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 a tree species that is widely distributed in the northern Andes. In *Prunus*, fruit set and productivity appears to be 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 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). 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 often showed morphological alterations and arrested development, but for some (suspected) incompatible crosses, pollen tubes could reach the ovary. The latter indicates the possibility of a genotype-specific breakdown of GSI in the species. Overall, this 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.

1   **Gametophytic self-incompatibility in Andean capuli (*Prunus serotina* subsp.**

2   ***capuli*): allelic diversity at the S-RNase locus influences normal pollen-tube**

3   **formation during fertilization**

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Abstract

16   Capuli *(Prunus serotina* subsp*. capuli)* is a tree species that is widely distributed in the

17 northern Andes. In *Prunus*, fruit set and productivity appears to be limited by gametophytic

18 self-incompatibility (GSI) which is controlled by the S-Locus. For the first time, this research

19 reveals the molecular structure of the capuli S-RNase (a proxy for S-Locus diversity) and

20 documents how S-Locus diversity influences GSI in the species. To this end, the capuli S-RNase

21 gene was amplified and sequenced in order to design a CAPS (Cleaved Amplified Polymorphic

22 Sequence) marker system that could unequivocally detect S-alleles by targeting the highly

23 polymorphic C2-C3 S-RNase intra-genic region. The devised system proved highly effective.

24 When used to assess S-Locus diversity in 15 *P. serotina* accessions, it could identify 18 S-

25 alleles; 7 more than when using standard methodologies for the identification of S-alleles

26 in *Prunus* species. CAPS marker information was subsequently used to formulate experimental

27 crosses between compatible and incompatible individuals (as defined by their S-allelic identity).

28 Crosses between heterozygote individuals with contrasting S-alleles resulted in normal pollen

29 tube formation and growth. In crosses between individuals with exactly similar S-allele

30 identities, pollen tubes often showed morphological alterations and arrested development, but for

31 some (suspected) incompatible crosses, pollen tubes could reach the ovary. The latter indicates

32 the possibility of a genotype-specific breakdown of GSI in the species. Overall, this supports the

33 notion that S-Locus diversity influences the reproductive patterns of Andean capuli and that it

34 should be considered in the design of orchards and basic propagation materials.

Introduction

36   The capuli (*Prunus serotina* subsp. *capuli*) is an allotetraploid woody perennial species,

37   taxonomically positioned in a clade with the *Padus* and *Laurocerasus* sub-genera of the *Prunus*

38   genus (Pairon et al., 2008; Bortiri et al., 2006; Guzmán et al., 2018). The species is native to

39   North America, but has been naturalized in the Andean highlands of Colombia, Ecuador, Peru

40   and Bolivia (Fresnedo-Ramírez et al., 2011; Guzmán et al., 2018). *P. serotina* was likely

41   introduced in the 17th century during the Spanish colonization of South America and has since

42   developed a rich ethnobotanical tradition in the region (Popenoe and Pachano 1922). Today,

43   local communities of the Andes cultivate *P. serotina* for its sweet black drupes. These are used to

44   produce traditional delicacies, ceremonial drinks and home-remedies (Popenoe and Pachano

45   1922).

46   *P. serotina* has the potential to contribute to the development of profitable, resilient and

47   biodiverse farming systems in the high Andes. In addition to its palatable flavor, the capuli drupe

48   possesses antimicrobial (Jiménez et al. 2011), antioxydant (Luna-Vázquez et al. 2013) and anti-

49   inflammatory properties (Vasco et al. 2009; Álvarez-Suárez et al. 2017). These characteristics

50   make it an attractive product for health-food and nutraceutical markets. The export potential of

51   the capuli fruit could serve as a socio-economic incentive towards the conservation, potentiation

52   and utilization of this semi-domesticated species, while simultaneously improving rural

53   livelihoods (National Research Council 1989).

54   The effective introduction of *P. serotina* in commercial orchards will require important

55   interventions in genetics and agronomy. In particular, understanding the species' gametophytic

56   self-incompatibility (GSI) system will prove crucial to the design of orchards that maximize fruit

57   set and yields. GSI is a mechanism that has evolved to inhibit self-pollination and to restrict

58   cross-pollination between genetically related individuals, thus preventing inbreeding depression

59   while promoting heterozygosity (Ushijima et al. 1998; de Nettancourt 2001). In *Prunus*, GSI is

60   controlled by a single, multi-allelic locus (*i.e.* the S-Locus). The S-Locus is composed of two

61   independent genes that are tightly linked: the style-specific ribonuclease (S-RNase) and the S-

62   locus F-box protein (SFB). These genes respectively encode for the pistil and pollen specificity

63   factors of the self-incompatibility response (Tao and Iezzoni 2010). Individuals showing the

64   same S-haplotype (*i.e.,* identical alleles for the S-RNase and SFB genes) belong to the same

65   incompatibility group (IG) and display cross-incompatibility. In numerous *Prunus* species, GSI

66   poses a restriction to free pollen flow and must be considered in the selection of cross-

67   compatible individuals to ensure fruit set in commercial orchards (Herrera et al. 2018). GSI can

68   also influence the selection of compatibility groups in breeding programs and seed multiplication

69   schemes (Cachi et al. 2017).

