (Fig. S4). The sequence of the PCR fragments was identical to the published *SFB3* sequence from the cultivar 'Cristobalina' (Fig. S4). Thus, the S3' haplotype was not detected in the studied cultivars.

Fig. 3 Comparison of two S_{17} -specific primer pairs. New S_{17} -specific primers amplify 398 bp long fragment specifically from the second intron of S-RNase of the S_{17} allele, whereas previously published S_{17} -specific reverse primer combined with EM-PC2consFD amplifies also other S-locus alleles of different sizes. **a** Primers EM-PC2consFD (Sutherland et al. 2004) and PavS17R2 (Szikriszt et al. 2012) amplify S_{17} (505 bp), but also S_3 (613 bp) and S_4 (796 bp). **b** Primers PaS17_F3 and PaS17_R3 designed within this study amplify specifically only S_{17}

Discussion

Sweet cherry (*Prunus avium* L.) is a self-incompatible species, which requires a suitable cultivar to be planted in the vicinity for successful pollination and fruit-setting. The DNA locus responsible for sweet cherry self-incompatibility has been previously identified and genetic testing has been developed for determining S-locus alleles. We used two different sets of consensus primers (Wiersma et al. 2001; Sonneveld et al. 2003) together with allele-specific primers and sequencing to successfully identify all S-genotypes of 50 sweet cherry cultivars grown in Estonia. All genotypes were confirmed with allele-specific primer pairs (Sonneveld et al. 2001, 2003; Szikriszt et al. 2012; Table S5).

Estonian Sweet Cherry Cultivars Possess Rare S-Alleles

S-allele frequencies in Estonian sweet cherry cultivars were considerably different from allele frequencies reported among 545 European sweet cherry cultivars (Schuster 2012) (Fig. 2). The most striking difference is the high frequency of allele S_{17} in Estonia (present in 24 out of 50 cultivars). Allele S_{17} is extremely rare (allele frequency 0.01%) among

European sweet cherry cultivars (Schuster 2012), but it is the most common S-allele in sweet cherries grown in Estonia, with allele frequencies of 26.6% and 19.4% in Estonian and foreign cultivars, respectively. Allele S_{17} was first described in Belgium, where 7 accessions with S_{17} were found among 65 wild sweet cherries (De Cuyper et al. 2005) and later as the third most common S-allele among 168 trees in a wild cherry population in Northern Germany (Schueler et al. 2006). It was found in a Sicilian sweet cherry cultivar ‘Moscatella’ (Marchese et al. 2007), in a Turkish wild sweet cherry accession (Coll.71) from among 28 landraces and wild sweet cherries (Szikriszt et al. 2012), in two landraces in a collection of 207 sweet cherries of diverse background (Mariette et al. 2010) and in three from among 94 cultivars mainly of Ukrainian origin (Ivanovych 2016). Allele S_{17} was listed among the rarest alleles with allele frequency of 0.006% among 153 Italian accessions (Marchese et al. 2017). Among cultivated sweet cherries, allele S_{17} is so rare that it has been reported in only six cultivars in a list of 1483 cultivars, including two Russian cultivars putatively with allele S_{17} , and are usually designated to the incompatibility group 0 of universal donors (Schuster 2020). Four of these cultivars have S-genotype $S_6|S_{17}$. The notable spread of S_{17} among Estonian sweet cherry cultivars likely originates from

Table 2 Recommended pollinators, self-fertility and parentage (if data available) of studied sweet cherries grown in Estonia combined with S-genotype data from this study. Pollination data has been collected from several empirical studies (Jaama and Jaama 1992; Jänes 2016)

