Gametophytic self-incompatibility in Andean capuli (*Prunus serotina* subsp. *capuli*): allelic diversity at the S-RNase locus influences normal pollen-tube formation during fertilization

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Capuli *(Prunus serotina* subsp. *capuli*) is an arboreal species that is widely distributed in the northern Andes and that is primarily cultivated for its sweet black cherries. In *Prunus*, fruit-set and productivity is limited by gametophytic self-incompatibility (GSI) which is controlled by the S-Locus. For the first time, this research reveals the molecular structure of the capuli S-RNase - a proxy for S-Locus diversity - and documents how S-Locus diversity influences GSI in the species. To this end, the Andean capuli S-RNase gene was amplified and sequenced in order to design a CAPS (Cleaved Amplified Polymorphic Sequence) marker system that could unequivocally detect S-alleles by targeting the highly polymorphic C2-C3 S-RNase intra-genic region. The devised system proved highly effective. When used to assess S-Locus diversity in 15 *P. serotina* accessions it could identify 18 S-alleles; 7 more than when using standard methodologies for the identification of S-alleles in *Prunus* species. CAPS marker information was subsequently used to formulate experimental crosses between compatible and incompatible individuals (as defined by their S-allelic identity). While crosses between heterozygote individuals with contrasting S-alleles resulted in normal pollen tube formation and growth, in crosses between individuals with exactly similar S-allele identities, pollen tubes showed morphological alterations and arrested development. The evidence presented here supports the notion that S-Locus diversity influences the reproductive patterns of Andean capuli and that it should be considered in the design of orchards and basic propagation materials.

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Abstract

Capuli *(Prunus serotina* subsp. *capuli)* is an arboreal species that is widely distributed in the northern Andes and that is primarily cultivated for its sweet black cherries. In *Prunus*, fruit-set and productivity is limited by gametophytic self-incompatibility (GSI) which is controlled by the S-Locus. For the first time, this research reveals the molecular structure of the capuli S-RNase - a proxy for S-Locus diversity - and documents how S-Locus diversity influences GSI in the species. To this end, the Andean capuli S-RNase gene was amplified and sequenced in order to design a CAPS (Cleaved Amplified Polymorphic Sequence) marker system that could unequivocally detect S-alleles by targeting the highly polymorphic C2-C3 S-RNase intra-genic region. The devised system proved highly effective. When used to assess S-Locus diversity in 15 *P. serotina* accessions it could identify 18 S-alleles; 7 more than when using standard methodologies for the identification of S-alleles in *Prunus* species. CAPS marker information was subsequently used to formulate experimental crosses between compatible and incompatible individuals (as defined by their S-allelic identity). While crosses between heterozygote individuals with contrasting S-alleles resulted in normal pollen tube formation and growth, in crosses between individuals with exactly similar S-allele identities, pollen tubes showed morphological alterations and arrested development. The evidence presented here supports the notion that S-Locus diversity influences the reproductive patterns of Andean capuli and that it should be considered in the design of orchards and basic propagation materials.

Introduction

The capuli (*Prunus serotina* subsp. *capuli*) is a North American arboreal species that has been naturalized in the Andean highlands of Colombia, Ecuador, Peru and Bolivia. The species was introduced in the 17th century during the Spanish colonization of South America and has since developed a rich ethnobotanical tradition in the region (Popenoe and Pachano 1922). Today, local communities of the Andes cultivate *P. serotina* for its sweet black cherries. These are used to produce traditional delicacies, ceremonial drinks and home-remedies (Popenoe and Pachano 1922).

P. serotina has the potential to contribute to the development of profitable, resilient and biodiverse farming systems in the high Andes. In addition to its palatable flavor, the capuli berry possesses antimicrobial (Jiménez et al. 2011), antioxidant (Luna-Vázquez et al. 2013) and anti-inflammatory properties (Vasco et al. 2009; Álvarez-Suárez et al. 2017). These characteristics make Andean *P. serotina* an attractive product for health-food and nutraceutical markets. The export potential of Andean capuli berries could serve as a socio-economic incentive towards the conservation, potentiation and utilization of this semi-domesticated species, while simultaneously improving rural livelihoods (National Research Council 1989).

The effective introduction of *P. serotina* in commercial orchards will require important interventions in genetics and agronomy. In particular, understanding the species' gametophytic self-incompatibility (GSI) system will prove crucial to the design of orchards that maximize fruit set and yields. GSI is a mechanism that has evolved to inhibit self-pollination and to restrict cross-pollination between genetically related individuals, thus preventing inbreeding depression while promoting heterozygosity (Ushijima et al. 1998; de Nettancourt 2001). In *Prunus*, GSI is controlled by a single, multi-allelic locus (*i.e.* the S-Locus). The S-Locus is composed of two independent genes that are tightly linked: the style-specific ribonuclease (S-RNase) and the S-

locus F-box protein (SFB). These genes respectively encode for the pistil and pollen specificity factors of the self-incompatibility response (Tao and Iezzoni 2010). Individuals showing the same S-haplotype (*i.e.,* identical alleles for the S-RNase and SFB genes) belong to the same incompatibility group (IG) and display cross-incompatibility. In numerous *Prunus* species, GSI poses a restriction to free pollen flow and must be considered in the selection of cross-compatible individuals to ensure fruit set in commercial orchards (Herrera et al. 2018). GSI can also influence the selection of compatibility groups in breeding programs and seed multiplication schemes (Cachi et al. 2017).

To our knowledge, GSI in *P. serotina* remains largely unexplored. In a previous study, Gordillo et al. (2015) investigated the allelic diversity of the S-Locus of Andean *P. serotina* by evaluating the degree of polymorphic variation in the Intron-I region of the S-RNase gene in 80 accessions from the Ecuadorian highlands. However, this study did not evaluate if and how this molecular diversity controls GSI in the species. Therefore, the main objective of this research was to study whether the *P. serotina* S-Locus controls sexual incompatibility in the species. To this end, we characterized the molecular structure of the *P. serotina* S-RNase gene in order to design an efficient molecular marker system that would enable the analysis of the allelic diversity of the S-Locus in Andean capuli populations. Subsequently, S-allelic information was used to set-up experimental crosses between cross-compatible and cross-incompatible haplotypes in order to evaluate pollen tube-formation patterns *in-vivo*. Ultimately, we aimed to demonstrate that *P. serotina* individuals from the same IG exhibit obstructed fertilization; the latter would support the notion that the S-Locus influences the reproductive patterns of the species.

Materials & Methods

Plant material

For the S-RNase gene characterization and the CAPS marker system design and validation, a pool of 15 *P. serotina* accessions harboring the 11 S alleles reported by Gordillo et al. (2015) was selected. These accessions were: H25, Azu15, Pic2, Pic19, Car7, I11, Car11, Can9, Car5, H14, Can22, Can11, Car3, Pic23, Car12. DNA samples were obtained from the *P. serotina* collection stored at the Plant Biotechnology Laboratory, Universidad San Francisco de Quito, Ecuador (Guadalupe et al. 2015). For the pollination assays, 7 capuli trees with flowers in balloon stage and located within a private orchard in Cayambe, Ecuador were selected. Young leaf tissue of each individual was employed for DNA extraction and subsequent S-genotyping using the CAPS marker system developed.

DNA extraction

Total genomic DNA was isolated from young leaves using the CTAB methodology (Xin and Chen 2006). DNA concentration and quality were assessed by spectrophotometry using Nanodrop 2000 (Thermo Scientific).