70   To our knowledge, GSI in *P. serotina* remains largely unexplored. Donovan (1969) studied

71   pollen extension and fruit-set patterns in selfings and crosses of North American *P. serotina*

72   populations. The results of this study provided support for GSI and some cross-incompatibility in

73   the species, but did not exclude the possibility for genotype-specific self-compatibility. At the

74   molecular level, Gordillo et al. (2015) investigated the allelic diversity of the S-Locus of Andean

75   *P. serotina* by evaluating the degree of polymorphic variation in the Intron-I region of the S-

76   RNase gene in 80 accessions from the Ecuadorian highlands. However, this study did not

77   evaluate if and how this molecular diversity controls GSI in the species. Therefore, the main

78   objective of this research was to study whether the *P. serotina* S-Locus controls sexual

79   incompatibility in the species. To this end, we characterized the molecular structure of the *P.*

80   *serotina* S-RNase gene in order to design an efficient molecular marker system that would enable

81   the analysis of the allelic diversity of the S-Locus in Andean capuli populations. Subsequently,

82   S-allelic information was used to set-up experimental crosses between cross-compatible and

83   cross-incompatible haplotypes in order to evaluate pollen tube-formation patterns *in-vivo*.

84   Ultimately, we aimed to demonstrate that *P. serotina* individuals from the same IG exhibit

85   obstructed fertilization; the latter would support the notion that the S-Locus influences the

86   reproductive patterns of the species.

Materials & Methods

Genetic and Plant Material

The characterization of the *P. serotina* S-RNase gene and the development of a CAPS (Cleaved Amplified Polymorphic Sequence) marker system were performed on a set of 15 *P. serotina* accessions for which S-RNase Intron-I polymorphisms had been previously described (Gordillo et al. 2015). Leaf samples for these accessions were originally collected by Guadalupe et al. (2015) from 6 provinces across the highlands of Ecuador. The geographical coordinates for the collection site of each accession are detailed in Table S1. DNA samples for all 15 accessions are stored at the Plant Biotechnology Laboratory of Universidad San Francisco de Quito (Cumbayá, Ecuador) and were used in this investigation.

For pollination assays, we used an independent set of 7 capuli trees located in a private orchard in Cayambe, Ecuador. These trees were selected because of their proximity to the Plant Biotechnology Laboratory of Universidad San Francisco de Quito. For each individual tree, genomic DNA was isolated from young leaves using the CTAB method (Xin and Chen 2006). DNA concentration and quality were assessed by spectrophotometry using a Nanodrop 2000 spectrophotomer (Thermo Scientific).

S-RNase gene amplification and DNA sequencing

The amplification of the S-RNase gene of 15 *P. serotina* accessions previously studied by Gordillo et al. (2015) was performed using the PaConsI-F (MCTTGTTCTTGSTTTYGCTTTCTTC; Sonneveld et al. 2003) and EM-PC5consRD (CAAAATACCACTTCATGTAACARC; Sutherland et al. 2004) primers. These primers respectively target the Signal Peptide and C5 regions of the *Prunus* S-RNase gene. PCR reactions consisted of 1X PCR Buffer (Invitrogen), 0.2 mM dNTPs, 1.5 mM MgCl2, 0.3 μM of

110   each primer, 1 U of Taq DNA polymerase (Invitrogen) and 40 ng of DNA in a 25 µl final

111   volume. Amplification conditions were as described by Ortega et al. (2006), but with a 58 ºC

112   annealing temperature. Amplified products were separated by gel electrophoresis in 1.5%

113   agarose gels at 80 volts for 90 minutes using TBE 1X as running buffer. DNA fragments were

114   visualized using SYBR-Safe (Invitrogen).

115   Finally, amplified products were excised from agarose gels and purified using the Wizard

116   SV Gel and PCR Clean-Up System Protocol (Promega). Purified products were subsequently

117   reamplified by PCR using the amplification conditions described above. Reamplified products

118   were sequenced at Macrogen Inc. (Seoul, Korea).

119   **Analysis of the *P. serotina* S-RNase sequences**

120   Consensus nucleotide sequences from forward and reverse read of all amplified fragments

121   were obtained using PREGAP and GAP4 (Staden 1996). The search for homologous sequences

122   in the GenBank database was performed using the NCBI BLAST tool (Altschul et al. 1990).

123   Intraspecific and interspecific alignments were performed using the CLUSTAL W algorithm of

124   MEGA7 (Kumar et al. 2016). Deduced amino acid sequences were obtained by translating the

125   coding sequence corresponding to each of the alleles using the online ExPASY Tool (Gasteiger

126   et al. 2003). Alignments of the deduced amino acid sequences were performed using the

127   CLUSTAL Omega algorithm of the European Bioinformatics Institute (Madeira et al., 2019).

128   Ka/Ks ratios calculation and the sliding window analysis with a window length of 20 codons

129   were performed using the DnaSp6 Software (Rozas and Rozas 1999). Shannon Entropy indexes

130   were calculated for each of the conserved regions (C1, C2, C3, RC4) and the RHV, using the

131   online Protein Variability Server (García-Boronat et al. 2008).

132 **Design and validation of the CAPS marker system**

133   The CAPS marker system was designed *in-silico* using the Genome Compiler Software

134   Package (Amirav-Drory et al., 2015). The digest function was employed for the screening of

135   restriction sites located within the analyzed sequences using a pool of 100 available enzymes,

136   and the gel simulation function was employed for the visualization of the restriction patterns in

137   virtual gels after the *in-silico* digestions.

