MASTER THESIS

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BUDAPEST

Variability of the *S-RNase* alleles in the hexaploid European plum (*Prunus domestica* L.)

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TABLE OF CONTENTS

Erika Brigitte Chonata Jiménez

Erika Brigitte Chonata Jiménez

1. USED ABBREVIATIONS

bp: Base pair

CTAB: Cetyl trimethylammonium bromide

DNA: Deoxyribonucleic acid

FAO: Food Agriculture Organization

GSI: Gametophytic self-incompatibility system

IC: Incompatibility

IEF: Isoelectric focusing

ILP: Intron length polymorphism

Myr: Million years

PM: Molecular weight

SC: Self-compatible

SI: Self-incompatible

SSI: Sporophytic self-incompatibility system

PCR: Polymerase chain reaction

pI: Isoelectric point

PVDF: Polyvinylidene difluoride

µL: Microliter

Erika Brigitte Chonata Jiménez

2. INTRODUCTION

European plum (*P. domestica* L.) is a stone fruit crop that belongs to the *Prunus* genus of the Rosaceae family, together with Japanese plum (*P. salicina* L.) are currently the most important and globally cultivated plum species, offering a wide range of variation in regards to fruit size and shape, flavor, aroma, texture, and color, greater than in any other fruit crop (Faust et al., 2011; Gago et al., 2022). Plum fruit is usually commercially sold canned, dried, processed into juice, or for confectionery uses, jam making, spirit production, and baking products. Nonetheless, the interest in plums as fresh fruit has increased in the last years driven by the consumer's interest in these fruits as sources of biologically active compounds and antioxidant content (Sottile et al., 2022; Wolf et al., 2020).

P. domestica is an allopolyploid (hexaploid) species ($2n = 6x = 48$) that has been formed because of a polyploidization event, different findings agree about the origin of *P. domestica* as an interspecific hybrid of *P. cerasifera* (2n) and *P. spinosa* (4n). It is also speculated that some species may have undergone secondary hybridization (Reales et al., 2010). It is believed to have originated from the Middle East and been introduced into Europe after extensive cultivation and human selection in the Caucasus mountainous area (Zhebentyayeva et al., 2019). Polyploid species exhibit increased vigor, higher yield level and product quality, and increased tolerance and resilience to both biotic and abiotic stressors (Sattler et al., 2016).

On the other hand, genetic information in plums is still limited, the level of polyploidy of European plums makes genome analyses more difficult compared to diploid species, besides its long juvenility period and high degree of heterozygosity (Fernandez i Marti et al., 2021). Moreover, plums are mainly self-incompatible species which allows them to avoid self- and cross-fertilization with close relatives, this behavior is governed by the Gametophytic Self-Incompatibility System (GSI). This system involves the cells of the pistil, which will recognize and reject self-related pollen, and it is under the control of a specific multiallelic locus known as *S-locus*. Fertilization is only possible between individuals carrying different *S*-alleles, which stimulates outcrossing (Halász et al., 2021a).

Self-incompatibility can be diagnosed by evaluating the fruit set, following controlled pollinations in the field, by observing pollen tube growth under fluorescence microscopy in self- and cross-pollinated flowers, by protein based methods through stylar ribonuclease electrophoresis and by DNA based methods using PCR and analytic and/or capillary electrophoresis, as in the case of *Prunus* species *S*-allele genotyping is facilitated by the intron length polymorphism (ILP) shown by both *S-RNase* introns (Halász, et al., 2021a; Herrera et al., 2018).

For most *Prunus* species, PCR *S*-allele typing methods have been developed, detecting a high *S-RNase* allelic diversity, however, studies are scarcer in polyploidy accessions in comparison with diploid species such as almond, apricot, peach and sweet cherry. Molecular studies of the allelic variation at the two loci within the *S*-

Erika Brigitte Chonata Jiménez

haplotype will provide sequence information for evolutionary studies and analysis of pollination compatibility for orchard design in European plum. The aim of this study was to identify allelic diversity of *S-RNase* alleles in the hexaploid plum *Prunus domestica* using three different specific primers spanning the *S-RNase* introns.

3. OBJECTIVES

- Evaluate three consensus primer pairs flanking the *S-RNase* intron regions for the identification of allelic diversity in 29 accessions of European plum (*Prunus domestica L.)*.
- Determine the best primer performance for the identification of the highest number of *S-RNase* alleles.
- Analyze the *S-RNase* alleles sequences from Hungarian landraces.
- Evaluate the *S*-locus based diversity of the GenBank collection at the university.

Erika Brigitte Chonata Jiménez

4. LITERATURE REVIEW

5.1 Plum origin, taxonomy and production

Plum is an economically important stone fruit worldwide that belongs to the Rosaceae family, genus *Prunus.* sub-family *Prunoideae*. *Prunus'* origin has been described in eastern Asia at about 61 Myr, and its major diversification could have been a result of the global warming period of the early Eocene. It includes most commercially representative stone fruits such as peach and nectarine (*P. persica* L.), European plum (*P. domestica* L.), Japanese plum (*P. salicina* Lindl.), apricot (*P. armeniaca* L.), sour cherry (*P. cerasus* L.) and sweet cherry (*P. avium* L.) (Ricci et al., 2020; Sottile et al., 2022).