S-RNase gene PCR amplification and DNA sequencing

The amplification of the S-RNase gene of the 15 *P. serotina* samples was performed using the PaConsI-F (Sonneveld et al. 2003) and EM-PC5consRD (Sutherland et al. 2004) primers targeting the signal peptide region and the C5 conserved region, respectively. The PCR reaction consisted of 1X PCR Buffer (Invitrogen), 0.2 mM dNTPs, 1.5 mM MgCl2, 0.3 µM of each primer, 1 U of Taq DNA polymerase (Invitrogen) and 40 ng of DNA in a 25 µl final volume. The PCR cycling conditions were the same as described by Ortega et al. (2006), but using 58 ºC

101 during the annealing stage. PCR products were separated by gel electrophoresis in 1.5% agarose
102 gels. Running conditions were 80 volts for 90 minutes using TBE 1X as running buffer. DNA
103 fragments were visualized using SYBR-Safe (Invitrogen).
104 PCR fragments were excised from the agarose gels and purified using the Wizard SV Gel
105 and PCR Clean-Up System Protocol (Promega). Purified products were reamplified by PCR
106 using the same amplification conditions and 20 ng of purified DNA as template. Reamplified
107 products were sequenced using the services from Macrogen.

108 **Analysis of the *P. serotina* S-RNase sequences**
109 Consensus nucleotide sequence for each of the sequenced fragments was obtained from the
110 forward and reverse reads using pre-Gap and Gap4 software (version 2.0). The search for
111 homologous sequences in the GenBank database was performed using the BLAST tool of the
112 NCBI (Altschul et al. 1990). Intraspecific and interspecific alignments were performed using the
113 CLUSTAL W algorithm of MEGA7 software (Kumar et al. 2016). Deduced amino acid
114 sequences were obtained by translating the coding sequence corresponding to each of the alleles
115 using the online ExPASY Tool (Gasteiger et al. 2003). Alignments of the deduced amino acid
116 sequences were performed using the CLUSTAL Omega algorithm of the European
117 Bioinformatics Institute (EMBL-EBI). Ka/Ks ratios calculation and the sliding window analysis
118 with a window length of 20 codons were performed using the DnaSp6 Software (Rozas and
119 Rozas 1999). Shannon Entropy indexes were calculated for each of the conserved regions (C1,
120 C2, C3, RC4) and the RHV, using the online Protein Variability Server (García-Boronat et al.
121 2008).

122 **Design and validation of the CAPS marker system**
123 The CAPS marker system was designed *in-silico* using the Genome Compiler Software

Package. The digest function was employed for the screening of restriction sites located within the analyzed sequences using a pool of 100 available enzymes, and the gel simulation function was employed for the visualization of the restriction patterns in virtual gels after the *in-silico* digestions.

For the *in-vitro* validation of the CAPS marker system, C2-C3 regions of the 15 *P. serotina* accessions were amplified using the primers Ps1C2Fw (ATY-CAT-GGC-CTR-TGG-CCA-AG) and Ps2C3Rv (TGY-TTR-TTC-CAT-TCV-CBT-TCC) specifically designed for the amplification in this species. Primers were designed from the C2 and C3 sequences obtained from the S-RNase gene characterization. Reagents concentrations for the PCR reaction were the following: 1X PCR Buffer, 3 mM MgCl2, 0.2 mM dNTPs, 0.5 µM of each primer, Taq polymerase Platinum (Invitrogen) 1U and 20 ng of DNA in a 25 µl final volume. Cycling conditions consisted in: 2 minutes of initial denaturation at 94°C, followed by 35 cycles of 1 minute at 94 °C, 2 minutes at 58°C, 4 minutes at 68°C and 10 minutes of final extension at 68°C. PCR products were separated by gel electrophoresis in 1.5% agarose gels. Running conditions were 80 volts for 90 minutes using TBE 1X as running buffer. DNA fragments were visualized using SYBR-Safe (Invitrogen). All the resulting bands were excised from the agarose gel and the DNA was recovered using the Wizard® SV Gel and PCR Clean-Up System (Promega). Purified DNA was reamplified using the same amplification conditions as described before, using 40 ng of DNA as template. Reamplified fragments were digested with RsaI, MboI, HinfI from the Anza line of ThermoFisher Scientific. The digestion reactions consisted in individual digestions containing 9 µl of nuclease-free water, 2 µL of digestion Buffer 10X, 1 µL of enzyme and 8 µL (<0.2 µg) of the reamplified fragments. Restriction reactions were incubated at 37°C for 20 minutes. Restriction patterns were visualized by gel electrophoresis in 2% agarose gels. Running

147 conditions were 80 volts for 1 hour using TBE 1X as running buffer. DNA fragments were
148 visualized using SYBR-Safe (Invitrogen).

149 **Pistils staining and fluorescence microscopy analysis.**

150 For evaluating the self-incompatibility phenotypic response, 7 *P. serotina* trees in flowering
151 stage were S-haplotyped using the CAPS marker system. According to the S-haplotyping results,
152 hand pollinations were carried out proposing 2 types of crosses: incompatible crosses (*i.e.* self-
153 pollinations or crosses between 2 heterozygote individuals carrying the exact same S-alleles) and
154 compatible crosses (*i.e.* between heterozygote individuals presenting completely different S-
155 alleles). Fertilized pistils were stained following the aniline blue protocol (Jefferies & Belcher,
156 1974) and pollen tube formation and growth patterns were analyzed via fluorescence
157 microscopy.

158 For the hand pollination assays, branches with flowers at "G" stage of reproductive
159 landmark stages (Ramírez and Davenport, 2016) were taken to the laboratory and placed in water
160 with plant food. For the flowers selected as pollen donors, pollen extraction was performed. This
161 procedure consisted in flower emasculation and anthers removal. Anthers were dried for 24
162 hours at room temperature for adequate pollen release. Pollen was collected and kept at 4 °C
163 until its use. For the pollen receptor flowers, each flower was emasculated and pollinated *in-vitro*
164 by hand using a paint brush, 24 hours after emasculation. 25 pistils per cross were collected after
165 48 hours since the hand pollination was performed.

166 For the aniline blue staining protocol, pollinated pistils were fixed in 70% ethanol for 7 days.
167 As preparation for microscopy, pistils were incubated at 62°C in 10 M NaOH for 40 min, to
168 soften the tissue and to enhance staining. Softened pistils were washed in distilled water and
169 stained with aniline blue for 1 hour. Pollen tubes were observed by a fluorescent microscopy

BX50 (Olympus). Multiple Image Analysis software (Amscope) was used to obtain an image of the style and for counting pollen tubes. A total of 78 pistils were analyzed. All protocols were previously standardized specifically for *P. serotina* subsp. *capuli*.

Data analysis of pollen tubes growth assay

In the style, pollen tube growth was scored as the number of pollen tubes that reached the base of the style. To compare pollen tube growth and development in the different type of crosses, the data was subjected to a normality test and to a Levene's test for assessing the homocedasticity of the data. Subsequently, a one-way ANOVA was carried out and the means were separated using the Fisher Pairwise comparisons, grouping information using the Fisher LSD Method and 95% of confidence. Analysis were performed using Minitab 17 Software package (2010).