138   For the *in-vitro* validation of the developed CAPS marker system, we designed species

139   specific primers to amplify the C2-C3 intergenic region of the *P. serotina* S-RNase gene:

140   Ps1C2Fw (ATY-CAT-GGC-CTR-TGG-CCA-AG) and Ps2C3Rv (TGY-TTR-TTC-CAT-TCV-

141   CBT-TCC). Primer design was based on S-RNase full-length sequences produced in this study.

142   The C2-C3 regions of the 15 *P. serotina* accessions were amplified using the Ps1C2Fw/

143   Ps2C3Rv primers. Reagents concentrations for the PCR reaction were the following: 1X PCR

144   Buffer, 3 mM MgCl2, 0.2 mM dNTPs, 0.5 µM of each primer, Taq polymerase Platinum

145   (Invitrogen) 1U and 20 ng of DNA in a 25 µl final volume. Cycling conditions consisted in: 2

146   minutes of initial denaturation at 94°C, followed by 35 cycles of 1 minute at 94 °C, 2 minutes at

147   58°C, 4 minutes at 68°C and 10 minutes of final extension at 68°C. PCR products were separated

148   by gel electrophoresis in 1.5% agarose gels. Running conditions were 80 volts for 90 minutes

149   using TBE 1X as running buffer. DNA fragments were visualized using SYBR-Safe

150   (Invitrogen). All the resulting bands were excised from the agarose gel and the DNA was

151   recovered using the Wizard® SV Gel and PCR Clean-Up System (Promega). Purified DNA was

152   reamplified using the same amplification conditions as described before, using 40 ng of DNA as

153   template. Reamplified fragments were digested with RsaI, MboI, HinfI from the Anza line of

154   ThermoFisher Scientific. The digestion reactions consisted in individual digestions containing 9

155   µl of nuclease-free water, 2 µL of digestion Buffer 10X, 1 µL of enzyme and 8 µL (<0.2 µg) of

156   the reamplified fragments. Restriction reactions were incubated at 37°C for 20 minutes.

157   Restriction patterns were visualized by gel electrophoresis in 2% agarose gels. Running

158   conditions were 80 volts for 1 hour using TBE 1X as running buffer. DNA fragments were

159   visualized using SYBR-Safe (Invitrogen).

160   **Pollination Assays: pistil staining and analysis by fluorescence microscopy**

161   To evaluate whether S-Locus allelic variation influences self-incompatibility responses in *P.*

162   *serotina*, we studied pollen tube formation and growth patterns in experimental crosses. Seven *P.*

163   *serotina* trees at flowering stage were S-haplotyped using the CAPS marker system developed in

164   this study. Based on S-haplotype results, we conducted incompatible crosses (*i.e.* self-

165   pollinations or crosses between 2 heterozygote individuals carrying the exact same S-alleles) and

166   compatible crosses (*i.e.* between heterozygotes presenting completely different S-alleles) by

167   hand pollination in the laboratory. To this end, branches with flowers at stage 59 of the BBCH

168   scale (Ramírez and Davenport 2016) were cut and transported to the Plant Biotechnology

169   Laboratory of Universidad San Francisco de Quito and placed in water with plant food. To

170   extract pollen from donor flowers, anthers were manually removed and dried for 24 hours at

171   room temperature to facilitate adequate pollen release. Collected pollen was kept at 4 °C until

172   further use. For pollination experiments, receptor flowers were emasculated and pollinated (*in-*

173   *vitro*) using a paint brush 24 hours after emasculation. Per experimental cross, 25 pistils were

174   collected 48 hours following hand pollination.

175   Subsequently, pollinated pistils were stained using the aniline blue protocol described by

176   Jefferies and Belcher (1974), and pollen tube formation and extension patterns were analyzed via

177   fluorescence microscopy. For the aniline blue staining protocol, pollinated pistils were fixed in

178   70% ethanol for 7 days. As preparation for microscopy, pistils were incubated at 62°C in 10 M

179   NaOH for 40 min to soften the tissue and to enhance staining. Softened pistils were washed in

180   distilled water and stained with aniline blue for 1 hour. Pollen tube extension patterns were

181   observed using a BX50 Fluorescent Microscope (Olympus). The Multiple Image Analysis

182   software package (Amscope) was used to obtain images of the style and for counting pollen

183   tubes. A total of 78 pistils were analyzed. All protocols were previously standardized specifically

184   for *P. serotina* subsp. *capuli*.

185   **Data analysis of pollen tubes growth assay**

186   In the style, pollen tube growth was scored as the number of pollen tubes that reached the

187   lower-third section of the style. To compare pollen tube growth and development in the different

188   type of crosses, the data was subjected to a normality test and to a Levene's test for assessing the

189   homocedasticity of the data. Subsequently, a one-way ANOVA was carried out and the means

190   were separated using the Fisher Pairwise comparisons, grouping information using the Fisher

191   LSD Method and 95% of confidence. Analysis were performed using Minitab 17 Software

192   package (2010).

Results

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

In this study, we amplified, sequenced and characterized 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 S2).

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

215   hypervariable region located between C2 and C3 (Fig. 1). Shannon entropy indices (*H*) were

216   employed to determine the degree of sequence variability for each of the gene's structural

217   regions amongst the 11 evaluated alleles. Our results indicated that the least variable regions

218   were C1, C2 and C3 (*H*=0.05, 0.049 and 0.09, respectively), while the RC4 region presented a

219   higher variability index (*H*=0.28). The C5 region was not included in the analysis as it

220   corresponded to the primer anchoring site (used for sequencing); therefore, its sequence could

221   not be retrieved. As anticipated, the most variable region was the C2-C3 intragenic region (*H*

222   =1.28), containing the RHV region. Our results also indicated that the RHV sequence was unique

223   for each of the 11 examined S-RNase alleles (Fig. 1). In addition, Ka/Ks ratios were used to

224   identify regions with a higher likelihood for the occurrence of non-synonymous mutations along

225   the S-RNase coding sequence. A sliding-window analysis of Ka/Ks ratios for the 11 examined

226   alleles showed that the highest Ka/Ks ratio (1.34) was located within the RHV region.

227   Conversely, the C2 and C3 regions showed the lowest Ka/Ks ratios (0.37 and 0.17, respectively)

228   (Fig. 2).