Cultivar	Recommended pollinators	Self-fertility	Parentage
Adelina (S ₃ S ₅)	Arthur (S ₃ S ₆), Tõmmu (S ₃ S ₁₇), Meelika (S ₄ S ₁₇)	No	Slava zhukova × Valerii chkalov (S ₃ S ₉)
Anne (S ₃ S ₆) ^a	Arthur (S ₃ S ₆) ^b , Dönisseni kollane (S ₆ S ₁₇), Leningradskaya tchernaya (S ₆ S ₁₇)	No	Zorka (S ₁₃ S ₁₇)
Anu (S ₅ S ₁₇)	Arthur (S ₃ S ₆), Meelika (S ₄ S ₁₇), Leningradskaya tchernaya (S ₆ S ₁₇)	No	Leningradskaya tchernaya (S ₆ S ₁₇)
Arthur (S ₃ S ₆)	Leningradskaya tchernaya (S ₆ S ₁₇), Mupi (S ₄ S ₁₇), Polli murel (S ₃ S ₆) ^b	No	Krassavitsa
Dönisseni kollane (S ₆ S ₁₇)	Meelika (S ₄ S ₁₇), Norri (S ₄ S ₁₇), Zorka (S ₁₃ S ₁₇), Nõmme liivakirss	Partly self-fertile	
Elle (S ₅ S ₁₃)	Arthur (S ₃ S ₆), Karmel (S ₄ S ₆), Leningradskaya tchernaya (S ₆ S ₁₇)	No	Juku (S ₆ S ₁₃)
Elo (S ₅ S ₁₇)		No	Leningradskaya tchernaya (S ₆ S ₁₇)
Elton (S ₆ S ₁₇)	Arthur (S ₃ S ₆), Dönisseni kollane (S ₆ S ₁₇) ^b , Leningradskaya tchernaya (S ₆ S ₁₇) ^b	Partly self-fertile	
Ene (S ₁₃ S ₁₇)	Anu (S ₅ S ₁₇), Arthur (S ₃ S ₆), Meelika (S ₄ S ₁₇)	No	Juku (S ₆ S ₁₃)
Gronkavaja (S ₄ S ₁₇)	Arthur (S ₃ S ₆), Dönisseni kollane (S ₆ S ₁₇), Elle (S ₅ S ₁₃), Iput (S ₃ S ₁₃)	No	
Iput (S ₃ S ₁₃)	Anu (S ₅ S ₁₇), Kaspar (S ₆ S ₁₇), Tjuttševka, Ovstuženka	No	
Irma (S ₃ S ₁₇)	Anu (S ₅ S ₁₇), Elle (S ₅ S ₁₃), Madissoni roosa (S ₄ S ₁₃), Meelika (S ₄ S ₁₇)	No	Leningradskaya tchernaya (S ₆ S ₁₇)
Jaama maguskirss (S ₄ S ₆)		No	Dönisseni kollane (S ₆ S ₁₇) × Kozlovskaya
Johan (S ₅ S ₆)	Arthur (S ₃ S ₆), Karmel (S ₄ S ₆), Tõmmu (S ₃ S ₁₇)	No	Leningradskaya tchernaya (S ₆ S ₁₇)
Juku (S ₆ S ₁₃)	Arthur (S ₃ S ₆), Meelika (S ₄ S ₁₇), Zorka (S ₁₃ S ₁₇)	No	
Jurgita (S ₆ S ₉)	Arthur (S ₃ S ₆), Karmel (S ₄ S ₆), Madissoni roosa (S ₄ S ₁₃)	No	
Kadrin (S ₅ S ₁₇)		No	Norri (S ₄ S ₁₇)
Kaie (S ₃ S ₆) ^a	Arthur (S ₃ S ₆), Leningradskaya tchernaya (S ₆ S ₁₇)	No	Zorka (S ₁₃ S ₁₇)
Karmel (S ₄ S ₆)	Arthur (S ₃ S ₆), Leningradskaya tchernaya (S ₆ S ₁₇), Norri (S ₄ S ₁₇)	No	Norri (S ₄ S ₁₇)
Kaspar (S ₆ S ₁₇)	Arthur (S ₃ S ₆), Dönisseni kollane (S ₆ S ₁₇) ^b , Meelika (S ₄ S ₁₇)	No	Norri (S ₄ S ₁₇)
Leningradskaya tchernaya (S ₆ S ₁₇)	Meelika (S ₄ S ₁₇), Polli murel (S ₃ S ₆), Zorka (S ₁₃ S ₁₇), Tõmmu (S ₃ S ₁₇)	No	
Madissoni roosa (S ₄ S ₁₃)	Irma (S ₃ S ₁₇), Leningradskaya tchernaya (S ₆ S ₁₇), Meelika (S ₄ S ₁₇)	Partly self-fertile	
Meelika (S ₄ S ₁₇)	Dönisseni kollane (S ₆ S ₁₇), Leningradskaya tchernaya (S ₆ S ₁₇), Norri (S ₄ S ₁₇) ^b	Partly self-fertile	Leningradskaya tchernaya (S ₆ S ₁₇)
Mupi (S ₄ S ₁₇)	Arthur (S ₃ S ₆), Norri (S ₄ S ₁₇) ^b , Polli murel (S ₃ S ₆), Tontu (S ₄ S ₆)	No	Kati
Norri (S ₄ S ₁₇)	Karmel (S ₄ S ₆), Meelika (S ₄ S ₁₇) ^b , Piret (S ₅ S ₁₇)	No	Leningradskaya tchernaya (S ₆ S ₁₇)
Piret (S ₅ S ₁₇)	Leningradskaya tchernaya (S ₆ S ₁₇), Meelika (S ₄ S ₁₇), Polli rubiin (S ₃ S ₁₇)	No	Norri (S ₄ S ₁₇)
Polli murel (S ₃ S ₆) ^a	Arthur (S ₃ S ₆) ^b , Leningradskaya tchernaya (S ₆ S ₁₇), Kaspar (S ₆ S ₁₇)	No	Zorka (S ₁₃ S ₁₇) × Zolotaja lashitskaja (S ₃ S ₆)
Polli rubiin (S ₃ S ₁₇)	Arthur (S ₃ S ₆), Piret (S ₅ S ₁₇), Tõmmu (S ₃ S ₁₇) ^b	No	Leningradskaya tchernaya (S ₆ S ₁₇) × Zolotaja lashitskaja (S ₃ S ₆)
Raimu (S ₆ S ₁₇)		No	Arthur (S ₃ S ₆)