Results

Molecular structure of the *P. serotina* S-RNase gene

In this study, we amplified and sequenced the S-RNase gene of 15 distinct *P. serotina* accessions. The amplification process generated a total of 22 DNA fragments ranging in size from 1023 to 2144 base pairs (Fig. S1). The identity of these 22 amplified fragments was evaluated by comparing their Intron-I sequence with Intron-I sequences reported by Gordillo et al. (2015). These comparisons revealed that 19 of the 22 amplicons corresponded to the 11 alleles previously described by Gordillo et al. (2015). The remaining 3 sequences were identified as new S-RNase alleles. We adopted standard nomenclature rules to catalogue the 14 detected alleles as *P. serotina* S_1 – *P. serotina* S_{14}. DNA sequences for all identified alleles were submitted to the NCBI GenBank database with the following accession numbers: MN098833-MN098846 (Table S1).

Deduced amino acid sequences for 11 of the 14 identified *P. serotina* S-RNase alleles identified were aligned and compared amongst them (intraspecific comparisons), as well as with S-RNase sequences retrieved for other *Prunus* species (interspecific comparisons) (Fig. 1). Alleles S_2, S_6 and S_{11} were not included in these analyses given that only a small fraction of their coding sequences could be retrieved. Overall, intraspecific homologies ranged from ~70% to ~90%, while interspecific homologies ranged from ~81% to ~99%.

Furthermore, we confirmed that the *P. serotina* S-RNase gene exhibits the characteristic molecular structure of *Prunus* T2/S-type RNases. When comparing the deduced amino acid sequences of *P. serotina* S-RNases with S-RNases reported for other *Prunus* species, we were able to identify the signal peptide region, Introns I and II, the five conserved regions and the

hypervariable region located between C2 and C3 (Fig. 1). Shannon entropy indices (*H*) were employed to determine the degree of sequence variability for each of the gene's structural regions amongst the 11 evaluated alleles. Our results indicated that the least variable regions were C1, C2 and C3 (*H*=0.05, 0.049 and 0.09, respectively), while the RC4 region presented a higher variability index (*H*=0.28). The C5 region was not included in the analysis as it corresponded to the primer anchoring site (used for sequencing); therefore, its sequence could not be retrieved. As anticipated, the most variable region was the C2-C3 intragenic region (*H* =1.28), containing the RHV region. Our results also indicated that the RHV sequence was unique for each of the 11 examined S-RNase alleles (Fig. 1). In addition, Ka/Ks ratios were used to identify regions with a higher likelihood for the occurrence of non-synonymous mutations along the S-RNase coding sequence. A sliding-window analysis of Ka/Ks ratios for the 11 examined alleles showed that the highest Ka/Ks ratio (1.34) was located within the RHV region. Conversely, the C2 and C3 regions showed the lowest Ka/Ks ratios (0.37 and 0.17, respectively) (Fig. 2).

Development of a CAPS marker system for analyzing the allelic diversity of the S-Locus in *P. serotina*

Given its high degree polymorphism in size and sequence, the C2-C3 intragenic region was used as a target for the development of a CAPS (Cleaved Amplified Polymorphic Sequence) marker system for the identification of S-RNase alleles in *P. serotina*. The CAPS marker system entails the amplification of a specific DNA region followed by enzymatic restriction to generate unique and distinctive restriction patterns (Shavrukov 2016).

The design of the CAPS marker system was performed *in-silico* using the Genome Compiler Software Package. Sequences corresponding to the C2-C3 regions (isolated from full-length

DNA sequences of the 11 S-RNase alleles) were screened using 100 restriction enzymes. From these, we identified 9 restriction enzymes with the ability to cleave all or the majority (> ~80%) of the 11 sequences analyzed. Based on these results, 99 *in-silico* restrictions (11 alleles x 9 enzymes) were simulated and the digestion patterns were visualized using the virtual simulator of Genome Compiler. These analyses demonstrated that to unequivocally identify the 11 S-alleles, at least 3 restriction enzymes were necessary (RsaI, MboI, HinfI). Each of these enzymes generated individual digestion patterns (Table 1) that when read simultaneously, produced a unique fingerprint for each S-allele. Based on this selection of enzymes and the digestion patterns, we generated a CAPS guide for the rapid identification of *P. serotina* S-RNase alleles (Table 2).

In-silico results were then validated *in-vitro* by amplifying the C2-C3 region corresponding to the 15 *P. serotina* accessions, followed by independent enzymatic restrictions with RsaI, MboI and HinfI. While the expectation was to obtain a maximum of 14 alleles for these 15 accessions (based on DNA sequence analysis), 4 additional (unexpected) bands were obtained for accessions H25, Azu15, Pic19, Car7, Car5, Car3, and Car12 during the C2-C3 amplification (Fig. 3). After the enzymatic digestion, the restriction patterns of the already characterized alleles matched with the *in-silico* predictions. Digestion patterns of the 4 unexpected bands did not match with any of our *in-silico* predictions, therefore we presumed that these bands corresponded to new uncharacterized S-alleles, which were designated as S_{15}-S_{18}. CAPS restriction patterns corresponding to the S_1-S_{18} alleles are presented in Fig. 4.

Influence of S-locus allelic variation on pollen tube formation patterns

To evaluate whether S-Locus allelic variation influences self-incompatibility responses in *P. serotina*, we studied pollen tube formation and growth patterns in experimental crosses. For

these experimental crosses, we selected 7 *P. serotina* tress from a private orchard in Cayambe (Ecuador) based on their S-allelic composition (as revealed with our CAPS marker system). The 7 individuals, which were in full bloom and exhibited flowers in the balloon stage, harbored 9 distinct S-alleles. Six of these alleles had been previously characterized (S_1, S_4, S_6, S_8, S_9, S_{10}), but the remaining 3 patterns corresponded to new uncharacterized alleles; the latter were designated as S_{19} - S_{21} (Table 3).

Based on these results, 2 types of crosses were set-up: incompatible crosses (*i.e.* self-pollinations or crosses between 2 heterozygote individuals carrying the exact same S-alleles) and compatible crosses (*i.e.* between heterozygote individuals presenting completely different S-alleles). Crossing assays are summarized in Table 4. To evaluate incompatible and compatible responses, we analyzed pollen tube formation and growth patterns via fluorescence microscopy. Fluorescence microscopy images clearly indicated that pollen tubes develop differently depending on the type of cross (Fig. 5). In incompatible crosses, pollen tube growth was inhibited in the first-third segment of the style, whereas in compatible crosses, pollen tubes grew along the style to reach the ovary. One-way analyses of variance confirmed that differences in pollen tube growth (based on arrested development vs. full development to reach the ovary) between incompatible and compatible crosses were statistically significant at $p<0.001$ Furthermore, it was also interesting to note that in addition to pollen tube growth inhibition, the tip of pollen tubes in incompatible crosses showed swelling (5a).

268 ## Discussion

269 **The S-RNase gene in *P. serotina* resembles those of related species**

270 The main objective of this research was to elucidate whether the S-Locus of *P. serotina*
271 controls sexual incompatibility patterns in the species. To this end, we used the S-RNase gene as
272 a proxy for the identification of S-haplotypes in *P. serotina* and evaluated whether S-RNase
273 allelic diversity correlates with sexual incompatibility patterns.