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

231   Given its high degree polymorphism in size and sequence, the C2-C3 intragenic region was

232   used as a target for the development of a CAPS (Cleaved Amplified Polymorphic Sequence)

233   marker system for the identification of S-RNase alleles in *P. serotina*. The CAPS marker system

234   entails the amplification of a specific DNA region followed by enzymatic restriction to generate

235   unique and distinctive restriction patterns (Shavrukov 2016).

236   The design of the CAPS marker system was performed *in-silico* using the Genome Compiler

237   Software Package. Sequences corresponding to the C2-C3 regions (isolated from full-length

238 DNA sequences of the 11 S-RNase alleles) were screened using 100 restriction enzymes. From

239 these, we identified 9 restriction enzymes with the ability to cleave all or the majority (> ~80%)

240 of the 11 sequences analyzed. Based on these results, 99 *in-silico* restrictions (11 alleles x 9

241 enzymes) were simulated and the digestion patterns were visualized using the virtual simulator

242 of Genome Compiler. These analyses demonstrated that to unequivocally identify the 11 S-

243 alleles, at least 3 restriction enzymes were necessary (RsaI, MboI, HinfI). Each of these enzymes

244 generated individual digestion patterns (Table 1) that when read simultaneously, produced a

245 unique fingerprint for each S-allele. Based on this selection of enzymes and the digestion

246 patterns, we generated a CAPS guide for the rapid identification of *P. serotina* S-RNase alleles

247 (Table 2).

248   *In-silico* results were then validated *in-vitro* by amplifying the C2-C3 region corresponding

249 to the 15 *P. serotina* accessions, followed by independent enzymatic restrictions with RsaI, MboI

250 and HinfI. While the expectation was to obtain a maximum of 14 alleles for these 15 accessions

251 (based on DNA sequence analysis), 4 additional (unexpected) bands were obtained for

252 accessions H25, Azu15, Pic19, Car7, Car5, Car3, and Car12 during the C2-C3 amplification

253 (Fig. 3). After the enzymatic digestion, the restriction patterns of the already characterized alleles

254 matched with the *in-silico* predictions. Digestion patterns of the 4 unexpected bands did not

255 match with any of our *in-silico* predictions, therefore we presumed that these bands corresponded

256 to new uncharacterized S-alleles, which were designated as S_{15}-S_{18}. CAPS restriction patterns

257 corresponding to the S_1-S_{18} alleles are presented in Fig. 4.

258

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

260   To evaluate whether S-Locus allelic variation influences self-incompatibility responses in *P.*

261 *serotina*, we studied pollen tube formation and growth patterns in pollination experiments. To

262 this end, we selected 7 *P. serotina* tress from a private orchard in Cayambe (Ecuador) based on

263 their S-allelic composition (as revealed with our CAPS marker system). The 7 individuals, which

264 were in full bloom and exhibited flowers at stage 59 of the BBHC scale, harbored 9 distinct S-

265 alleles. Six of these alleles had been previously characterized (S_1, S_4, S_6, S_8, S_9, S_{10}), but the

266 remaining 3 patterns corresponded to new uncharacterized alleles; the latter were designated as

267 S_{19} - S_{21} (Table 3).

268   Based on these results, 2 types of crosses were established: incompatible crosses (*i.e.* self-

269 pollinations or crosses between 2 heterozygote individuals carrying the exact same S-alleles) and

270 compatible crosses (*i.e.* between heterozygote individuals presenting completely different S-

271 alleles). Crossing assays are summarized in Table 4. To evaluate incompatible and compatible

272 responses, we analyzed pollen tube extension patterns via fluorescence microscopy. These

273 results clearly indicated that pollen tubes develop differently depending on the type of cross (Fig.

274 5). In compatible crosses, we found pollen tubes that reached the ovary in all pollinated pistils

275 (Fig. 5A). By contrast, in the pistils of incompatible crosses, pollen tube growth was typically

276 inhibited in the upper- and middle-third sections of the style (Fig. 5B). Furthermore, pollen tubes

277 in incompatible crosses showed swelling at the tip (Fig. 5C) – this morphological alteration was

278 not observed in compatible crosses. For specific incompatible crosses, several pollinated pistils

279 displayed a variable number of pollen tubes that reached the bottom-third section of the style, but

280 in consistently lower proportions relative to compatible crosses (Table S3). The occurrence of

281 this phenomenon was especially common in crosses *22x17* and *17x17*. It is important to

282 highlight, nevertheless, that pollen tubes that did reach the lower-third segment of the style in

283   incompatible crosses always showed swollen tips. A one-way analysis of variance confirmed

284   that differences in pollen tube growth (based on arrested development vs. full development to

285   reach the ovary) between incompatible and compatible crosses were statistically significant at

286    $p<0.001$.

Discussion

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

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

Detailed analysis of the amino acid sequences of 11 *P. serotina* S-RNase alleles confirms that the *P. serotina* S-RNase gene has a high degree of amino-acid sequence homology (~81% to ~99%) with stylar ribonucleases from related species *Prunus* (Ushijima et al. 1998; Yaegaki et al. 2001; Vaughan et al. 2008; Gao et al. 2012). For instance, the *P. serotina* S_8 and *P. webbii* S_3 (NCBI: ABY19368) alleles displayed a protein identity of ~99% (Fig. S2). A high degree of interspecific amino acid sequence homology between S-RNase alleles in *Prunus* has been reported earlier (Banovic et al. 2009; Ortega et al. 2006; Surbanovski et al. 2007). These findings support the hypothesis that *Prunus* S-alleles with very high interspecific identities (>99%) may have originated from a common ancestor before speciation, and that these alleles have been conserved ever since in the derived lineages (Ortega et al. 2006; Surbanovski et al. 2007; Sassa et al. 1996).

