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Identification and Characterization of *S-RNases* in Japanese Plum Genotypes

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Abstract

Many species of the genus *Prunus* exhibit the gametophytic self-incompatibility system, governed by the *S-locus*, that encode *S-Ribonucleases* in the pistil, and are able to degrade the RNA of the pollen tubes when the *S-haplotypes* of both gametophytes are the same, preventing the fertilization of the oosphere. The objective of this study was to identify and characterize the *S-alleles* of Japanese Plum genotypes to verify groups of reproductive compatibility. Isolation, amplification and DNA sequencing were performed to obtain an allelic profile of the genotypes. Combinations of PC1 and PC2 primers identified 95% of the genotypes. After sequencing, the 'S^c', 'S^i', 'S^a' and 'S^h' alleles were obtained with an identity greater than 90%, compared to the NCBI sequences. PC2 was more extensive in identifying the *S-alleles* in the *S-RNases* coding region, generating larger fragments than PC1. In this way, it was possible to generate three groups of genotypes with *S-alleles* of the same size with PC2: Group 1 (Selection Embrapa A19, Selection Embrapa A28, Black Amber Black, Fortune, Roysum, SC-7 and Zafira); and Group 2: (Selection Embrapa A7, Carazinho, Sanguínea, Laroda, SC-15 and Rebelatto) and Group 3 (Selection Ameixa 86-13, Golden King, Letícia and Robusto). Each primer combination amplified only one allele per genotype, suggesting the development of specific primers to amplify the *S-alleles* in each genotype. The identification and characterization of the alleles allow the use of genotypes compatible with each other, considering the floral synchrony of the genotypes, besides providing information for the management of the breeding processes in Japanese Plums.

Keywords: plant reproduction, genetics, Rosaceae, *Prunus salicina* Lindl

1. Introduction

Sexual reproduction is an important aspect achieved by the Plant Kingdom. During this process, evolutionary mechanisms involving the union of half the genome of both parents, through sexual gametes [haploid (n) cells] and genetic recombination, allows the generation of segregating individuals, which is an important characteristic for species survival in different environmental pressures. Moreover, the use of reproductive structures (male and female gametophytes) favored genetic diversity when compared with asexual reproduction (Gonçalves & Lorenzi, 2011).

Vegetables developed two strategies of sexual reproduction: autogamy and allogamy. Autogamous plants have developed the ability to self-pollinate and allogamous plants evolved the reproductive process for the need of cross-pollination. This strategy allowed for the development of sexual self-incompatibility (sporophytic and gametophytic) to favor genetic variability, avoiding inbreeding (De Nettancourt, 2001; Schifino-Wittmann & Dall'agnol, 2002).

Sporophytic Self-Incompatibility (SSI) can be observed for example in the family Passifloraceae (Madureira, 2009) and Brassicaceae (Havlícková et al., 2014). The specificity in SSI regarding the rejection of the male gametophyte in pollination depends on the maternal tissue, the carpet [diploid (2n) tissue covering the pollen grain]. Pollen deposited on stigma carpet comes in contact with their cells, signaling for rejection or acceptance of the pollen tube growth. Enzymes contained in the pistil will prevent the growth of the pollen tubes if they have the same genetic characteristics of the pistil (Schifino-Wittmann & Dall'agnol, 2002).

The system of Gametophytic Self-Incompatibility (GSI) can be found in the families Solanaceae, Scrophulariaceae, and Rosaceae, and is characterized by the recognition of the pistil cells when they come in contact with pollen grains (n), cells or pollen tubes. Moreover, the enzymes produced during GSI interaction degrade the cells of the male gametophytes (Aguiar et al., 2015). Furthermore, the enzymes or female haplotype that causes cytotoxicity and disrupts the growth of pollen tubes are called *S-ribonucleases* enzymes (*S-RNases*) and are produced from the expression of genes involved in the sterility of sex cells in many plant species.

RNases are composed of N-glycosylated protein sites and histidine residues (conserved amino acid blocks) that act as a peptide signal (Matsuura et al., 2001; Mcclure et al., 2011), and due to the active site CAS I and CAS II, they are able to cleave single strand RNA (substrate) (Luhtala & Parker, 2010) carrying out a role of self-pollen rejection as well as defense against the growth of infectious structures (Mittler et al., 2011).