Worldwide plum production in 2019 exceeded the 12 million tons, with an increase of 20% in the last decade, being China, Romania and Serbia the leading producers (FAO, 2019). Chile is the largest plum exporter in the world being China, the United States, and Brazil considered its main importers. Hungarian plum production is mostly destined for internal consumption and for processing, it represents 9.2 % (51 000 tons) of the national fruit production (Makovicsné Zsohár, 2019).

The majority of these species are cultivated for their edible fruit, which has common reproductive organs, including a superior ovary position and a drupe as its fruit. They share a common genome with a base chromosome number of 8 (Ryugo, 1988). Due to spontaneous hybridization presents several cases of polyploidy and is widely regarded as the most diverse and numerous group of tree species which exists in nearly every temperate zone in the world. It may be grown in subtropical and cooler climates, with a wide variation in flavor, nutritional content, aroma, fruit size and shape, texture, and color. More than 6000 cultivars from different species have been bred (Blažek, 2007; Wolf et al., 2020). Despite this fact, only two species are the most significant for commercial production, the hexaploid European plum (*P. domestica* L.) and the diploid Japanese plum (*P. salicina* Lindl.) (Topp et al., 2012)**.** Other pomological groups generally small-fruited are also well known and have been used as scion or rootstock such as cherry plum (*P. cerasifera* Ehrh), blackthorn (*P. spinosa*), the sub-specie of *P. domestica* damson plum (*P. insititia* L.), as well as Asian species (*P. simonii* Carr.) and the native American *(P. americana* Marsh) (Sottile et al., 2022).

5.2 European plum (*Prunus domestica* L.)

5.2.1 Origin and taxonomy

European Plum origin has been described in Western Asia, specifically in the Caucasus region between the Black Sea and the Caspian Sea, and its domestication in China. From there it was dispersed to Western Europe more than 2,000 years ago. Its biological origin is still disputed and uncertain, due to its complexity, recurrent hybridizations and allopolyploidy phenomena. Earliest findings by Rybin (1936) established that domestic plum is a natural hybrid created from the diploid cherry plum (*P. cerasifera*) (2n = 2x = 16) and tetraploid blackthorn

Erika Brigitte Chirata Jiménez

(*P. spinosa*) (2n = 4x = 32). This allohexaploid genome statement has been supported recently by Zhebentyayeva et al. (2019), in his investigation chloroplast markers were used, *P. cerasifera* was identified as the most probable progenitor in the maternal lineage, while nuclear DNA markers, indicated a potential contribution of *P. spinosa* to the *P. domestica* genome. On the other hand, the other theory describes its genome number origin as autohexaploid, which means that hexaploid possesses the triplet set of chromosomes of a diploid species (Makovicsné Zsohár, 2019). As a matter of fact, Zohary (1992) states that the only ancestor is cherry plum (*P. cerasifera*). The taxonomical classification of European plum is presented in **Table 1**.

Table 1 *Taxonomy of European plum (P. domestica* L.*)*

Kingdom	Plantae
Subkingdom	*Viridiplantae*
Infrakingdom	*Streptophyta*
Superdivision	*Embryophyta*
Division	*Tracheophyta*
Subdivision	*Spermatophytina*
Class	*Magnoliopsida*
Superorder	*Rosanae*
Order	*Rosales*
Family	Rosaceae
Genus	*Prunus* L.
Species	*P. domestica* L.

Source: Bahrin et al., 2022

In order to preserve germplasm, exchange information and promote breeding programs, the European Cooperative Program for Plant Genetic Resources (ECPGR) and the European Fruit Research Institutes Network (EUFRIN) collaborated to create the online catalog EURISCO. This database contains information about 5078 samples of *P. domestica* diversity from ex-situ plant collections conserved in Europe (Weise et al., 2017).

Moreover, the main European plum-producing countries including but not limited to Serbia, Romania, Italy, and Germany, have worked on developing extensive breeding programs focusing on common traits for improving productivity, fruit taste and quality, resistance to PPV, as well as specific traits: late or early blooming, self-fertility, short growing period, different maturing times, flesh firmness, winter hardiness resistance to diseases and frost (Butac et al., 2012; Neumuller et al., 2021).

Polyploidization is an important process in plant evolution that promotes speciation, variability, and adaptation to various environmental factors (Trojak-Goluch et al., 2021). Polyploidy plants are recognized for their increased vigor and resistance to frost and microorganism infection in comparison with diploid species (OCDE, 2002). In contrast, the level of polyploidy of European plum makes genome analyses challenging being one of the least

Erika Brigitte Chonata Jiménez

studied species within the *Prunus* genus. In **Figure 1** the botanical illustrations of the tetraploid *P. spinosa* and the hexaploid *P. domestica* L. *and P. insititia* L. are shown.