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

To investigate GSI-controlled incompatibility patterns in *P. serotina*, we required an effective marker system that would enable the unequivocal identification of S-RNase allelic diversity. The standard methodology for S-alleles identification in *Prunus* species involves the 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

310   methodology often fails to discriminate between allelic variants exhibiting similar amplicon

311   length but distinct genetic sequences (Halász et al. 2008; Gordillo et al. 2015; Kodad et al. 2008;

312   López et al. 2004).

313   To address this limitation, we developed a CAPS molecular marker system designed to

314   identify sequence-level polymorphisms in the S-RNase C2-C3 intragenic region. This region was

315   selected as it includes the RHV sequence; the latter presumed to mediate the recognition of

316   genetically related S-RNase/SFB complexes (Matton et al. 1997; Ushijima et al. 1998). The

317   RHV region is located at the surface of the S-RNase protein and is thought to play a key role in

318   the recognition of self- and non self-pollen (Matton et al. 1997; Ushijima et al. 1998); it is

319   therefore considered an adequate proxy of the self-incompatibility response in *P. serotina*.

320   Furthermore, our results indicated that the C2-C3 intragenic region represents the most

321   polymorphic segment of the *P. serotina* S-RNase gene. In fact, Shannon entropy indices and

322   Ka/Ks ratios revealed that the highest degree of amino acid sequence variation in the gene

323   concentrates in the RHV region.

324   The CAPS marker system designed in this study proved highly effective in the identification

325   of S-RNase alleles. When employed to genotype an initial core-set of 15 *P. serotina* accessions

326   representing a comprehensive sample of the species' S-RNase allelic diversity in the Ecuadorian

327   highlands (Gordillo et al. 2015), the system enabled the unequivocal identification of 18 S-

328   RNase alleles. From these, seven alleles (S_{12}-S_{18}) could not be identified when using the Intron I

329   molecular marker system (Gordillo et al. 2015), and four (S_{15}-S_{18}) could not be detected when

330   amplifying, extracting and sequencing the full-length S-RNase genes for the aforementioned 15

331   individuals. Furthermore, an additional three alleles (S_{19}-S_{21}) were identified when characterizing

332   a set of 7 individuals used for pollen-tube formation analyses. The effectiveness of our CAPS

333   marker system in the determination of S-RNase diversity can be ascribed to three important

334   factors. In the first place, the use of species-specific primers to amplify the C2-C3 intragenic

335   region allowed for a higher resolution and differentiation of length-based allelic variants. Size

336   differences in the C2-C3 intragenic region are attributable to differences in the lengths of the

337   Intron II and RHV sequences (i.e. the most polymorphic regions of the S-RNase gene) which are

338   easier to resolve via gel electrophoresis as low-molecular weight products. By contrast, the

339   amplification of the full-length S-RNases yield high-molecular weight amplicons of similar size

340   which are not easily isolated. The inability to purify these amplicons independently may lead to

341   the masking-off of allelic differences resulting in allele-misidentification during sequencing (*i.e.*

342   pooling of two or more allelic variants yields one consensus sequence as seen in this study). In

343   second place, the use of 3 restriction enzymes is a powerful tool to exploit and elucidate the

344   intrinsic, polymorphic nature of the C2-C3 intragenic region. This feature is especially useful

345   when two S-alleles exhibit similar amplicon lengths (*i.e.* gel electrophoresis does not allow the

346   clear resolution of size-differences of a few base pairs). Accordingly, it offers an opportunity to

347   identify heterozygotes when an individual's S-alleles are similar in sequence length (Moriya et

348   al. 2007). Finally, the *in-silico* design of our CAPS molecular system allowed us the possibility

349   to simulate multiple digestions in order to identify the most informative restriction enzymes. The

350   efficiency of our 3 enzyme CAPS marker system is evident when comparing it to CAPS systems

351   developed for haplotyping the S-Locus in other species from the Rosacea family. For instance,

352   for the identification of 17 S-alleles in European pear cultivars it was necessary to employ 11

353   different enzymes (Moriya et al. 2007), whereas for the identification of 22 S-alleles in Japanese

354   apple cultivars, 17 different enzymes were required (Kim et al. 2008).

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

356   As evidenced in this study, the S-RNase of *P. serotina* shares a high degree of identity with

357   S-RNases from related species for which the S-Locus has been reported to control GSI. This

358   finding would suggest that the S-Locus also plays a crucial role in determining the reproductive

359   patterns of *P. serotina*. To add evidence to this theory, we investigated pollen-tube formation in

360   crosses between compatible and incompatible individuals (as defined by their S-allelic

361   composition via CAPS).

362   Our results demonstrated that crosses between heterozygote individuals with contrasting S-

363   alleles result in normal pollen tube formation and growth (*i.e.* pollen tubes extend along the style

364   and reach the ovary). In these crosses, the number of pollen tubes reaching the ovary was

365   abundant. By contrast, in pistils from crosses between heterozygote individuals with exactly

366   similar S-allele identities (including self-pollinations), the vast majority of pollen tubes showed

367   morphological alterations (*i.e.* swelling of pollen tube tips) and arrested development (Fig. 5). In

368   a number of incompatible crosses, a variable number of pollen tubes could reach the lower-third

369   section of the style, albeit at a comparatively lower density than in compatible crosses. These

370   pollen tubes also showed swollen tips, a morphological alteration that is caracteristic of aberrant

371   pollen tubes (Oukabli et al. 2000; Ludwig et al. 2013; Cachi et al. 2014; Estaji et al. 2016;

372   Radunić et al. 2017). For two specific incompatible crosses (*i.e. 22x17* and 17x17), however, a

373   substantial number of pollen tubes could reach the ovary. This unexpected result could be

374   evidence of a full or partial breakdown of the *P. serotina* GSI system.