Table 2 (continued)

Cultivar	Recommended pollinators	Self-fertility	Parentage
Taki (S ₃ S ₁₇)	Arthur (S ₃ S ₆), Kaspar (S ₆ S ₁₇)	No	Meelika (S ₄ S ₁₇)
Tiia (S ₆ S ₁₇) ^a		No	Leningradskaya tchernaya (S ₆ S ₁₇)
Tiki (S ₄ S ₁₃)	Arthur (S ₃ S ₆), Leningradskaya tchernaya (S ₆ S ₁₇), Mupi (S ₄ S ₁₇)	No	Meelika (S ₄ S ₁₇)
Tõmmu (S ₃ S ₁₇)	Kaspar (S ₆ S ₁₇), Leningradskaya tchernaya (S ₆ S ₁₇), Madissoni roosa (S ₄ S ₁₃)	No	Krassavitsa
Tontu (S ₄ S ₆)	Arthur (S ₃ S ₆), Dönisseni kollane (S ₆ S ₁₇), Madissoni roosa (S ₄ S ₁₃)	No	Norri (S ₄ S ₁₇)
Veidenbergi maguskirss (S ₃ S ₁₇)		Partly self-fertile	
Zorka (S ₁₃ S ₁₇)	Leningradskaya tchernaya (S ₆ S ₁₇), Meelika (S ₄ S ₁₇), Norri (S ₄ S ₁₇)	No	

^aCultivars with unexpected parental data^bCultivars which pollinate a cultivar of the same incompatibility group

Russian cultivar ‘Leningradskaya tchernaya’ S₆|S₁₇, and its offspring ‘Norri’ S₄|S₁₇, which have been extensively used in Estonian sweet cherry breeding. Estonian cultivar ‘Meelika’ S₄|S₁₇ was previously listed as S₄|S₆ (Lacis et al. 2008), but was now confirmed to be S₄|S₁₇. This suggests that there could be more cultivars with S₁₇ that were originally genotyped to have more common alleles.