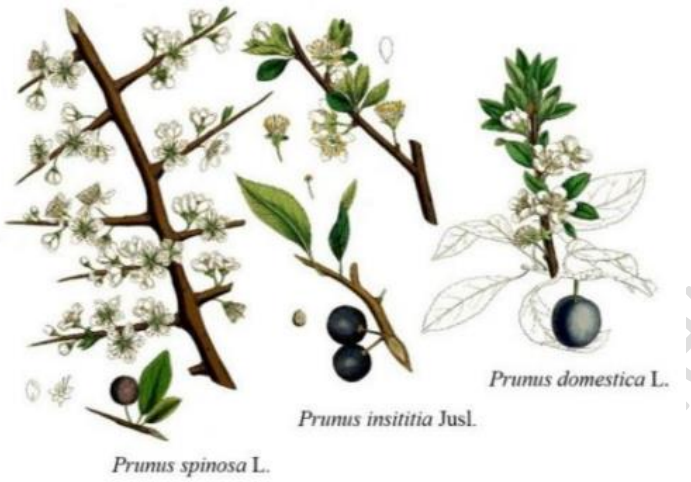

Figure 1 *Botanical graphics of P. spinosa, P. domestica L. and P. insititia L.*
Source: Makovicsné Zsohár, 2019

5.2.2 Use and health benefits

Most of the plum production is used for fresh consumption, dried fruit, preserves, and liquor making. The benefits of consumption have become more widely recognized as a result of the knowledge of its proven high nutritional and health value, as it is a source of macronutrients, vitamins, fiber, and minerals (primarily potassium, calcium, magnesium, and phosphorus), in addition to its high content of phenolic acids and flavonoids which contributes to its antioxidant potential and anti-inflammatory effects (Igwe & Charlton, 2016; Shukla & Kishan, 2021). The positive effects of plums on cognitive improvement, bowel movement, infection as well as allergy control were also examined in the study. A compressive review study carried out by Wallace (2017) presents its bone protective effects, while its anticancer properties have been also demonstrated by different authors (Bahrin et al., 2022). These characteristics make it an attractive product also for nutraceutical markets.

5.3 Self-incompatibility systems

5.3.1 Background of self-incompatibility

The achievement of a high fruit set and yield is dependent on reproduction biology, which at the same time has a genetic and environmental influence (Sassa, 2016). Self-incompatibility (SI) is a plant reproduction mechanism that restricts the creation of a zygote after self-pollination in a fertile hermaphrodite plant with both female and male reproductive systems, as well as pollination among closely related species. (Furstenau & Cartwright, 2017; Watanabe et al., 2012)**.** Occurs in approximately 40% of species including important crops such as canola, potato, pome and stone fruits, olive, cocoa, tea and coffee (Igic et al., 2008).

Erika Brigitte Chonata Benez

Hermaphroditic plant species have evolved mechanisms to inhibit self-pollination and to restrict cross-pollination between related individuals, which is either a morphologically- or a molecularly self-incompatibility (SI) system. The first type, also called heteromorphic the male and female reproductive organs are spatial or temporal separated. Whereas the molecularly enforced or homomorphic SI involves the interaction of gene products expressed in the pollen and in specific pistil cells, in this way self-incompatible plants will reject pollen that expresses an allele that is the same presented in the pistil as presented in **Figure 2.** The term SI was presented to replace "*self-sterility"* early in 1917 (Cappadocia, 2003; Furstenau & Cartwright, 2017).

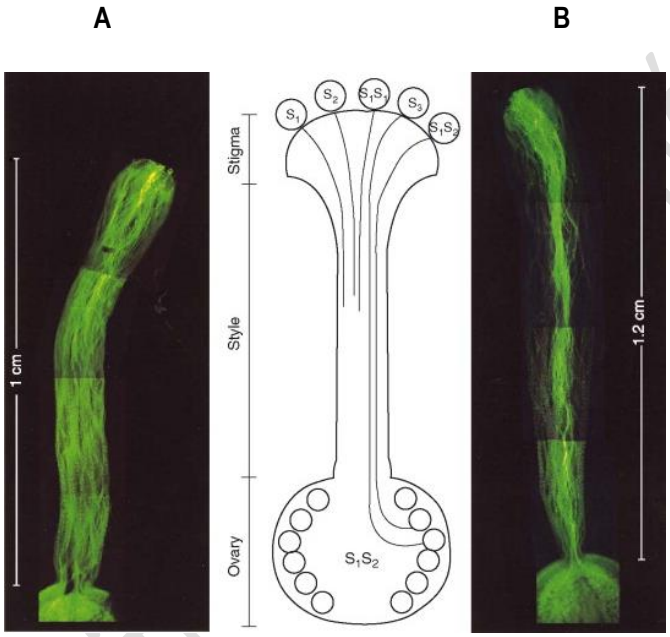

Figure 2 *Representation of a RNase-based GSI pollen behavior in Solanum chacoense (genotype S_1S_2) observed by epifluorescence 48 h after pollination.*
(A) Incompatible cross, pollen tube fluorescence stops its growth at the middle style. (B) Compatible cross, pollen tube enters the ovary for fertilization.
Source: Cappadocia, 2003

This self-incompatibility system is genetically controlled at the multiallelic *S-locus* by two genes with male and female specificity which are tightly linked and inherited as an *S*-haplotype (Casselman et al., 2000; Vieira et al., 2003)**.** The term haplotype is used to refer the two variants of the *S-locus*, while the term allele is used to describe either pistil or pollen *S-locus* genes (Sun et al., 2018).