375   The breakdown of GSI is not an uncommon phenomenon in *Prunus* (Yamane et al. 2001;

376   Hauck et al. 2002; Tobutt et al. 2004; Zarrinbal et al. 2018; Guerra et al. 2019). In fact, in a

377   previous investigation of GSI in *P. serotina*, Donovan (1969) reported that selfings of specific

378   genotypes led to normal fertilization, embryo formation and fruit set. Donovan (1969) concluded

379   that the incompatibility reaction in *P. serotina* was not as strong as in other members of the

380   genus, and the author could not rule out self-compatibility in the species. In other *Prunus*

381   species, the partial or full loss of self-incompatibility has been primarily ascribed to the presence

382   of non-functional S-haplotypes caused by mutations in the S-RNase (Hauck et al.2002; Hauck et

383   al. 2006; Li et al. 2020) or SFB genes (Wünsch and Hormaza 2004; Tao et al. 2007; Wu et al.

384   2013; Muñoz-Espinoza et al.2017) that lead to loss of functionality. In this study, we have

385   focused exclusively on investigating the phenotypic behavior of pollen tube formation patterns in

386   compatible and incompatible crosses using S-RNase diversity as proxy for S-Locus identity.

387   However, GSI in *Prunus* is attributed to the interaction and recognition of self/non-self S-

388   RNase/SFB complexes (Matsumoto and Tao 2016), and a clearer understanding of GSI and self-

389   compatibility in *P. serotina* will also require a profound investigation of SFB diversity.

390   Furthermore, a concise understanding of the extent of GSI in *P. serotina*, and the extent to which

391   the S-Locus influences the reproductive patterns of the species, will also need to explored and

392   validated with fertilization and fruit set experiments *in vivo*.

Conclusions

393

394   In this study, our objective was to investigate if the *P. serotina* S-Locus controls

395   gemetophytic sexual incompatibility (GSI) in the species. To this end, we characterized the

396   molecular structure of the *P. serotina* S-RNase gene and developed a CAPS molecular marker

397   system to unequivocally determine S-RNase alleles. Experimental crosses between cross-

398   compatible and cross-incompatible S-haplotypes (as determined by CAPS) were then used to

399   evaluate pollen tube-formation patterns *in-vivo*. Our results demonstrate that crosses between

400   heterozygote individuals with contrasting S-haplotypes result in normal pollen tube formation

401   and growth (i.e. pollen tubes extend along the style and reach the ovary). Accordingly, crosses

402   between individuals with the same S-haplotype typically lead to the morphological alteration of

403   pollen tubes and arrested development during fertilization. Notwithstanding, we also report that

404   some (suspected) incompatible crosses, pollen tubes can reach the ovary. The latter hints at the

405   possibility of genotype-specific breakdown of GSI in the species.

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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.

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		

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).

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

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.

	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

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.

Pollen donor		Pollen Receptor	Expected phenotype	Observed phenotype
22 (S_1, S_4)	X	17 (S_1, S_4)	Incompatible	Undetermined
12 (S_4, S_9)	X	13 (S_4, S_9)	Incompatible	Incompatible
17 (S_1, S_4)	X	1 (S_8, S_{19})	Compatible	Compatible
14 (S_{20}, S_{21})	X	15 (S_{10}, S_6)	Compatible	Compatible
17 (S_1, S_4)	X	17 (S_1, S_4)	Incompatible	Undetermined
13 (S_4, S_9)	X	13 (S_4, S_9)	Incompatible	Incompatible
1 (S_8, S_{19})	X	1 (S_8, S_{19})	Incompatible	Incompatible
15 (S_{10}, S_6)	X	15 (S_{10}, S_6)	Incompatible	Incompatible

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. 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)
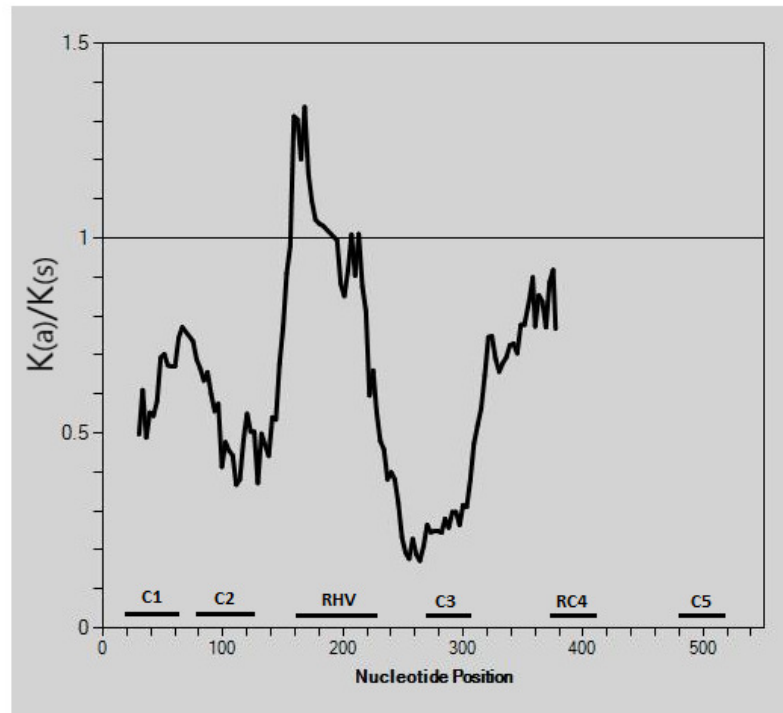
Figure 3