Alleles S₁ and S₂ are very common in Europe (Schuster 2012), but were found in none of the Estonian cultivars, and either of them were found only in one foreign cultivar genotyped in this study (‘Lapins’ S₁|S₄ and ‘Kompaktnaja’ S₂|S₄). Allele S₃ is also less common in cultivars grown in Estonia than overall in Europe (Fig. 2). Allele S₁₃, which is rare in Europe (2.0% allele frequency as reported by Schuster, 2012), was found in seven cultivars out of 50 (allele frequency 7.0%).

S-Genotyping Helps Differentiate Landraces and Old Cultivars

Sweet cherry cultivar ‘Dönisseni kollane’ S₆|S₁₇ appears distinct from ‘Dönissens gelbe knorpelkirsche’ S₃|S₆ (Schuster 2020). In addition to the difference in S-locus genotype, there is a potential difference in appearance as ‘Dönisseni kollane’ stone fruits have a slightly pink tone, which is visible in Estonian fruit catalogues but is not seen in pictures of ‘Dönissens gelbe knorpelkirsche’ in German fruit catalogues. Further research is needed to determine whether the Estonian ‘Dönisseni kollane’ is an offspring of ‘Dönissens gelbe knorpelkirsche’.

‘Elton’ is an old cultivar originating from England. According to the previously published genotyping, ‘Elton heart BC’ is S₄|S₆, ‘Elton heart EM’ is S₃|S₆ and ‘Black elton’ is S₁|S₃ (Wiersma et al. 2001; Wang et al. 2010; Schuster 2020). The ‘Elton’ clone grown in Estonia and used in this study was S-genotyped as S₆|S₁₇ (Table 1), matching

with none of the previously published ‘Elton’ -named cultivars. We hypothesize that the ‘Elton’ grown and propagated in Estonia might be the progeny but not the direct clone of the ‘Elton’ originating from England.

When comparing cultivars with their reported breeding parents (Table 2), the cultivars generally have one S-allele in common, which is the expected result for self-incompatible sweet cherry. An exception is ‘Zorka’ S₁₃|S₁₇, which shares neither of its S-alleles with its three reported offspring. Our S-genotyping results show that the sweet cherry collected as ‘Zorka’ S₁₃|S₁₇ is unlikely the breeding parent for ‘Polli murel’ S₃|S₆, ‘Anne’ S₃|S₆, and ‘Kaie’ S₃|S₆. ‘Zorka’ originates from Russian Federation bred in 1938 and is unable to survive very cold winters. A possible explanation could be that the original ‘Zorka’ has not survived and has been replaced by another cultivar of different S-genotype.

Comparing Empirical and Genotypical Cross-Compatibility of Sweet Cherry Cultivars

Crosspollination tests have been carried out in Estonia for a long period of time, and based on the accumulated empirical data (Jaama and Jaama 1992; Jänes 2016), a recommendation list of suitable pollinators has been created for sweet cherry cultivars grown in Estonia (Table 2). Many recommended cross-pollinating cultivars carried one allele in common with the cultivar being pollinated, meaning that it is generally sufficient for one allele to be different to achieve good pollination and yield. Intriguingly, in nine cases, the recommended cultivar had both alleles in common, thus belonging to the same incompatibility group; hence, its pollen should theoretically not be able to fertilize the pistil (Fig. 4 and Table 2).

For example, ‘Arthur’ and ‘Polli murel’ are both S₃|S₆ and at the same time recommended as suitable pollinators for each other. However, most cultivars with matching

Fig. 4 Schematic overview of sweet cherry blossom and the role of S-alleles in self-incompatibility. **a** Pollination in self-incompatible sweet cherry can only occur when pollen is of a different S-allele than either of the alleles of the pistil. **b** Pollen with self allele triggers matching S-RNase to become cytotoxic and does not allow pollen tube to form. **c** Several sweet cherry cultivars grown in Estonia were found to be pollinated by (♀) or to pollinate (♂) other cultivars carrying the same S-alleles (Table 2). Some cultivars belong to both groups

recommended pollinators have allele S₁₇: ‘Elton’ S₆|S₁₇, ‘Kaspar’ S₆|S₁₇, ‘Norri’ S₄|S₁₇, ‘Polli rubiin’ S₃|S₁₇, ‘Meelika’, S₄|S₁₇ and ‘Mupi’ S₄|S₁₇ (Fig. 4c and Table 2). This implies that either these cultivars are partly self-fertile or there is more than one allele that appears as S₁₇. We sequenced the PCR products of allele-specific primers of the cultivars with pollination discrepancies and confirmed our S-genotypes to be correct. We confirmed that the S-alleles of these cultivars are not different from each other; hence, the discrepancy must be due to self-fertility or possibly due to some environmental clue.