Under favorable conditions of temperature and humidity, pollen tubes grow within the stigma, and a range of biochemical interactions take place between the pollen tubes and the cells of female tissue. In compatible interactions, RNases act on the cellular apoplastic, especially on the stigma papillae, and they are massively absorbed by the clathrin vesicles of the pollen tube (Williams, Wu, Li, Sun, & Kao, 2015). When there is a positive interaction for reproduction, F-box proteins (SFB) contained in the male gametophyte facilitate the fertilization of the oosphere. The F-box proteins degrade RNases by ubiquitination via 26 S proteasomes and favor the growth of the pollen tube (Zang et al., 2007). Both RNases and F-Box are the product of the expression of GSI genes contained in a single multi-allelic gene locus called *S-alleles* or Sterility loci encoding the female *S-haplotype* (*S-RNase*) and the male *S-haplotype* (*S-Fbox*) (Hua et al., 2008; Williams et al., 2015).

The female *S-haplotype* encodes the RNases present conserved regions (C): (C1, C2, C3, C4, and C5). Moreover, between C2 and C3 there is a common in Rosaceae Hypervariable region (RHV) and there are two histidine residues responsible for the enzymatic activity and degradation of RNA from pollen grains (Takayama & Isogai, 2005). In Rosaceae, the conserved regions have been investigated for the identification of genes encoding RNases and for polymorphism of the allelic series in the *S-locus* within the species (Ushijima et al., 1998).

The Prunoideae tribe, which belongs to the Rosaceae family, share the GSI trait and several studies have identified the RNases in *Prunus dulcis* (López et al., 2004) and *Prunus salicina* (Carrasco et al., 2012). These studies have intensified in recent years, highlighting the importance and confirmation of *S-haplotypes* in reproduction. Genotypes with desired agronomic characteristics have been investigated to access the breeding capabilities and to select self-fertility genotypes or uncover alleles that are possibly associated with self-fertility (Marchese et al., 2008; Fernández i Martí et al., 2014).

The identification and characterization of the allelic series (*S-alleles*), which is named 'S^1', 'S^2', 'S^3', 'S^4', and 'S^5' or 'S^a', 'S^h', 'S^i', 'S^c', and 'S^e', that encode the RNases in the stigma of the Japanese Plum cultivars, can help to manage orchards, since the cultivation of genotypes with different alleles may increase fertilization and effective fruit set (Ushijima et al., 1998; De Nettancourt, 2001; Kondo et al., 2002; De Conti et al., 2013; Sassa, 2016). Molecular studies to understand the reproductive process in *Prunus* species are focused on the identification and characterization of *S-RNases* genes through tools like Polymerase Chain Reaction (PCR) and DNA sequencing.

The aim of this study was to identify and characterize the allelic series of the *S-RNases* for *Prunus salicina* Lindl. cultivars to verify the possible reproductive (in)compatibility among genotypes under study.

2. Material and Methods

2.1 Plant Material for Total DNA Isolation

Twenty genotypes of *P. salicina* were used for *S-RNases* analysis. Young, expanded and healthy leaves were collected from Japanese Plum genotypes: 'Selection Embrapa A19', 'Selection Embrapa A28', 'Selection Embrapa A7', 'Black Amber', 'Carazinho', 'Sanguínea', 'Bruce', 'Laroda', 'Piamontesa', 'Selection Ameixa 86-13' (SA 86-13), 'Golden King', 'Chatard', 'Letícia', 'Robusto', 'Fortune', 'Roysum', 'SC-15', 'SC-7', 'Zafira' and 'Rebelatto'. The plant material was collected from the EPAGRI collection-Videira Experimental Station (Videira, Santa Catarina-Brazil).

The DNA was extracted according to the method proposed by Doyle & Doyle (1991). DNA from the samples was quantified in NanoVue® spectrophotometer to verify purity and concentration. After, we used 1% agarose gel electrophoresis to confer band integrity. Moreover, the gel was stained with ethidium bromide and visualized under UV light on the Vilber Lourmat® E-Box-1000 transilluminator.

2.2 Polymerase Chain Reaction (PCR) for S-allele Amplification

For the PCR reactions, 20 ng of DNA was taken from each sample and PROMEGA® kit reagents were used in the following concentrations: GoTaq (1.25U Taq DNA polymerase), 1x PCR Buffer (25 mM Tris-HCl, pH 9.0), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.4 μM of each primer (Table 1), and Milli-Q water to bring the final volume to 25 μl. The thermocycler (Biocycler MG96G) was used to amplify DNA at an initial temperature of 94 °C for 5 minutes, followed by 40 cycles of 94 °C for 1 minute, 59 °C for 2 minutes, at 72 °C for 3 minutes, and a final cycle of 72 °C for 10 min. The amplified PCR product was subjected to 1.5% agarose gel electrophoresis at 5 V cm^{-1}, and we used the DNA ladder 100 bp as a molecular marker (Ludwig®), and the samples were stained with ethidium bromide and visualized under UV light.