PCR amplification of the *P. serotina* C2-C3 intragenic region using the Ps1C2Fw and Ps2C3Rv 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
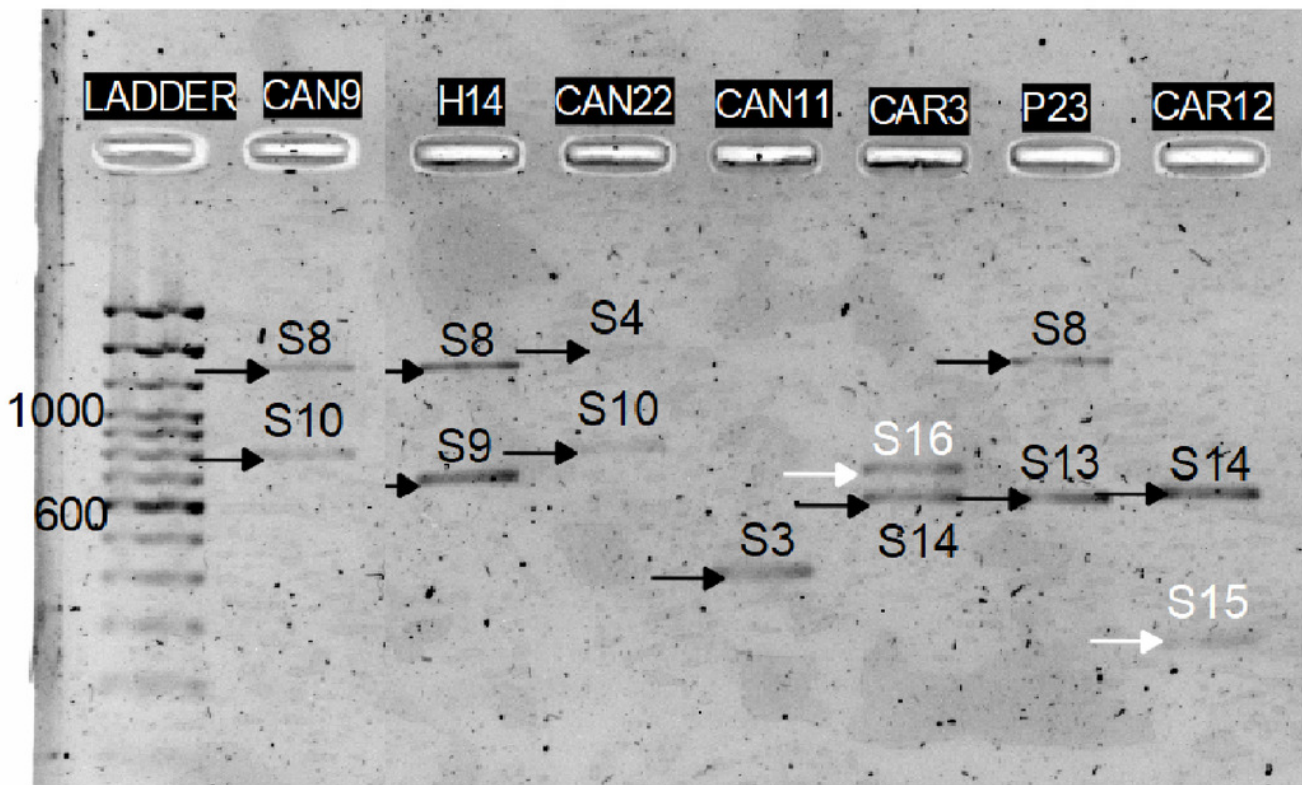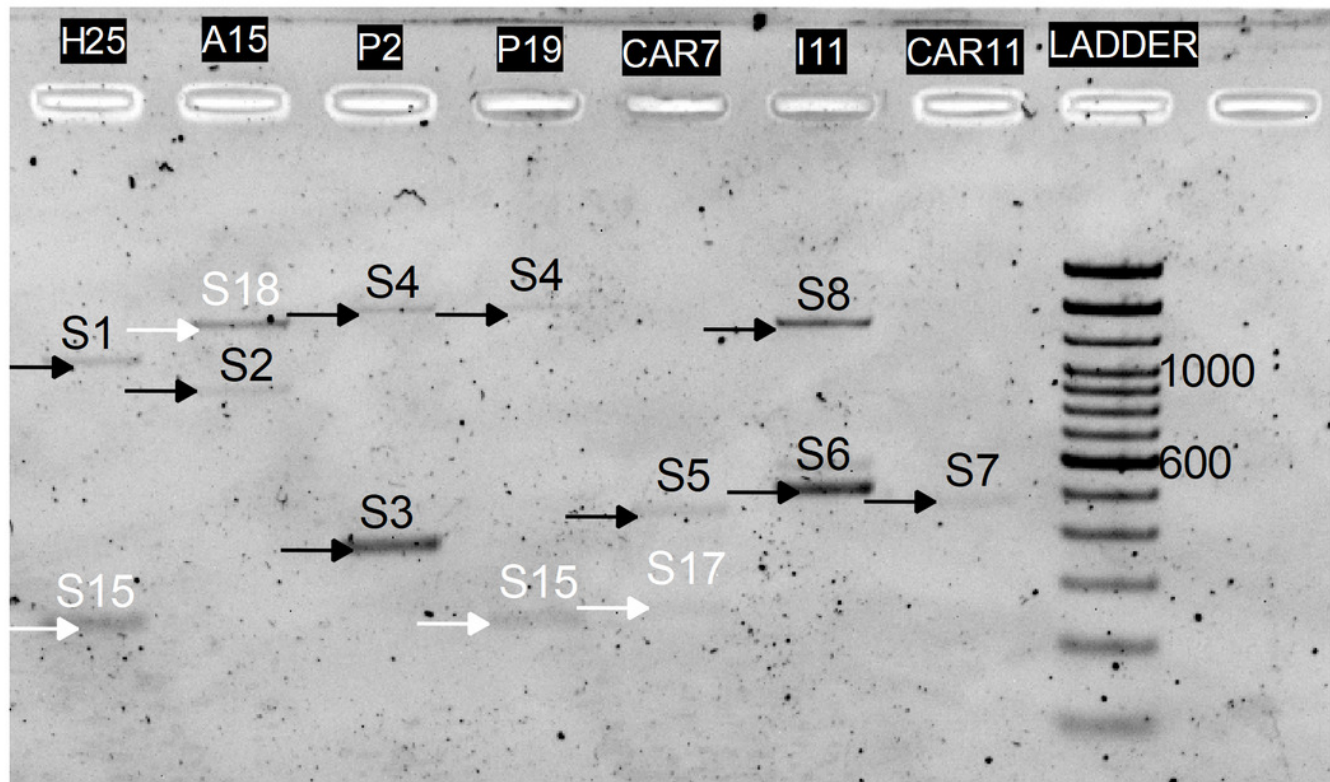
Figure 4