There exists a known self-fertile allele of S₄’, which carries a deletion causing an early stop codon and degradation of SFB protein (Muñoz-Espinoza et al. 2017), which is found in ‘Stella’ and ‘Lapins’. Partly self-fertile ‘Meelika’

and ‘Madissoni roosa’ both have S₄, which could be responsible for the self-fertility. Cultivars ‘Meelika’, ‘Mupi’ and ‘Norri’ were tested for the S₄’ deletion but were found not to have the deletion (Fig. S3).

The cultivars with the ability to be pollinated by or to pollinate a cultivar of the same incompatibility group all carried alleles S₃|S₆ or S₁₇. Out of five partly self-fertile cultivars, four have S₁₇: ‘Dönisseni kollane’ S₆|S₁₇, ‘Elton’ S₆|S₁₇, ‘Meelika’ S₄|S₁₇ and ‘Veidenbergi maguskirss’ S₃|S₁₇ (Table 2). However, there are another 20 cultivars with S₁₇, which are self-incompatible. ‘Leningradskaya tchernaya’ S₆|S₁₇, which has been extensively used as a breeding parent, is not officially a self-fertile sweet cherry but has occasionally been reported to be partly self-fertile in home gardens. ‘Leningradskaya

tchernaya' $S_6|S_{17}$ is also a breeding parent to partly self-fertile 'Meelika' $S_4|S_{17}$. 'Tiia' $S_6|S_{17}$ is a curious case as it shares both of its S-alleles with its reported parent 'Leningradskaya tchernaya' S_{17} , possibly confirming that 'Leningradskaya tchernaya' could have self-pollinated and produced a viable offspring. However, no cultivars have been found to be $S_{17}|S_{17}$; thus, it is not likely a self-compatible allele. The self-compatibility is also only partial, and yield is greatly improved when other cultivars are planted nearby. Thus, it could be a heritable reduced effectiveness of the gametophytic self-incompatibility but not a self-fertility allele in itself.

It is intriguing that several empirically identified recommended pollinators for Estonian sweet cherry cultivars belong to the same incompatibility group as the pollinated sweet cherry. Many of these discrepancies are with cultivars carrying S_{17} , with four S_{17} cultivars being partly self-fertile. Cultivar 'Madissoni roosa' $S_4|S_{13}$ is also partly self-fertile but neither of the breeding parents are known. Hence, this possibly suggests a segregating mutation affecting self-compatibility in addition to the S-locus. The mechanism behind this is yet to be uncovered.

Sweet cherries grown in Estonia have very different S-allele distribution compared to the rest of Europe. Semi-wild S-allele S_{17} is vastly more common and S_{13} is fairly common, whereas S_1 and S_2 are not found in any Estonian cultivars or breeding lines. This makes Estonian cultivars excellent pollinators for sweet cherry all across Europe. Alternatively, the frequency of S_{17} could be underestimated in Eastern Europe populations, and this finding could elucidate S-genotypes for cultivars with previously unresolved S-genotype.

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Author Contribution K.L. conceived, initiated, and directed the study. A.K. designed the experiments and carried out the S-genotyping of sweet cherries. K.K. designed the genotype list, provided majority of the samples and provided empirical data about flowering and pollination. L.J. designed and carried out DNA sequence analyses and designed new allele-specific primers. A.K., K.L. and L.J. wrote the manuscript with revisions from K.K. All the authors contributed to and approved the final version of the manuscript.

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Availability of Data and Material DNA sequences have been submitted to NCBI Nucleotide database. All relevant data has been included in the tables and supplementary tables of the manuscript.

Declarations

Competing Interests The authors declare no competing interests.

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