Table 1. Primers used in the amplification of *S-RNases* alleles by PCR of 20 genotypes of *Prunus salicina*

PRIMERS*	**SEQUENCE (5'→3')**	**T (°C)**	**REFERENCE**
PRU-C2	F: CTATGGCCAAGTAATTATTCAAACC		Tao et al. (1999)
PCE-R	R: TGT TTG TTC CAT TCG CYT TCC C	59	Yamane et al. (2001)
PRU-C5	R: TACCACTTCATGTAACAACTGAG		Tao et al. (1999)

Note. *Primer Combination: PC1 = PRU-C2 + PCE-R; PC2 = PRU-C2 + PRU-C5; F: forward; R: reverse.

2.3 Sequencing of S-RNases From the PCR Product and in Silico Analysis

The PCR products (bands) were purified with Thermo Scientific® Gene JET PCR Purification kit. The DNA samples were standardized with concentrations of 50 ng ul^{-1} and sent for sequencing. Sanger sequencing method was performed using the Genetic Analyzer 3500 apparatus (Applied Biosystems, USA) from the Laboratório de Genômica Estrutural of UFPEL.

Sequencing result analyzes were run *in silico* to investigate the quality of the bases, and to remove the low-quality nucleotides the Chromas® software was used. The data were aligned in Clustal X2 software (Thompson et al., 1997) to observe consensus sequences. Finally, the sequences were compared in the database of the National Center for Biotechnology Information (NCBI) with nucleotide sequences already deposited through the tool BLASTN.

3. Results

3.1 PCR Analysis and Identification of Female S-haplotypes of Japanese Plum Genotypes

DNA amplification in Japanese Plum genotypes showed differences in the sizes of the fragments (alleles) produced from PRU-C2+PCE-R (PC1) and PRU-C2+PRU-C5 (PC2) primer combinations. These primers were designed from the conserved regions (C2 through C5) and were used in many species of the genus *Prunus* to present the genome amplification capacity of the conserved region of *S-RNases* and RHV (Tao et al., 1999).

The combination of PRU-C2+PCE-R (PC1) primers allowed the amplification of *S-RNases* alleles in 95% of the analyzed cultivars, except for 'Bruce'. The selections and cultivars evaluated presented alleles or bands with fragment sizes varying between 250 and 1200 base pairs (bp). It was verified that most of the genotypes amplified only one allele, except the 'Selection Embrapa A7' and 'Carazinho', which presented two alleles each (300 and 250 bp, 400 and 300 bp, respectively). The genotypes 'Embrapa A19', 'Embrapa A28', and cultivars Fortune, SC-7, Zafira, and Black Amber presented 1200 bp bands. The genotypes SA 86-13, Golden King, Letícia, Robusto and Piamontesa presented bands with approximately 400 bp and Chatard had shown 350 bp. The cultivars Sanguínea, Laroda, SC-15, SC-7, and Rebelatto showed allele with 300 bp (Figure 1).

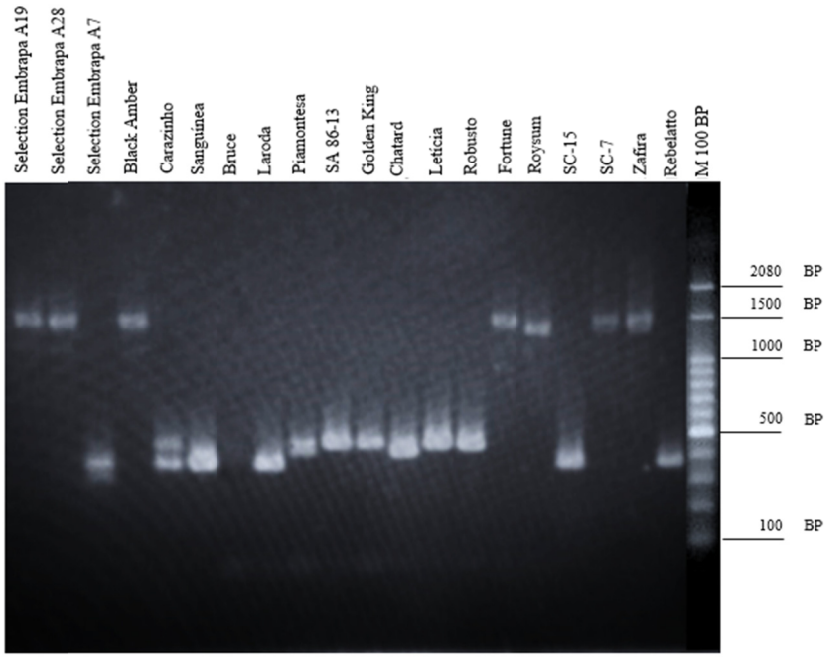

Figure 1. Identification of *S-RNases* through PC1 (PRU-C2 + PCE-R) used in Japanese Plum genotypes; M: 100 bp Marker