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}, S[sub]16

Letters indicate the restriction patterns reported for each enzyme in Table 1: RsaI, MboI and HinfI, respectively
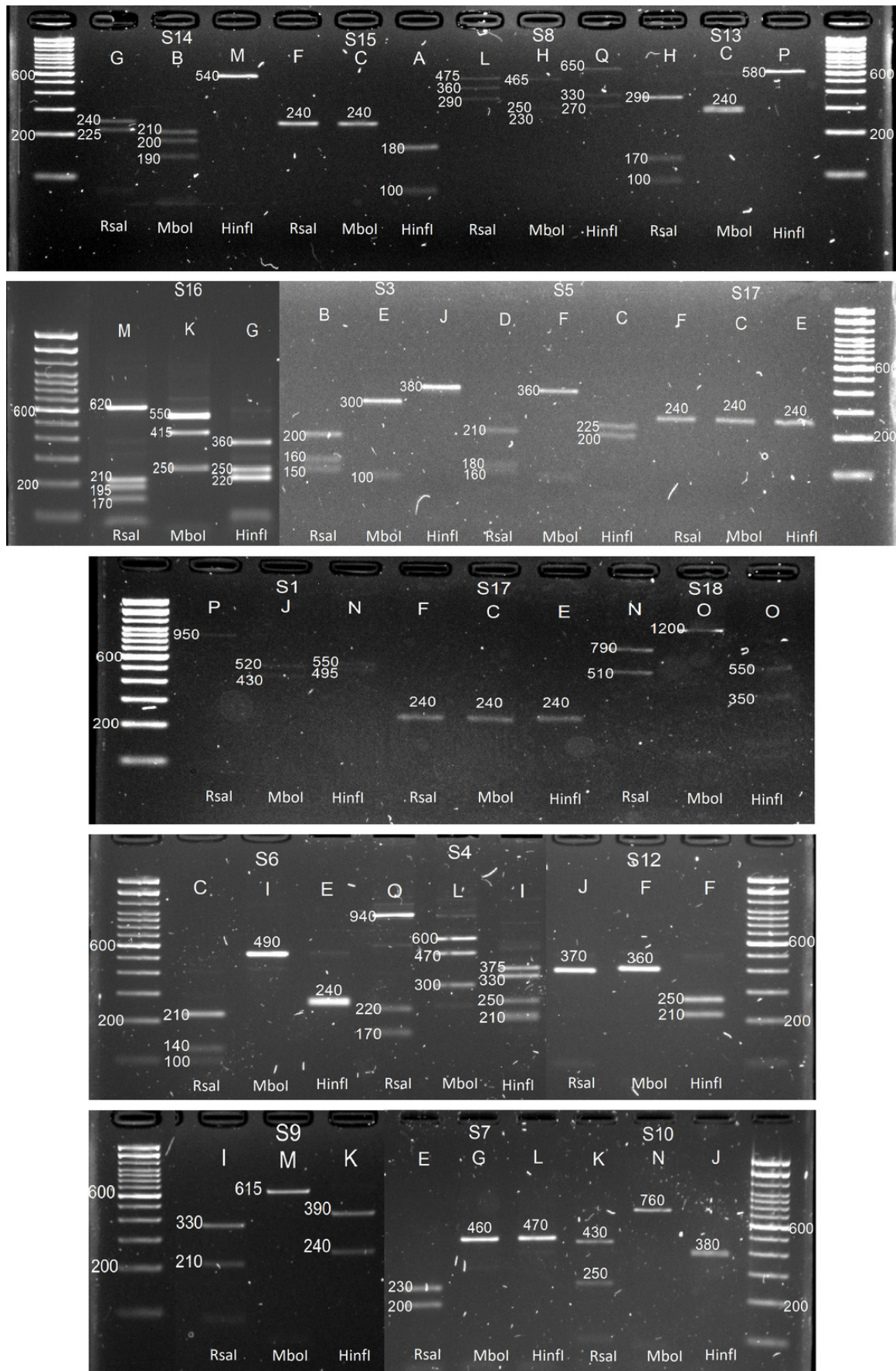
Figure 5

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) 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. Pollen tubes growth was inhibited in the upper-third section of the style showing swollen tips. B) Incompatible cross: Self-pollination of Individual 1 (S_8 – S_{19}). Pollen tubes growth was inhibited in the upper-third section of the style. C) Swollen tips of Self-pollination of Individual 13 (S_4 – S_9)