Three mechanisms can be used to categorize self-incompatibility. The sporophytic self-incompatibility (SSI), is studied mainly in Brassicaceae where the determinant genes encode a pollen ligand and a stigma receptor kinase, their interaction results in incompatible signaling inside the stigma papilla cells (Takayama & Isogai, 2005)**.** The gametophytic self-incompatibility (GSI) found mainly in Solanaceae and Rosaceae, is a *S-RNase* based mechanism whose determinants are a single pistil-specific *S-RNase* gene and a group of pollen-specific

S-locus F-box (*SLF*) genes (Lee et al., 1994). Finally, the Papaver system exhibits a more complicated signaling network including calcium fluxes, actin rearrangements that cause death of incompatible pollen, known as programmed cell death (PCD) (Muñoz-Sanz et al., 2020; Silva & Goring, 2001).

As the name suggests in the GSI system, the genotype of the gamete (haploid pollen) determines its incompatibility type, whereas in SSI, the genotype of the sporophyte (diploid parental plant) determines the incompatibility type. Additionally, rejection of incompatible pollen in GSI often occurs in the style while in SSI occurs at the stigma surface as shown in **Figure 3** (Ahmad et al., 2022)**.**

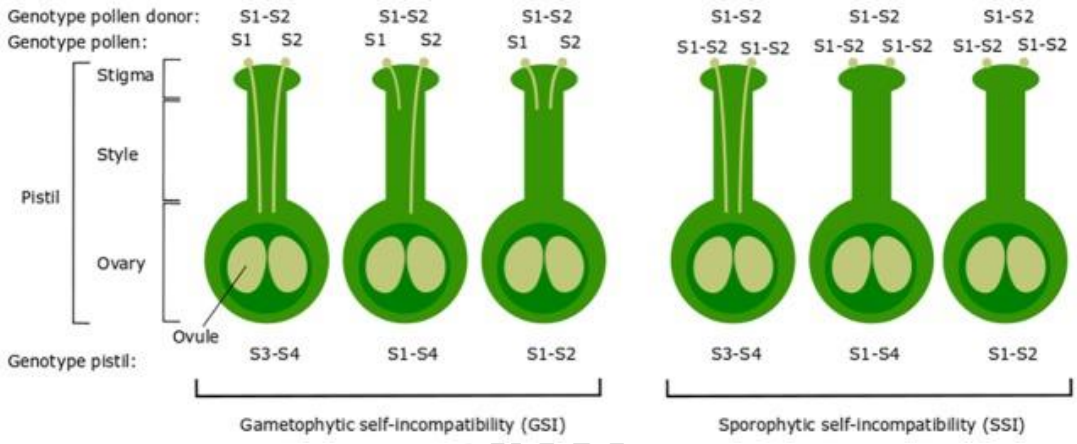

Figure 3 *Representation of an RNase-based GSI pollen behavior in Solanum chacoense (genotype S_1S_2), observed by epifluorescence 48 h after pollination.*
Source: Claessen et al., 2019

5.3.2 Prunus-specific gametophytic self-incompatibility

Among different taxa where GSI is the mechanism that controls self-incompatibility, similar linked genes are present at the *S-locus*, however, the detailed mechanisms show differences.

In *Prunus*, *S-locus* is less than 70 kb, located on chromosome 6, and contains two related genes involved in the pollen-pistil interaction. One expressed on the pistil, the *S-ribonuclease* (*S-RNase*) gene that encodes glycoproteins with ribonuclease activity, acting as cytotoxin and causing rejection of pollen when its *S*-allele is the same. And the other expressed in the pollen, is an *S-haplotype-specific F-box* (*SFB*) gene that determines the pollen allele specificity (Herrera et al., 2018; Fernandez i Marti et al., 2021).

Studies in pollen have shown its association with self/cross *S-RNases*, moreover, F-box proteins are known for their roles in the 26S ubiquitin/proteasome pathway of protein degradation which implies a mechanism of inactivation through this pathway (Qiao et al., 2004). Accordingly, non-self *S-RNases* are degraded in compatible pollinations, but in self-pollinations these proteins avoid degradation and destroy the pollen RNA (Sun et al., 2015)**.**

The effective operation of the GSI system requires co-evolution between pollen and stigma parts of the *S*-locus, through complemented mutations in both parts and this co-evolution expects high polymorphism at both loci (Muñoz-Sanz et al., 2020). Genetically, genotypes with the same *S*-haplotype are incompatible with one another and are members of the same incompatibility group (IG), whereas cultivars with different *S*-alleles are inter-compatible belonging to a different IG. Therefore, the assignment of each cultivar to its corresponding group is essential to create the right crosses in orchard design and breeding programs (Abdallah et al., 2019; Halász et al., 2021a).