274 Detailed analysis of the amino acid sequences of 11 *P. serotina* S-RNase alleles indicated
275 that the *P. serotina* S-RNase gene displays a T2/S-type protein structure; the latter characteristic
276 of stylar ribonucleases in *Prunus* (Ushijima et al. 1998; Yaegaki et al. 2001; Vaughnan et al.
277 2008; Gao et al. 2012). In fact, the *P. serotina* S-RNase gene showed a high degree of amino-
278 acid sequence homology (~81% to ~99%) with S-RNases from related species in the genus. For
279 instance, the *P. serotina* S_8 and *P. webbii* S_3 (NCBI: ABY19368) alleles displayed a protein
280 identity of ~99% (Fig. 6). A high degree of interspecific amino acid sequence homology between
281 S-RNase alleles in *Prunus* has been reported earlier (Banovic et al. 2009; Ortega et al. 2006;
282 Surbanovski et al. 2007). These findings support the hypothesis that *Prunus* S-alleles with very
283 high interspecific identities (>99%) may have originated from a common ancestor before
284 speciation, and that these alleles have been conserved ever since in the derived lineages (Ortega
285 et al. 2006; Surbanovski et al. 2007; Sassa et al. 1996).

286 **CAPS as a molecular marker for determining the allelic diversity of the S-Locus**

287 To investigate GSI-controlled incompatibility patterns in *P. serotina*, we required an
288 effective marker system that would enable the unequivocal identification of S-RNase allelic
289 diversity. The standard methodology for S-alleles identification in *Prunus* species involves the
290 amplification of the S-RNase Intron I and Intron II, followed by amplicon size differentiation via

gel electrophoresis (Ortega et al. 2006; Sonneveld et al. 2003; Gao et al. 2012). However, this methodology often fails to discriminate between allelic variants exhibiting similar amplicon length but distinct genetic sequences (Halász et al. 2008; Gordillo et al. 2015; Kodad et al. 2008; López et al. 2004).

To address this limitation, we developed a CAPS molecular marker system designed to identify sequence-level polymorphisms in the S-RNase C2-C3 intragenic region. This region was selected as it includes the RHV sequence; the latter presumed to mediate the recognition of genetically related S-RNase/SFB complexes (Matton et al. 1997; Ushijima et al. 1998). The RHV region is located at the surface of the S-RNase protein and is thought to play a key role in the recognition of self and non-self pollen (Matton et al. 1997; Ushijima et al. 1998); it is therefore considered an adequate proxy of the self-incompatibility response in *P. serotina*. Furthermore, our results indicated that the C2-C3 intragenic region represents the most polymorphic segment of the *P. serotina* S-RNase gene. In fact, Shannon entropy indices and Ka/Ks ratios revealed that the highest degree of amino acid sequence variation in the gene concentrates in the RHV region.

The CAPS marker system designed in this study proved highly effective in the identification of S-RNase alleles. When employed to genotype an initial core-set of 15 *P. serotina* accessions (representing the species' S-RNase allelic diversity in the Ecuadorian highlands), the system enabled the unequivocal identification of 18 S-RNase alleles. From these, 7 alleles (S_{12}-S_{18}) could not be identified when using the Intron I molecular marker system (Gordillo et al. 2015), and four (S_{15}-S_{18}) could not be detected when amplifying, extracting and sequencing the full-length S-RNase genes for the aforementioned 15 individuals. Furthermore, an additional three alleles (S_{19}-S_{21}) were identified when characterizing a set of 7 individuals used for pollen-tube

formation analyses. The effectiveness of our CAPS marker system in the determination of S-RNase diversity can be ascribed to three important factors. In the first place, the use of species-specific primers to amplify the C2-C3 intragenic region allowed for a higher resolution and differentiation of length-based allelic variants. Size differences in the C2-C3 intragenic region are attributable to differences in the lengths of the Intron II and RHV sequences (i.e. the most polymorphic regions of the S-RNase gene) which are easier to resolve via gel electrophoresis as low-molecular weight products. By contrast, the amplification of the full-length S-RNases yield high-molecular weight amplicons of similar size which are not easily isolated. The inability to purify these amplicons independently may lead to the masking-off of allelic differences resulting in allele-misidentification during sequencing (*i.e.* pooling of two or more allelic variants yields one consensus sequence as seen in this study). In second place, the use of 3 restriction enzymes is a powerful tool to exploit and elucidate the intrinsic, polymorphic nature of the C2-C3 intragenic region. This feature is especially useful when two S-alleles exhibit similar amplicon lengths (*i.e.* gel electrophoresis does not allow the clear resolution of size-differences of a few base pairs). Accordingly, it offers an opportunity to identify heterozygotes when an individual's S-alleles are similar in sequence length (Moriya et al. 2007). Finally, the *in-silico* design of our CAPS molecular system allowed us the possibility to simulate multiple digestions in order to identify the most informative restriction enzymes. The efficiency of our 3 enzyme CAPS marker system is evident when comparing it to CAPS systems developed for haplotyping the S-Locus in other species from the Rosacea family. For instance, for the identification of 17 S-alleles in European pear cultivars it was necessary to employ 11 different enzymes (Moriya et al. 2007), whereas for the identification of 22 S-alleles in Japanese apple cultivars, 17 different enzymes were required (Kim et al. 2008).

S-Locus allelic diversity and its influence over GSI in P. serotina

As evidenced in this study, the S-RNase of *P. serotina* shares a high degree of identity with S-RNases from related species for which the S-Locus has been reported to control GSI. This finding would suggest that the S-Locus also plays a crucial role in determining the reproductive patterns of *P. serotina*. To add evidence to this theory, we investigated pollen-tube formation in crosses between compatible and incompatible individuals (as defined by their S-allelic composition via CAPS).

Our results demonstrated that crosses between heterozygote individuals with contrasting S-alleles result in normal pollen tube formation and growth (*i.e.* pollen tubes extend along the style and reach the ovary). By contrast, in crosses between heterozygote individuals with exactly similar S-alleles identities (including self-pollinations), pollen tubes showed morphological alterations (*i.e.* swelling of pollen tube tips) and arrested development (Fig. 5). While the ultimate evidence for cross compatibility is the confirmation of fertilization, we have shown a clear correlative pattern between S-alleles identity and the physiological development of the fertilization process in *P. serotina*.

According to our results, tetraploidy in *P. serotina* did not appear to influence cross-compatibility patterns. As a tetraploid species, *P. serotina* heterozygous individuals can produce diploid homoallelic pollen (pollen grains harboring two copies of the same S-allele) or diploid heteroallelic pollen (pollen grains harboring two different S-alleles). In self-pollinations and assisted-crosses between individuals carrying the exact S-alleles, pollen tube formation and growth was consistently arrested (*i.e.* a clear sign of self-incompatible response). These results indicated that heteroallelic pollen of *P. serotina* did not resulted in GSI breakdown, contrary to the reports for polyploid Solanaceous species, in which diploid heteroallelic pollen confers self-compatibility, regardless of the S-allelic composition of the style due to a phenomenon known as

competitive interaction (Lewis and Modlibowska 1942; Golz et al. 1999; 2001; Luu et al. 2001). A plausible model to explain these observations is the "one-allele match model", which has been successfully adopted to explain self-incompatibility in other tetraploid *Prunus* species like *P. pseudocerasus* and *P. spinosa* (Hauck et al. 2006; Nunes et al. 2006). In this model, the self-incompatibility reaction occurs when at least one of the functional SFB alleles of the pollen grain matches with its cognate S-RNase allele expressed in the style (Hauck et al. 2006).