The size of the amplified alleles varied between 1300 and 600 bp with the PRU-C2+PRU-C5 primers, obtaining an amplification of 95% of the individuals evaluated. The absence of bands in the gel continued to be observed in 'Bruce'. The *S-RNase* allele amplification pattern with approximately 1300 bp was observed in the 'Selection Embrapa A19', 'Selection Embrapa A28', 'Fortune', 'Black Amber', 'Roysum', 'SC-7' and 'Zafira'. The genotypes 'SA 86-13', 'Golden King', 'Letícia' and 'Robusto' presented alleles with 700 bp. 'Piamontesa' and 'Chatard' presented alleles of 650 bp, and the allele with 600 bp were observed in 'Selection Embrapa A7', 'Carazinho', 'Sanguínea', 'Laroda' and 'SC-15' and 'Rebelatto' (Figure 2).

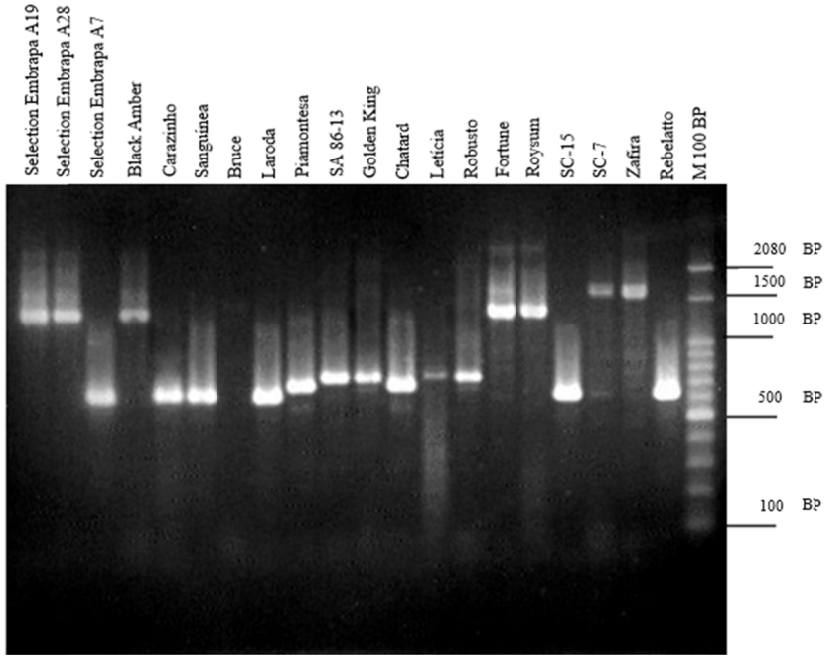

Figure 2. Identification of *S-RNases* alleles through PC2 (PRU-C2 + PRU-C5) used in Japanese Plum genotypes. M: 100 bp Marker

We have found that groups of *S-alleles* were formed based on the size of the amplified DNA fragments within the female *S-haplotypes* in the Plum genotypes (Table 2).

Table 2. Groups of Japanese Plum genotypes based on the size of the amplified fragments of the *S-RNases* for the combinations of primers 1 and 2 (PC1 and PC2, respectively)

Genotypes groups formed from the size of the *S-alleles*					
Groups formed from PC1			Groups formed from PC2		
G1	**G2**	**G3**	**G1**	**G2**	**G3**
Selection Embrapa A19	Sanguínea	Piamontesa	Selection Embrapa A19	Selection Embrapa A7	SA 86-13
Selection Embrapa A28	Laroda	SA 86-13	Selection Embrapa A28	Carazinho	Golden King
Black Amber	Rebelatto	Golden King	Black Amber	Sanguínea	Letícia
SC-7	*	Letícia	Fortune	Laroda	Robusto
Zafira	*	Robusto	Roysum	SC-15	*
*	*	*	*	Rebelatto	*

3.2 Analysis of the Sequencing of S-RNases of Japanese Plum Genotypes

Data obtained *in silico* allowed for the characterization of 15 genotypes and at least five *S-RNases* alleles: 'S^c' or 'S^4', 'S^i', 'S^a' and 'S^h' of the species *Prunus salicina* L. The 'S^c' presented 100% and 99% identity when the PC1 combination was used for the 'Selection Embrapa A19' and 'Selection Embrapa A28', respectively. When the combination PC2 was used, only the 'Selection Embrapa A28' showed 100% identity with the 'S^c' allele (Table A1).