5.4 Importance of self-incompatibility systems

This specific discrimination promotes outcrossing and avoids inbreeding, in this sense growers and breeders have used this system as a tool for manipulating domesticated crops (Muñoz-Sanz et al., 2020). Therefore, applications derived from knowledge of SI include crop production (yield and quality) and marker-assisted breeding,

5.4.1 Crop production

Self-incompatibility system has been considered to have a strong impact on the evolution and success of angiosperms through outbreeding, since stimulates gene flow and genetic diversity (DeNettancourt, 1977). Nevertheless, commercial orchards of SI fruit species aiming to ensure fruit set must simultaneously interplant cross-compatible cultivars from several pollen incompatibility groups. SI can lead to inadequate fruit sets and yield. In the case of *Prunus,* they are unable to bear fruit parthenocarpically which makes a successful fertilization necessary. Additionally, the success of cross-pollination depends on pollinators and environmental conditions (Claessen et al., 2019).

Therefore, untargeted and molecular-targeted strategies to overcome SI have been studied through the years. Some untargeted strategies are physical treatments, chemical agents, timing to avoid *S*-determinant expression, tissue culture techniques, mentor and mixed pollen (Kučera et al., 2006; Lewis & Crowe, 1954).

Growers and breeders have been interested in discovering and choosing SC cultivars for thousands of years. For instance, the John Innes Center sweet cherry breeding program induced SC in a cultivar through pollen x-ray irradiation in the 1940s, from this cultivar different commercial varieties have been created, and over time these cultivars were shown to possess a common defective allele (Ushijima et al., 2003). Also, molecular mechanisms using conventional breeding approaches demonstrated the introgression of *S-locus* inhibitor (Sli) from SC natural mutants of *Solanum chacoense* achieved SC diploid potatoes to that degrading all S-RNases (Kardile et al., 2022). Also, studies focusing on the down-regulation of pollen- and pistil-expressed genes as *S-RNase* or SI-related genes have successfully converted self-incompatible diploids to self-compatible ones, with increased vigor and plant morphology similar to the wild type (Ye et al., 2018).

Erika Brigitte Cronata Jiménez

5.4.2 Marker-assisted breeding

Much progress has been made through the years in identifying and characterizing the *S*-genotype through controlled pollinations, pollen tube growth tests, and enzymatic assays (Muñoz-Sanz et al., 2020). In fact, early in 1937, there was already information on 6 sweet cherry *S*-alleles obtained through cross-pollination tests (Sonneveld et al., 2003). Furthermore, in the last two decades, the knowledge of sequence polymorphism of the *S*-determinants genes (*S-RNase* and *SFB*) has allowed the development of molecular *S*-genotyping systems in various rosaceous fruit tree species which has accelerated the identification of new *S*-alleles over the time (Yamane & Tao, 2009).

5.5 Diagnostics of self-incompatibility

5.5.1 Field controlled cross-pollinations and evaluation of fruit set

Traditionally, self-incompatibility relationships have been identified by conducting field-controlled pollinations Incompatible and compatible groups are defined by monitoring the percentage of fruit set after four weeks of self- and cross-pollinations in orchards (Williams, 1970). This process involves the emasculation of flowers, followed by hand pollination, using small tools or only fingers (Guerra & Rodrigo, 2015).

The method's low cost and practicality have led to its continued application today. However, the evaluation of *S*-genotyping is extremely conditioned on environmental factors and these analyses include a series of crossings that take years long, because to the extended juvenility period of fruit tree species (Halász & Hegedűs, 2006; Larsen et al., 2016). Also, it has been found that pollination through this method has achieved low fruit set and, in some cultivars, a total lack of fruit. In fact, in Japanese plum crosses, the lack of fruit set could have been attributed to flower emasculation (Guerra et al., 2010). These findings could have led to incorrect diagnoses of self-incompatibility. Furthermore, in the case of hexaploid plum, data obtained in cross pollinations experiments cannot be accurate, since it is difficult to distinguish between full-compatible and semi-compatible genotypes specially in polyploid species (Larsen et al., 2016).

5.5.2 Pollen tube control tests

Self-incompatibility can also be determined by monitoring pollen tube elongation through the style, using microscopy after hand-pollinations in field or laboratory conditions. In self-compatible cultivars the elongation of the pollen tube through the style takes about 3 days. Within a day of pollination pollen grains begin to germinate on the moist surface of the stigma, consequently the pollen tube penetrates the stigma, crossing a compact nutritious transmitting tissue surrounded by vascular bundles in the style until reaching the ovary for fertilization. On the other hand, in the case of self-incompatible cultivars pollen tube growth is arrested in the style, interfering with ovary fertilization, as presented in **Figure 4** the microscopy images of pollen growth in SI (A) and SC (B) apricot pistils (Guerra et al., 2009; Herrera et al., 2018).

Erika Brigitte Chonata Jiménez

One of the first published techniques by Thiele & Strydom (1964) requires the collection of pollinated pistils from three several days after pollination, followed by fixation and observation of stained sections under the microscope. This method is based on the analysis of callose wall of the pollen tubes (which contains two layers of polysaccharides) that separates the cytoplasm with organelles from the other part of the pollen tube, in cases of self-incompatibility an increase in the callosic particles its observed, which is attributed to premature cytoplasm disintegration. Using this approach, the callose is selectively stained using water soluble substances such as blue aniline, and the structure of dead or living tissue is observed using fluorescent UV light through a conventional microscopy (Halász & Hegedűs, 2006).