As established earlier, GSI in *Prunus* is attributed to the interaction and recognition of self/non-self S-RNase/SFB complexes (Matsumoto and Tao 2016). In this study, we were able to predict the phenotype of the crosses based on the analysis of the S-RNase gene. Yamane et al. (2003) suggested that both genes are linked, inherited together and correspond one to another for each S-haplotype. This could explain why we were able to predict the phenotype of the crosses by only analyzing the S-RNase gene. However, to further elucidate the mechanism of GSI in *P. serotina*, the interaction between both SBF and S-RNase genes should be assessed.

Conclusions

We investigated GSI in *P. serotina*, a wild species, of which the GSI status had not been established previously. We characterized the S-RNase gene by sequence alignments and comparative analysis with sequences reported for other *Prunus* species. We also developed a CAPS molecular marker to efficiently discriminate between S-alleles and to propose compatible and incompatible crosses between the studied accessions. Subsequently, pollen tubes development was studied using fluorescence microscopy of pollinated pistils. Results indicated that compatible crosses are characterized by pollen tubes normal growth reaching the style, whereas incompatible crosses are characterized by pollen tube growth at the first third of the style. Based on this evidence, we conclude that GSI reported for the *Prunus* genus is operating in *P. serotina*. Furthermore, we also developed a powerful molecular tool for the rapid and precise identification of S-alleles in this species, which provides vital information for assisting in breeding programs and for the establishment of capuli productive orchards.

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528 protein with an F-box motif that is very tightly linked to a gene for S-RNase in two species
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530 https://doi.org/10.1093/pcp/pcg088

Table 1(on next page)

Individual digestion patterns expected for the digestion of C2-C3 amplicons with three different enzymes (RsaI, MboI and HinfI).

Composed patterns (formed by the simultaneous reading of the three individual digestion patterns) specific to each S-allele are presented in Table 2. **Note:** In order to facilitate the digestion pattern identification, fragment sizes presented in this table were rounded.

1

Restriction Enzymes

Pattern	RsaI				MboI				HinfI			
A	200				200				180	100		
B	200	160	150		210	200	190		200			
C	210	140	100		240				225	200		
D	210	180	160		300				230	190	100	
E	230	200			300	100			240			
F	240				360				250	210		
G	240	225			460				360	250	220	
H	290	170	100		465	250	230		365			
I	330	210	180		490				375	330	250	210
J	370				520	430			380			
K	430	250			550	415	250		390	240		
L	475	360	290		600	470	300		470			
M	620	210	195	170	615				540			
N	790	510			760				550	495		
O	940	220	170		1200				550	350		
P	950				290	180	100		580			
Q	280	120	90		600	500	400	380	650	330	270	
R	220	200	110		430	100			290	220		

2

3 **Note:** In order to facilitate the digestion pattern identification, fragment sizes presented in this table were rounded.

Table 2 (on next page)

Composed digestion patterns for the identification of *P. serotina* S-alleles (S_1-S_{18}) using three restriction enzymes.

Each letter makes reference to the restriction pattern shown in Table 1 for each restriction enzyme (RsaI, MboI and HinfI, respectively).

1

S-RNase Allele	Restriction Pattern
S1	PJN
S3	BEJ
S4	OLI
S5	DFC
S6	CIE
S7	EGL
S8	LHQ
S9	IMK
S10	KNJ
S12	JFF
S13	HCP
S14	GBM
S15	FCA
S16	MKG
S17	FCE
S18	NOO

2

Table 3(on next page)

S- allelic composition of the 7 *P. serotina* individuals selected for performing assisted crosses.

All individuals were heterozygotes and amplified 2 bands. S-alleles were determined using the CAPS marker system.

1

	C2-C3 CAPS Patterns				
Individual	**Band**	**RsaI**	**MboI**	**HinfI**	**S-allele**
1	1	L	H	Q	S_8
	2	Q	P	R	S_{19}
12	1	O	L	I	S_4
	2	I	M	K	S_9
13	1	O	L	I	S_4
	2	I	M	K	S_9
14	1	O	Q	I	S_{20}
	2	R	R	F	S_{21}
15	1	K	N	J	S_{10}
	2	C	I	E	S_6
17	1	O	L	I	S_4
	2	P	J	N	S_1
22	1	O	L	I	S_4
	2	P	J	N	S_1

2

Table 4(on next page)

Proposed crosses according to the S-allelic composition of the 7 *P. serotina* trees genotyped.

Crosses between two individuals carrying the exact same S-alleles are expected to be incompatible. Crosses between two individuals carrying completely different S-alleles are expected to be compatible. Self-pollinations of the pollen receptor trees are expected to be incompatible.

1

Pollen donor		Pollen Receptor	Expected phenotype
22 (S1, S4)	X	17 (S1, S4)	Incompatible
12 (S4, S9)	X	13 (S4, S9)	Incompatible
17 (S1, S4)	X	1 (S8, S19)	Compatible
14 (S20, S21)	X	15 (S10, S6)	Compatible
17 (S1, S4)	X	17 (S1, S4)	Incompatible
13 (S4, S9)	X	13 (S4, S9)	Incompatible
1 (S8, S19)	X	1 (S8, S19)	Incompatible
15 (S10, S6)	X	15 (S10, S6)	Incompatible

2

Figure 1

Alignment of the deduced amino acid sequences of *P. serotina* S-RNases with S-RNases reported for different *Prunus* species.

Alignment included: *P. serotina* (capuli), *P. tenella* (dwarf Russian almond), *P. armeniaca* (apricot), *P. salicina* (plum), *P. mume* (Japanese apricot), *P. avium* (sweet cherry) for determining the genetic structure of the *P. serotina* S-RNase gene. Aligning algorithm: Clustal Omega (EMLB-EBI). Asterisks denote conserved sites; dots denote conservative substitutions and dashes indicate gaps. Conserved cysteine residues are pointed out with a filled circle, whereas conserved histidine residues are pointed out with an open circle. The signal peptide, the conserved (C1-C5) and the hyper-variable (RHV) regions are underlined and arrows indicate the position of Intron I and II. GenBank accession numbers: P. ten S_{13} (KJ755355), P. sal S_c (AB084102), P. avi S_{31} (DQ266443), P. arm S_{36} (GU574198), P. arm S_1 (AY587561), P. arm S_2 (AY587562), P. mum S_1 (AB101438), P. mum S_7 (AB364468), P. avi S_3 (AB010306), P. sal S_a (AB252411), P. dul S_c (AB011470)