The identity of the 'S^c' allele deposited in the NCBI was found in the cultivars Black Amber, Fortune, and Roysum. With the PC1 combination, the percentages of identity of the 'S^c' allele identified and compared to the NCBI sequences were 96%, 94% and 98% in Black Amber, Fortune, and Roysum. Using the PC2 combination, Black Amber had the lowest percentage of identity (94%) with the 'S^c' allele compared to Fortune, and Roysum, with 99% and 97% identity with the 'S^c' allele, respectively (Table A1).

The cultivars Sanguínea, Laroda, SC-15, and Rebelatto presented common alleles called 'S^i' and their percentage of identity was 99%, 99%, 98%, and 96%, respectively when PC1 was used. of alleles amplified with PC2, the percentage of identity was lower than the alleles obtained with PC1 with values of 95%, 94%, 94% and 97% in the analyzed genotypes.

While comparing the data obtained in the present research and NCBI, the 'S^h' allele was identified in 'SA 86-13', 'Golden King', 'Letícia' and 'Robusto'. Using the combination of primers PC1 and PC2, the identity values were 96%, 95%, 97%, 98%, and 100%, 97%, 98%, 99%, respectively. Sequence analysis of the Piamontesa and Chatard genotypes showed that they had nucleotide sequences identical to the 'S^a' allele with 99% and 93% identity, respectively, when using PC1 and 99% identity in both cultivars using PC2. For all results obtained from the sequencing (Table A1), it was observed that the e-value was zero or close to zero, confirming the degree of identity and significance of the data obtained in comparison with the sequences already deposited in the NCBI.

Table 3. Groups based on the sequencing of *S-RNases* from PC2 in Japanese Plum genotypes

Genotypes groups with the same *S-alleles* based on PC2		
G1-Allele S^c	G2-Allele S^i	G3-Allele S^h
Selection Embrapa A19	Sanguínea	SA 86-13
Selection Embrapa A28	Laroda	Golden King
Black Amber	SC-15	Letícia
Fortune	Rebelatto	Robusto
Roysum	*	*

4. Discussion

4.1 PCR Analysis and Identification of S-RNases in Japanese Plum Genotypes

The PCR products generated from the Primers Combination 2 (PC2) were larger than those obtained with Primers Combination 1 (PC1) in all genotypes. PC1 amplified DNA fragments between the C2 and C3 regions (Yamane et al., 2001), and the PC2 anchors the C2 and C5 conserved regions, thus justifying the difference among DNA fragments of the female *S-haplotypes* identified in the study.

Gu et al. (2015) obtained an amplification pattern for the conserved regions of the *S-RNases* alleles similar to ours when they used the combinations of primers [PMT2 + PCE-R and PRU-C2 + Pp-SR]. The authors observed that the largest DNA fragments were produced from the combination that covered the C1 to C5 regions and the smallest fragments were observed covering the C1 to C3 regions. The PRU-C2 + Pp-SR primers showed a larger fragment amplified covering the region that coded for the *S-RNase* in both the *Prunus* and *Malus* genus, as a function of specificity and the capacity to amplified the *S-alleles* linked to GSI in this genus.

The amplification of *S-haplotype* alleles for *P. salicina* may present different results among the cultivars when using different Primers Combination (PC), as shown in this study. 'Bruce', for example, did not have the female *S-haplotypes* alleles amplified with the two PC. This condition may be associated with the specificity of the primers and the lack of complementarity among primers sequence with the nucleotides that coding for *S-RNases* in this genotype, because the primers were not specifically designed for *P. salicina* genome but based on the *Prunus cerasus* (Yamane et al., 2001) and *Prunus avium S-allele* sequences (Tao et al., 1999). In addition, most of the genotypes amplified a single band, according to the electrophoresis analysis (Figure 1 and 2), reinforcing the idea that the specificity of the primers is restricted for some species and genotypes of the genus *Prunus*.