Variations in this method have been implemented over time to increase staining efficiency. Nowadays, in most studies preparations of pistils are autoclaved to soften the tissues, followed of staining with aniline blue for subsequent observation under a fluorescence microscopy (Guerra et al., 2009). This approach has been applied to identify incompatibility relationships between different diploid *Prunus* species. Although, in the case of polyploidy varieties results have not been clear due to its hybrid complex genome (Guerra & Rodrigo, 2015). It is still a powerful technique to verify results obtained as S-genotyping methods based on PCR.

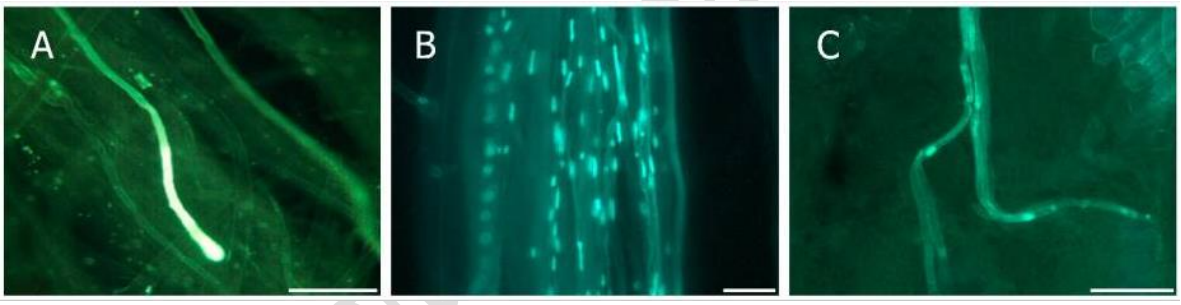

Figure 4 *Pollen tube growth in apricot pistils observed through a microscope using fluorescent UV light. (**A**)Self-incompatible cultivar, pollen tube growing stops at the middle of the style (**B**) Self-compatible cultivar, pollen tubes grow along the style (**C**) Self-compatible cultivar, pollen tubes at the base of the style.*
Source: Herrera et al., 2018

Self-fertility, partial self-fertility, and self-incompatibility have been detected in hexaploidy European plum cultivars (Kozma et al., 2003). In a study conducted in Serbia to examine self-compatibility in 18 European plum cultivars using fluorescence microscopy, 13 cultivars were found to be self-compatibles and only 5 self-incompatibles, self-incompatible cultivars were distinguished if the pollen tubes interrupted their growth in the style and swelling characteristics were observed on the tips, product of the accumulation of callose (Nikolić & Milatović, 2010).

5.5.3 Protein based method through stylar ribonuclease electrophoresis

McClure et al. (1989) identified stylar *S*-proteins as glycoproteins with ribonuclease activity, which were recognized as essential in pollen rejection in SI. A two-dimensional electrophoresis approach is used to identify

stylar glycoproteins representing *S*-allele products. Isoelectric focusing (IEF) is commonly applied in the first dimension to separate proteins according to their PI value, while in the second-dimension sodium dodecylsulfate electrophoresis is used to separate them according to their molecular weight. However, IEF is performed in the presence of urea, which has a buffering effect that restricts the correct pH gradient formation in the resolution of proteins with alkaline isoelectric points (Halász & Hegedűs, 2006). As a solution, O'Farrell et al. (1977) presented Nonequilibrium pH Gradient Electrophoresis (NEPHGE) as an alternative technique for IEF in some species for RNase identification.

Results of this approach are given as a pattern of spots, in a form of coordinate system with the separated proteins increasing according to PI values from left to right and decreasing according to molecular weight from top to bottom (**Figure 5**) (Halász & Hegedűs, 2006). Furthermore, this approach allows RNase specific localization by electroblotting using PVDF membranes to determine the N-terminal amino acid sequence or by immunoblotting analysis using antiserums produced against RNase molecules.

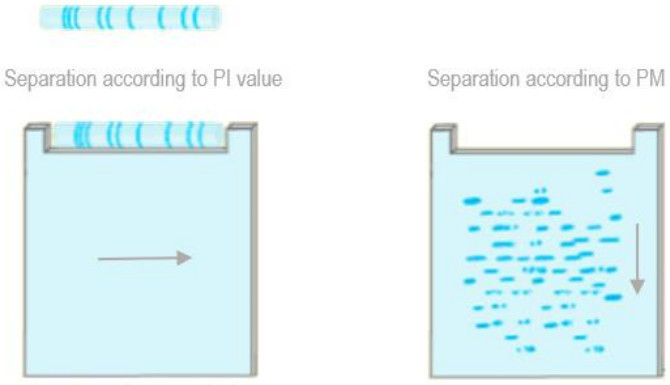

Figure 5 *Two-dimensional electrophoresis for protein separation based on isoelectric point and molecular weight value.*
Source: Büyükköroğlu et al., 2018