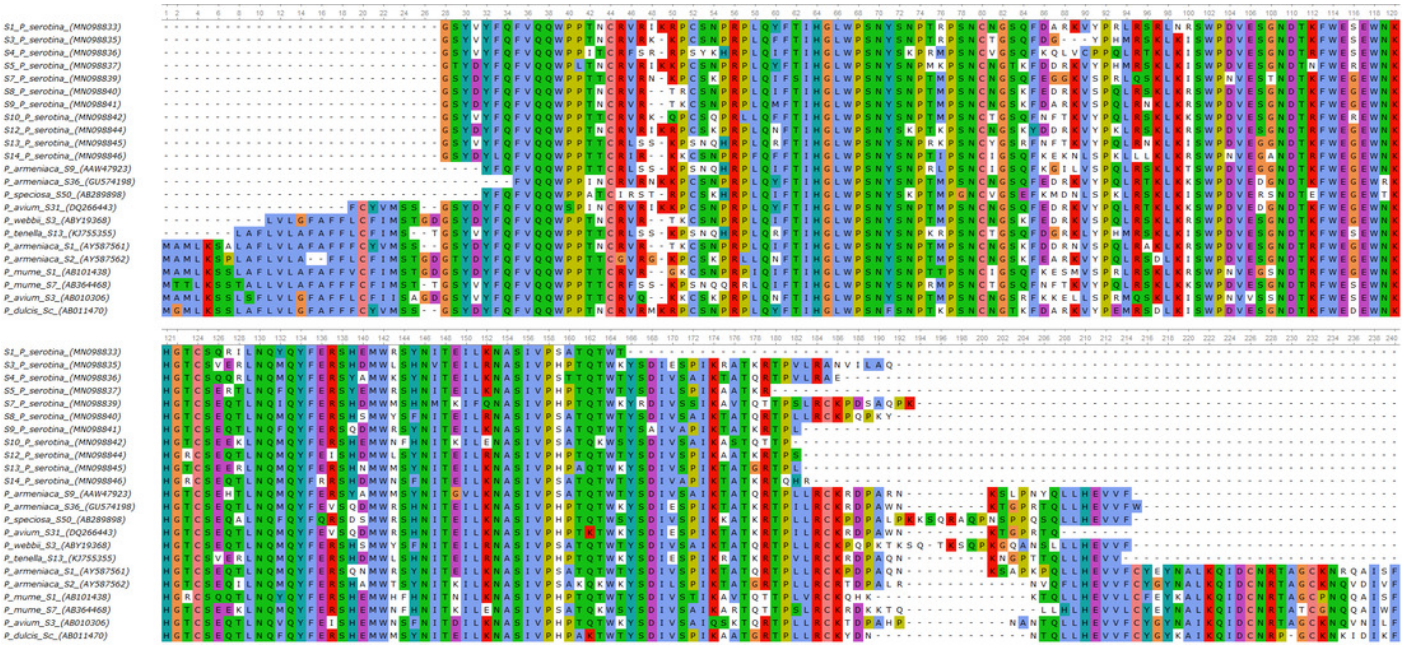


Figure 2

Graphical representation of the Ka/Ks ratios calculated along the coding sequence of the 11 *P. serotina* S-alleles based on a sliding window analysis of 20 codons.

The position of conserved regions (C1-C5) and RHV region are indicated. The plot was obtained using DnaSP6 (Rozas and Rozas 1999)

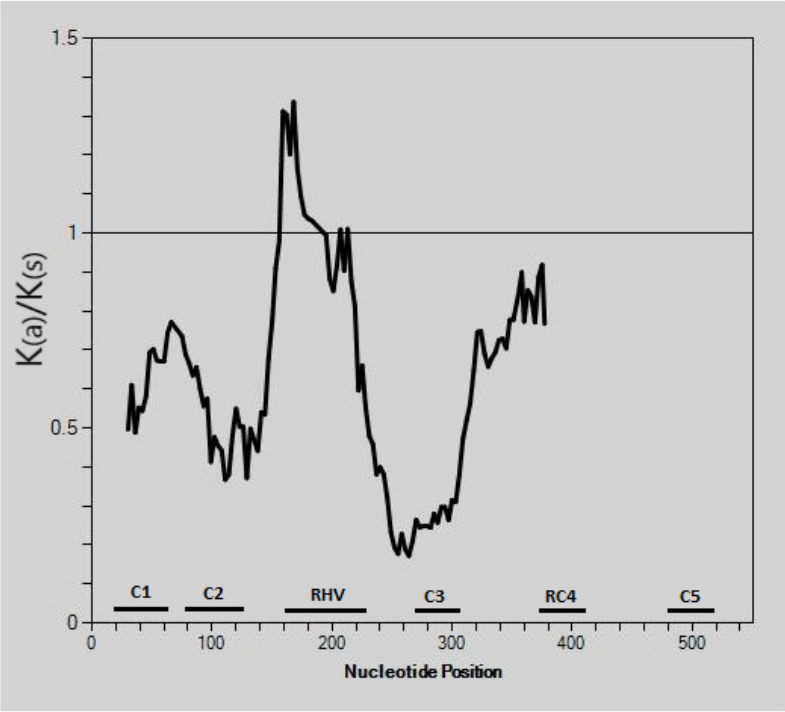
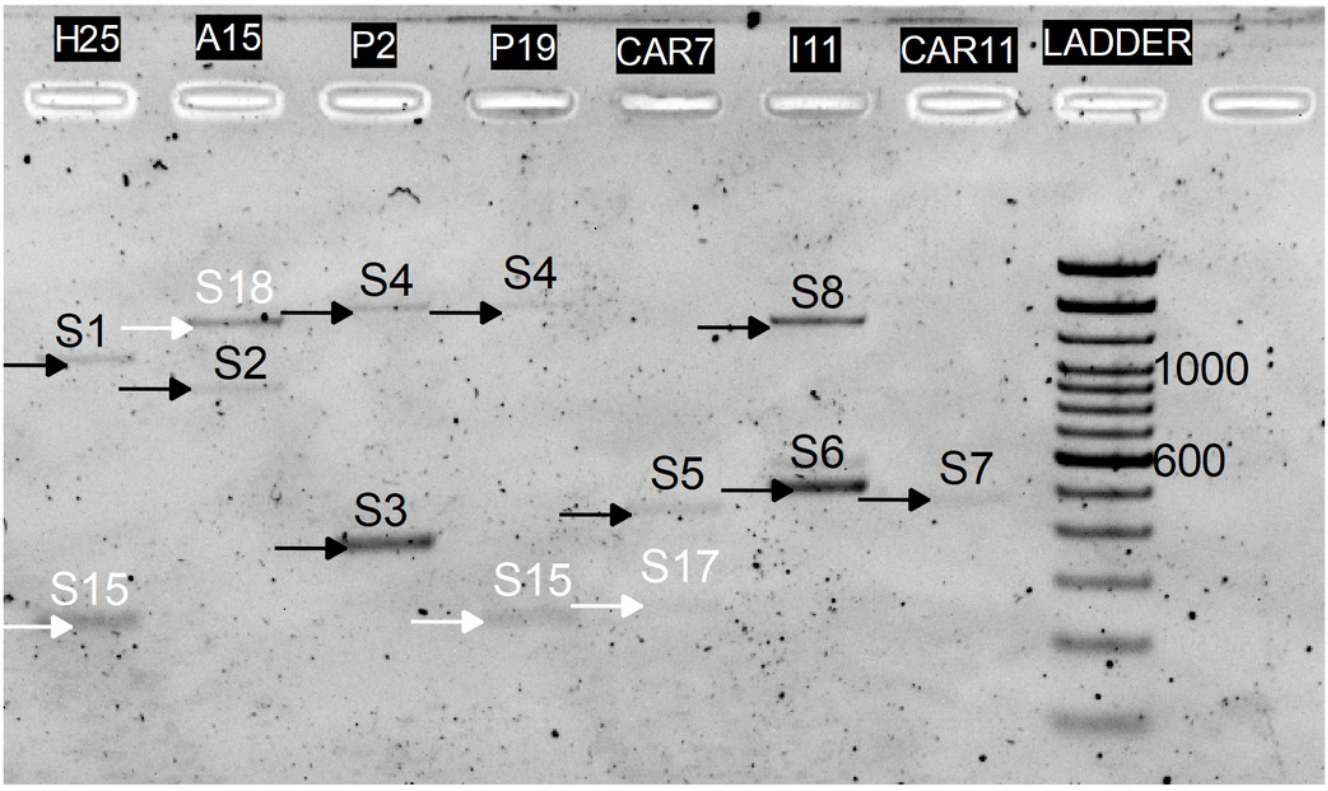

1.5
1
0.5
0
K(a)/K(s)
C1
C2
RHV
C3
RC4
C5
0
100
200
300
400
500
Nucleotide Position

Figure 3

PCR amplification of the *P. serotina* C2-C3 intragenic region using specific primers.