Studying the characteristics of GSI, Beppu et al. (2005) observed that at least two bands were amplified in cultivars of *P. salicina*, confirming with Guerra et al. (2010) that they identified the 'S^cS^b' alleles in the cultivars Black Amber, Fortune and Laroda and the 'S^eS^h' alleles in 'Laettitia' (called 'Letícia' in this research). However, in our research we could not identify the 'S^b' allele in Black Amber, Fortune and Laroda, nor the Allele 'S^e' in Letícia, when we performed the characterization of these same cultivars in the present study. Let us state that it is necessary to develop specific primers for Japanese plum *S-haplotypes* grown in brazilian orchards. It is important to highlight that the expression of genes is conditioned to internal factors such as phytosanitary status, metabolism and nutrition, and external factors such as temperature, humidity and location of genotypes, where they are inserted as generators and influences of responses such as the present research, not characterizing the second allele in Japanese plum genotypes.

Mota et al. (2010) obtained an allelic profile similar to the present study when they tested eleven Japanese plum cultivars with the same combinations of primers used in our research. However, they observed that the cultivars Pluma 7, The First and two distinct Gulfblaze genotypes (Guaíba clone and São Paulo clone) produced a single allelic band with PC1 and the same with 'The First' and 'Gulfblaze Guaíba' when using PC2, confirming the need to perform a new design of Japanese plum specific primers in order to avoid such distortions between the generated data. However, it was found that the fragments generated in all PC2 genotypes were larger in comparison to PC1.

It was observed that the occurrence of null alleles within the same gene locus may be an important factor to be considered (Mota et al., 2010). Null alleles are associated with modifications in DNA by deletion or addition of nucleotides at the sites where primers anchor, hampering primer binding in the target sequence and the amplification (Suzuki & Bird, 2008; Fernández et al., 2009; Dabrowski et al., 2015). Moreover, the overlap of amplified bands in the gel may be an impediment to the visualization of the second band in the gel, which was observed in the cultivar Shiro by Halász et al. (2007).

The appearance of an amplified fragment of DNA for self-incompatibility is common in almond cultivars (*Prunus dulcis*). Furthermore, Kodad et al. (2008) observed this in four of the fourteen almond cultivars. The same authors emphasized that this type of result can occur as a function of null loci for GSI in some species of the genus *Prunus*.

De Conti et al. (2013) obtained the *S-allelic* profile of Japanese Plum cultivars using the same PC of the present research. The authors verified the presence of a single allelic band in the cultivars Gulf Rubi, Amarelinha, Harry Pickstone, Selection 19, and Methley. They highlighted the possible homozygosis for the *S-alleles* within the loci chromosomes responsible for GSI expression, and these results are in agreement with the appearance of a single band per genotype identified in our study.

Mota et al. (2010) detected the presence of two alleles per cultivar in eight of ten Japanese Plum cultivars analyzed. They plotted a profile of reproductive compatibility among individuals, *i.e.*, the cultivars Santa Rosa ('S^cS^e'), Harry Pickstone ('S^bS^k'), and América ('S^cS^c'). Although the results of our study show only one fragment amplified by genotype, it was possible to produce groups of genotypes formed from the size of the *S-allele* fragments (Table 2) to verify the similarity between the genotypes studied for *S-alleles*.

Regarding the amplification of the DNA fragment from the *S-locus*, PC2 produced results that were more comprehensive in the identification groups, although the identification of *S-alleles* is sometimes difficult to understand in certain genotypes. Molecular studies performed by Mota et al. (2010) identified that the 'Harry Pickstone' and 'America' cultivars were incompatible with each other when comparing reproductive compatibility groups based on the molecular profile of *S-alleles*. However, according to EPAGRI (Unpublished data), these same cultivars are compatible with each other and used in various orchards of Santa Catarina. Such observed differences in reproductive compatibility based on the molecular profile and phenotypic profile suggests the presence of an additional Quantitative Trait Locus or QTLs (Empresa Brasileira de Pesquisa Agropecuária [EMBRAPA], 2006). Moreover, this trait influences the expression of *S-alleles* and consequently the proteins involved in GSI of Japanese Plum genotypes.

QTLs act on several genes associated with genetic characteristics of agronomic interest, contributing quantitatively to expression of genes linked to fruit weight, pulp tonality and firmness, fruit ripening time (Hernández Mora et al., 2017), grain numbers per pods (Faleiro et al., 2003), and that in recent years the possibility of influence of these QTLs may be associated with GSI (Fernández i Martí et al., 2011), specifically in the linkage group 6 in Prunus (Vilanova et al., 2003), contributing to the expression of *S-alleles*. As reported by Fernandez i Martí et al. (2014) *S-locus* can be influenced by epigenetics, characterized by DNA methylation in *Prunus* genus genotypes, possibly causing loss of *S-RNase* function and promoting reproductive self-compatibility. Therefore, the cultivars identified by Mota et al. (2010) as incompatible with each other, may be phenotypically compatible due to possible influences of DNA methylation, and the environmental conditions in which they are inserted.