The first results in *Prunus* have been found in the analyses of two *S*-RNases associated with GSI in almond, which allowed to identify only one *S*-glycoprotein, using an antiserum first used in Japanese pear (Tao et al., 1997), the fact that only one allele was recognized was attributed to a difference in the recognition site of the antiserum. Later, in a study on sweet cherry to verify incompatibility alleles in protein stylar extracts using 2D electrophoresis with IEF and staining for ribonuclease activity, when two cultivars from the same incompatibility group were evaluated, they produced identical zymograms, but cultivars from different incompatibility groups produced different zymograms (Bošković & Tobutt, 1996). Likewise, Tao et al. (1999) performed a molecular study of identification, characterization, and cDNA cloning of S-RNase from sweet cherry, six *S*-products were tested, and the results revealed only two alleles which was explained by the likelihood of sample overlapping, meaning that a spot could have been comprised of many proteins (Halász & Hegedűs, 2006). This method can

obtain the highest protein resolution among mixture of different type of proteins, however the requirements of trained professionals and laboratory skills are some disadvantages of this method.

5.5.4 DNA based methods: PCR. Sequencing

This method is based on isolating DNA from plant tissues. The CTAB method of DNA extraction uses a detergent that forms an insoluble compound with the DNA being separated from other cellular compounds. This complex is then separated through resuspension with NaCl. Nucleic acids are concentrated by ethanol precipitation, and a final ribonuclease treatment is applied. The methods that use kits are based on the strong negative charge of DNA that will connect to positively charged surfaces in a chromatography column. Following DNA extraction, a polymerase chain reaction (PCR) is carried out to amplify segments of DNA of interest (Halász & Hegedűs, 2006).

Molecular markers based on the polymerase chain reaction (PCR) for amplification of genomic DNA of conserved regions have been applied to determine *S*-genotypes in different species. The first PCR amplification of *S*-alleles was carried out in *Brassica oleracea,* a sporophytic self-incompatible specie. The alleles identified after PCR-products of known sequences were digested with different restriction enzymes (Brace et al., 1993).

After the discovery, cloning and characterization of *S-locus* genes determinants of self-incompatibility, S-RNase and F-box proteins for pistil and pollen, respectively, PCR genotyping methods based on the polymorphism of amplified fragment length have been developed in many *Prunus* species, which not only allow to identify self-compatible and self-incompatible cultivars but also provides sequence information for evolutionary studies. The ability of analysis of vegetative tissue to detect SC and SI cultivars is one of the main advantages of DNA-based *S*-genotyping, making it proper for seedling selection (Halász & Hegedűs, 2006).

In Rosaceae, the pollen *S*-determinant contains two variables (V1 and V2) and two hypervariable (HVa and HVb) regions, coded by pollen-specific *F-box* genes (*SFB)* (Ushijima et al., 2003). While the pistil *S*-determinant is composed of five highly conserved regions (C1–C5), with two highly polymorphic introns coded by the *S-RNase* gene (**Figure 6**). *S*-genotyping of *Prunus* accessions has been mainly based on the allele-specific intron length polymorphism (ILP) shown by both *S-RNase* introns, therefore consensus primers flanking *S-RNase* introns are used to amplify PCR different sized products that are *S*-genotype specific (Halász, et al., 2021b; Sutherland et al., 2004).

Erika Brigitte Cronat Jiménez

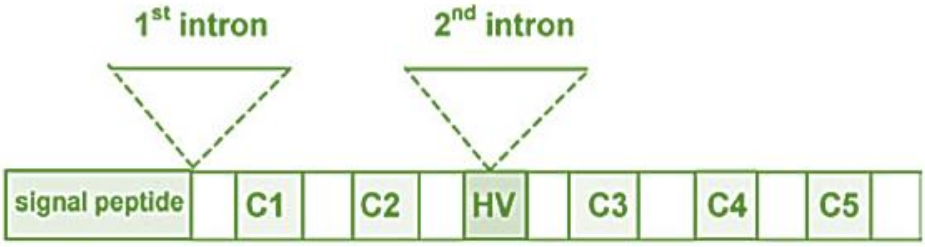

Figure 6 *Structure of a Prunus S-RNase gene.*
Source: Sonneveld et al., 2003

5.6 S-alleles in plums

The identification of the first pistil proteins associated to the RNase activity in Rosacea was possible in Japanese pear using biochemical methods (Sassa et al., 1993). The first dated sequence information in *Prunus* was the N-terminus sequence of almond (Tao et al., 1997)**.** In the future, this approach contributed to the development of PCR *S*-allele genotyping methods for the identification of self-incompatibility among cultivars in several *Prunus* species, where high *S-RNase* allelic diversity have been found for example Japanese plum (Yamane et al., 1999), almond (Tao et al., 1997), sweet cherry (Tao et al., 1999), Japanese apricot (Yaegaki et al., 2001), European apricot (Romero et al., 2004).

In the case of the hexaploid plum the first findings regarding the molecular genetics of SI were published by Sutherland et al. (2004). In the study, the isolation of stylar myrobalan *S-RNases* by RT-PCR allowed the design of consensus primers spanning the second intron using conserved regions of these found sequences. These primers were used in 15 diploid myrobalan and 19 hexaploid plum cultivars, obtaining size fragments ranging from 0.7 - 3.0 kb and 0.6 - 1.2 kb for myrobalan and plum cultivars, respectively. 23 of these isolated bands were sequenced and its comparison among *Prunus S-RNases* revealed close identity sequence in the coding region and allowed the design of 24 *S*-alleles specific primers.