Black arrows indicate the position of the expected alleles whereas white arrows highlight the unexpected alleles obtained for H25, Azu15, Pic19, Car7, Car3 and Car12


H25
A15
P2
P19
CAR7
I11
CAR11
LADDER
S1
S2
S18
S4
S4
S8
1000
600
S5
S6
S7
S3
S15
S15
S17

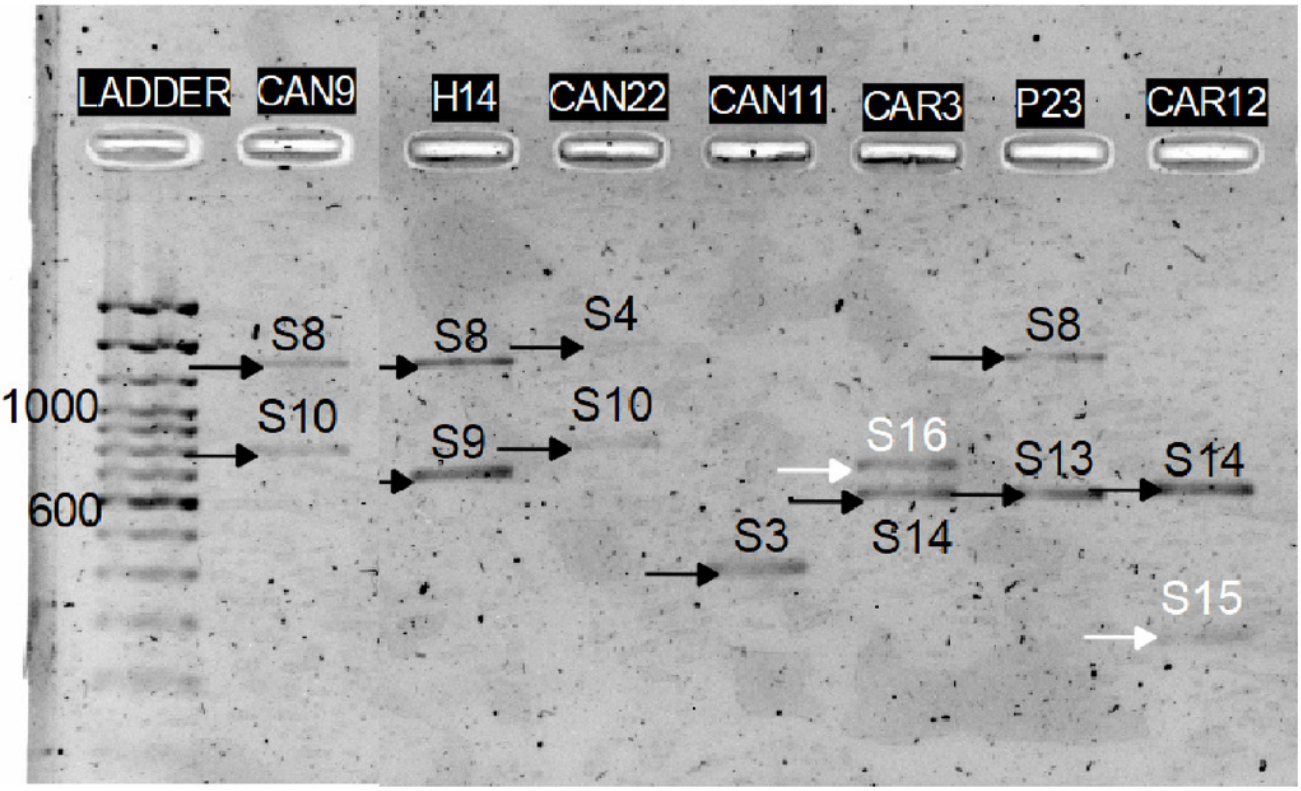
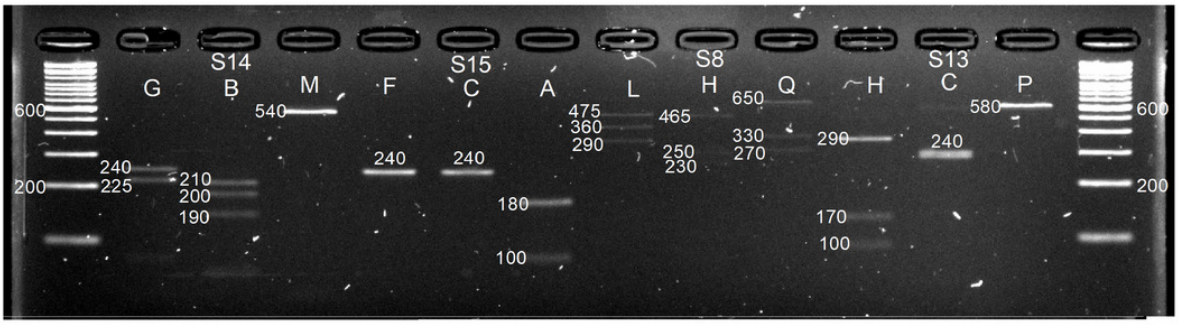
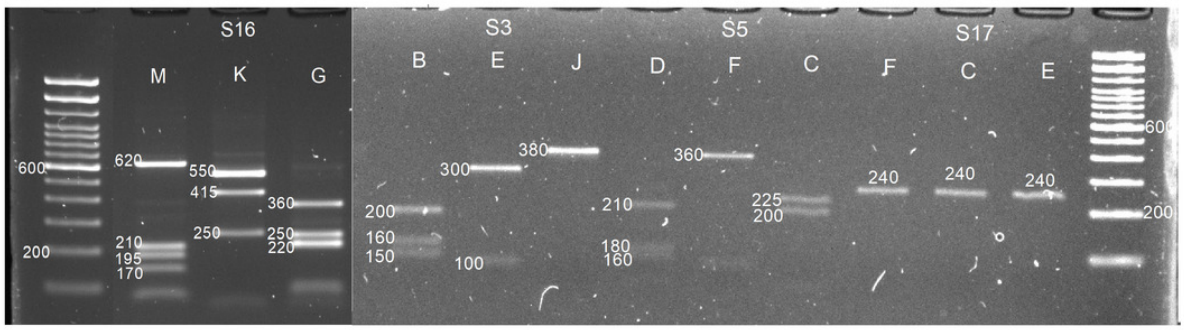