The *S-locus* is multi-allelic, highly polymorphic, and favored genetic variability within the *Prunus* genus. The complete characterization of the allelic series linked to GSI in the Japanese Plum can support the discovery of genes associated with self-fertility, but we could not sequence the second allele in the studied genotypes. Of the 20 genotypes, 15 genotypes were characterized from the single band identified by PCR. This sequencing allowed us to discover some alleles not yet identified in the Embrapa Selections and to confirm the other alleles studies in previous research, contributing to increase the data library for the *S-alleles* of the species *P. salicina*, from genotypes grown in Southern Brazil. In the following, we describe a little of the *S-alleles* characterized in the genotypes and the inheritances of the alleles by the parents.

The 'S^c' allele sequenced in 'Black Amber' may have been inherited from the 'Queen Rosa' cultivar (Table A2), and Halász et al. (2007) observed that Queen Rosa cultivar is one of the parents of Santa Rosa (self-compatible cultivar with alleles 'S^cS^e'). The cultivars Roysum and Fortune presented the 'S^c' allele, which originated from Mutant Late Santa Rosa and Laroda, respectively (Ramming & Tanner, 1993; Beppu et al., 2002; Carrasco et al., 2012), is shown in Table A2. Molecular information on *S-allele* segregation and the register of phenotypic observations are important for understanding GSI in genotypes in studies, although we have not been able to identify the second allele in most cultivars.

The 'S^i' allele was characterized in the cultivars Sanguínea, Laroda, SC-15, and Rebelatto. Among these cultivars, we highlight 'Rebelatto', because it presented the 'S^i' allele, which is associated with GSI (Beppu et al., 2002), but this cultivar is self-compatible. Possibly the second allele not amplified in this cultivar is the *S-determinant* linked to self-fertility. The *S-allele* linked to self-fertility in the cultivar Rebelatto may have been inherited from the parental 'Carazinho', which is a self-fertility cultivar that presented two *S-alleles* amplified with the PC1, but we were not able to sequence the alleles of this cultivar to conform the alleles linked to self-fertility (Fig. 1). In addition to the 'S^a' allele in 'Piamontesa' and described by Yamane et al. (1999) is associated with GSI, as well as the 'S^a' was featured in 'Chatard', and this allele appears to be involved with GSI (Halász et al. 2007).

Another important group of genotypes that had their DNA fragments sequenced was the SA 86-13, Golden King, Letícia and Robusto where the allele characterized was the 'S^h'. Letícia is one of the most cultivated genotypes in the southern region of Brazil (EMBRAPA, 2015), and presented the same allele found in Golden King and SA 86-13 (Leticia's sister selection). Klabunde et al. (2014) observed that the cultivar Golden King was possibly one of the parental donors of the *S-allele* associated with the GSI trait in Letícia (Laetitia®, originally from South

Africa), and the same occurred with the SA 86-13 genotype. The data suggests that the 'S^h' allele was related to self-incompatibility in these genotypes because they are effectively dependent on pollinators in order to get high fruit set.

The identification and characterization of *S-alleles* in Japanese Plum genotypes are complex and very important for an increased understanding of GSI, and also critical for understanding the influence of female *S-haplotypes* on the reproduction of the species. Furthermore, it is necessary to study the *S-determinant* since the *S-locus* governs the expression of RNases and F-Box proteins. In addition to the genomic studies, the characterization of physiological aspects related to pollen tube growth, fruit-set and floral synchrony are important for SGI understanding (EMBRAPA, 2008).

During the orchard planting, we have to consider other factors like the combination of cultivars, rootstock, training system, and spacing. The cultivar combination is highly important especially in Brazil, due to the instability of environmental conditions and the limited number of cultivars adapted to the local weather. Moreover, the characterization of Japanese Plum alleles is important for choosing a better cultivar combination to set an orchard and is also an important step in the development of new cultivars in breeding programs. The transfer of *S-alleles* associated with self-fertility in a Japanese Plum segregating population is necessary for the establishment of cultivars which do not require pollinating plants.

We concluded that the use of PCR for DNA amplification followed by sequencing of *S-haplotypes* allowed us to identify and characterize the *S-alleles* that encode the RNases in *P. salicina* genotypes. The information subsidized reproductive plant management in the field and the breeding programs of Japanese Plum worldwide. However, further studies should be conducted in order to verify if putative QTLs or events like DNA methylation, under different environmental conditions, have an influence on GSI. These complementary investigations may explain and support the breeding improvement of this species.

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Appendix A

Table A1. *S-alleles* identified in *P. salicina* genotypes, with two primer combinations; *S-RNAse* identified; (%) identity (ID); (%) Query Cover (QC); access to the sequences of the alleles, with the e-value and of the respective authors that deposited the data in NCBI

GENOTYPES	PC1	*S-RNase*	ID	QC	ACCESS	PC2	*S-RNase*	ID	QC	ACCESS
Sel. Embrapa A19	663	S^c	100	100	DQ646489.1	*	*	*	*	*
Sel. Embrapa A28	565	S^c	99	99	DQ646489.1	550	S^c	100	100	AB280791.1
Black Amber	790	S^c	96	99	AF432418.1	470	S^c	94	100	AF432418.1
Sanguínea	324	S^i	99	99	AB084149.1	492	S^i	95	100	AB084149.1
Laroda	299	S^i	99	100	AB084149.1	467	S^i	94	94	AB084149.1
Piamontesa	363	S^a	99	93	AB252411.1	522	S^a	99	100	AB252411.1
SA 86-13	414	S^h	96	99	DQ790374.1	613	S^h	100	98	AB084148.1
Golden King	275	S^h	95	85	DQ790374.1	381	S^h	97	100	AB084148.1
Chatard	204	S^a	93	100	AB252411.1	524	S^a	99	100	AB252411.1
Letícia	396	S^h	97	97	DQ790374.1	566	S^h	98	100	AB084148.1
Robusto	407	S^h	98	100	DQ790374.1	509	S^h	99	100	AB084148.1
Fortune	487	S^c	94	100	AF432418.1	516	S^c	99	95	AB280791.1
Roysum	481	S^c	98	100	AF432418.1	435	S^c	97	96	AB280791.1
SC-15	292	S^i	98	99	AB084149.1	480	S^i	94	92	AB084149.1
Rebelatto	304	S^i	96	99	AB084149.1	497	S^i	97	100	AB084149.1

Note. Combination of primers PC1 (Pru-C2 + PCE-R) and PC2 (Pru-C2 + Pru-C5) in base pairs. (*) Satisfactory sequencing data were not obtained for this cultivar and combination of primers.

Table A2. Identification of *Prunus salicina* Lindl genotypes with approximate PCR amplified pairs with the combination of PRU-C2 + PCE-R (PC1) and PRU-C2 + PRU-C5 (PC2) primers, in addition to the country of origin and the parents

Genotypes	BP (PC1)	BP (PC2)	Countries	Parents	References
Selection Embrapa A19	1200	1300	Brazil	Free pollination of Pluma 2	Unknown
Selection Embrapa A28	1200	1300	Brazil	Free pollination of Pluma 2	Unknown
Selection Embrapa A7	300 & 250	600	Brazil	Free pollination of Pluma 2	Unknown
Black Amber	1200	1300	USA	Friar × Queen Rosa	Halász et al., 2007
Carazinho	400 & 300**	600	Brazil	Unknown origin	Dalbó et al., 2010
Sanguínea	300	600	Brazil	Unknown origin	Klabunde et al., 2014
Bruce	*	*	USA	*Prunus salicina* × *Prunus angustifolia*	Zarrouk et al., 2006
Laroda	300	600	USA	Santa Rosa × Gaviota	Beppu et al., 2002
Piamontesa	400	650	Argentina	Unknown origin	Klabunde et al., 2014
SA 86-13	400	700	South Africa	Free pollination of Golden King	Klabunde et al., 2014
Golden King	400	700	Australia	Unknown origin	Okie & Ramming, 1999
Chatard	350	650	Argentina	Unknown origin	Bakarcic & De Santis, 1969
Letícia	400	700	South Africa	Free pollination of Golden King	Klabunde et al., 2014
Robusto	300	700	USA	(Queen Ann × Barstow) × (O. Premier × *P. angustifolia*)	Carrasco et al., 2012
Fortune	1200	1300	USA	Laroda × B65-11	Ramimg & Tanner, 1993
Roysum	1100	1300	USA	Mutante da Late Santa Rosa	Carrasco et al., 2012
SC-15	300	600	Brazil	Seleção 93-1-8-29 (Chatard × Angeleno)	Unknown
SC-7	1200	1500	Brazil	Letícia × Piamontesa	Unknown
Zafira	1200	1500	Brazil	SC-7 × Fortune	Unknown
Rebelatto	300	600	Brazil	Amarelinha × Carazinho	Unknown

Note. (*) the cultivars did not present bands amplification with the combination of primers tested. (**) the cultivars presented two amplified bands in our PCR conditions.

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