LADDER
CAN9
H14
CAN22
CAN11
CAR3
P23
CAR12
1000
600
S8
S10
S8
S4
S10
S9
S8
S16
S13
S14
S3
S14
S15

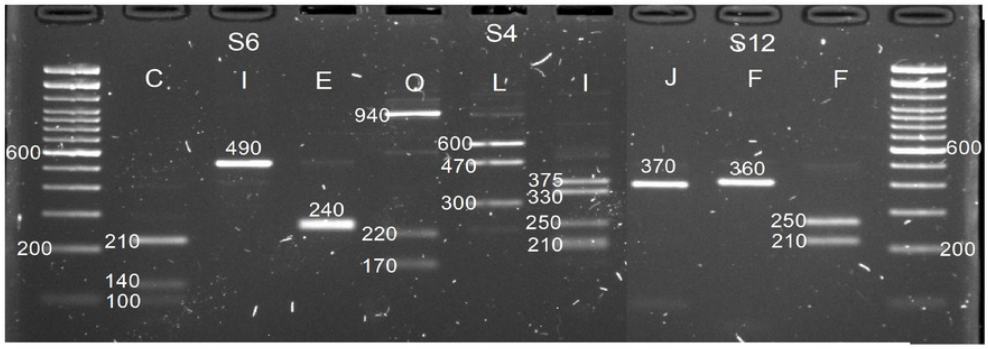
Figure 4

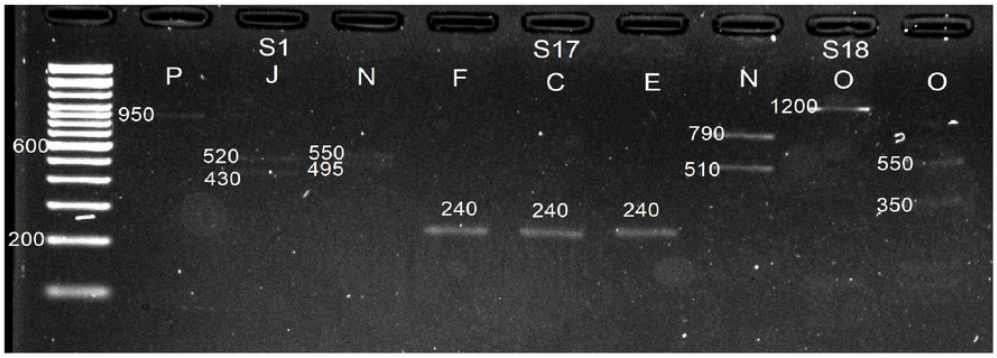
In-vitro CAPS patterns obtained for alleles S_1, S_3, S_4, S_5, S_6, S_7, S_8, S_9, S_{10}, S_{12}, S_{13}, S_{14}, S_{15},[sub] [su

Letters indicate the restriction patterns reported for each enzyme in Table 1


S14
G B M
600
540
240
225
210
200
190
200
S15
F C A
240
240
180
100
S8
L H Q
475
360
290
465
250
230
650
330
270
S13
H C P
290
240
580
170
100
600
200


S16
M K G
600
620
550
415
360
250
250
220
210
195
170
200
S3
B E J
380
300
200
160
150
100
210
180
160
S5
D F C
360
225
200
S17
F C E
240
240
240
600
200


S6
C I E
600
490
240
210
200
140
100
S4
Q L I
940
600
470
375
330
300
250
210
220
170
S12
J F F
370
360
250
210
600
200


S1
P J N
950
600
520
430
550
495
200
S17
F C E
240
240
240
S18
N O O
1200
790
510
550
350

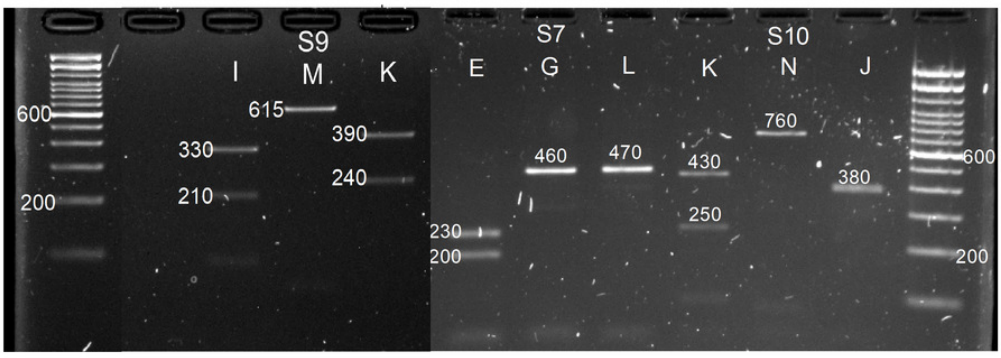

S9
I M K
600
615
330
390
240
210
200
S7
E G L
460
470
230
200
S10
K N J
430
760
380
250
600
200

Figure 5

Pollen tube growth analysis in incompatible and compatible assisted crosses using fluorescence microscopy.

Pollen tubes were stained following the aniline blue protocol after 48 hours of hand pollination. a) Incompatible cross: Individual 22 (S_1 – S_4) X Individual 17 (S_1 – S_4). Pollen tubes growth was inhibited in the first third of the style showing swollen tips. b) Incompatible cross: Self-pollination of Individual 17. Pollen tube growth was inhibited in the first third of the style. c) Compatible cross: Individual 17 (S_1 – S_4) X Individual 1 (S_8 – S_{19}). Pollen tubes grew along the style and reached the ovary

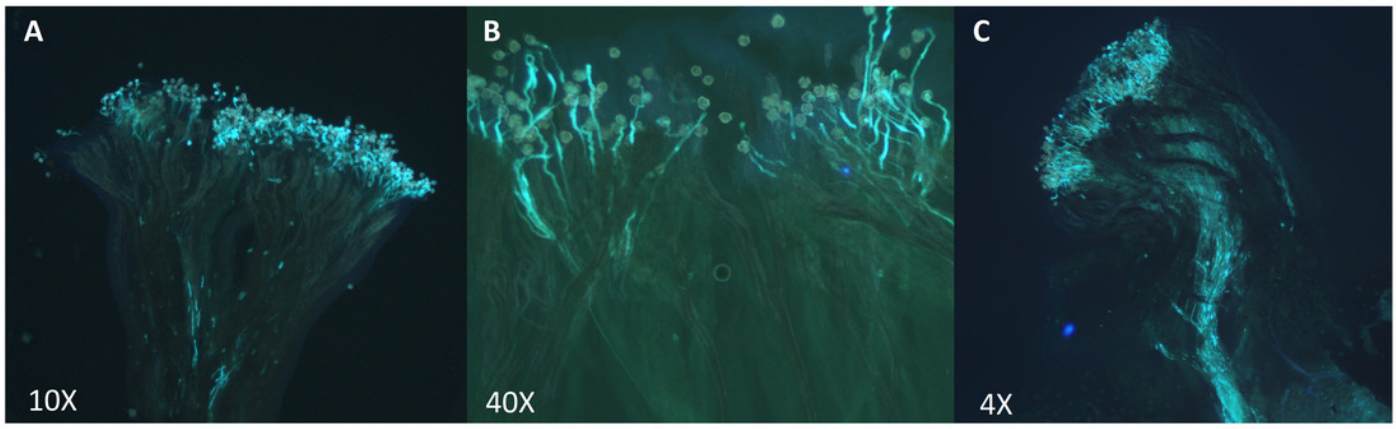


Figure 6

Inter-specific alignment between *P. serotina* S_8 and *P. webbii* S_3 (ABY19368) alleles.

Sequences are 99.38% identical. The conservative amino acid substitution of an arginine (R) by a lysine (K) located between C2 and C3 is pointed out with an arrow