Sonneveld et al. (2003) designed consensus primers from cDNA *S-RNase* sequences obtained from sweet cherry. The primer pair PaConsI-F and PaConsI-R, was designed to detect variability in the first intron, positioned between the region coding for the signal peptide and the mature protein, obtaining genomic amplification products between 303 and 523 bp, while for the second intron the primers PaConsII-F and PaConsII-R were designed to detect variability in the RHV region with genomic amplification products between 577 and 2383 bp (Figure 6). Later, this approach enabled the sequencing of the first three *P. domestica S-RNase* alleles (S_5, S_6, S_9) by Sutherland et al. (2008).

Erika Brigitte Chonata Jiménez

In the same way, Kota-Dombrovska & Lācis, (2013) used six consensus *S-locus* markers previously developed for other *Prunus* species flanking the first and second intron which allowed the identification of 14-37 alleles per marker on 33 European plum cultivars, the second intron was amplified using EM-PC2consFD and EM-PC3consRD primers which provided high level of variability. Likewise, Halász et al. (2014) obtained *S*-genotyping data from 16 important hexaploid plums grown in Hungary, using primers designed for diploid *Prunus* species flanking the first and second intron, in the case of the first intron, the fluorescent labelled PaConsI-F primer designed by Sonneveld et al. (2003) was used together with the primer EM-PC1consRD designed by Ortega et al. (2005), followed of a precise fragment size determination in a capillary sequencer. For the second intron the primers EM-PC2consFD and EM-PC3consRD, which revealed 18 different alleles with sizes between 232 and 416 bp, representing high genetic diversity.

On other hand, the combination of different methods also has been applied for the characterization of plum genetic resources, for instance SSR markers, *S*-locus markers and phenotypic data were combined in the study of Tunisian plum species as in the case of Baraket et al. (2019). They studied 19 Tunisian plum accessions and discovered that SSR markers did not allow a polyploidy identification, due to homozygosity, in contrast *S*-genotyping could identify cultivars with different polyploidy levels, but not cultivars with the same ploidy number. Based on the same approach, Abdallah et al. (2019) applied three different primer combinations, the first spanning the second intron (PruC2/PCER), the second pair was specific for the first intron of *S-RNase* gene (PaCons1F/PaCons1R2), and the third primer combination spanning the *SFB* gene intron (Fbox5'F/Fbox–IntronR). A total of 32 *S*-alleles were identified, 16 from *P. salicina*, 11 from *P. domestica* and 5 from P. *insititia*. It was observed that the primer spanning the second intron could identify the complete *S*-genotype, while the primers spanning the first *S-RNase* and *SFB* gene intron presented limited polymorphism. These S-genotyping results were validated at the laboratory though cross pollinations and followed of pollen tube growth detection to verify the results, concluding that the combination of the three primers can be complemented with the pollen tube analysis for an accurate characterization.

A recent study carried out in Hungary by Halász et al. (2021b) about the characterization of the *S-locus* of 17 *P. spinosa* and *P. domestica* subsp. *insititia* cultivar candidates could obtain 18 different alleles with 11 *S-RNase* alleles detected for the first time. In the same year, Fernandez i Marti et al. (2021) determined the *S*-alleles of 36 cultivars and selections using primers that revealed 17 new alleles. The primer set combination for the *S-RNase* locus flanking the second and fifth conserved domains (PruC2-PCER) obtained a higher number of amplified fragments per sample.

Erika Brigitte Chonata Jiménez

5. MATERIALS AND METHODS

6.1 Plant materials

Buds from a total of 29 *P. domestica* cultivars, landraces and hybrids were used for the analysis. These samples were conserved at the orchard of MATE university in Soroksár. Name of the cultivars and biological origin are listed in **Table 2**.

Table 2 *Summary of the 29 accessions of P. domestica used in this study, including their origins.*

Cultivar name	Origin
Ageni 707	It was selected in Agen by Benedictine monks in the XIII century.
Anna Spath	Seedling selected by Spath.
Bellamira	Mirabelle de Nancy × Cacaks Beste.
Besztercei	Its origin is uncertain, Middle Eastern or Carpathian Basin origins are also possible.
Bódi szilva	Hungarian landrace (Jászság, Heves, Tiszántúl).
Bühli korai	German landrace.
California blue	American cultivar, spontaneous seedling of Ageni.
Centenar	Romanian fajta.
Czar	Prince Englebert × Early Prolific.
Duránci	Hungarian landrace (Borsod, Alföld).
Fehérszilva	Hungarian landrace (Duna-Tisza-köz, Borsod, Bácska).
Giant	Ageni × Pond's Seedling.
Gömöri nyakas	It is originated from Gömör, found by József Pólya in 1870.
Grand Prize	Burbank produced by cross-breeding.
Kirke szilvája	Unknown origin, from XVI century.
Mirabelle de Nancy	Syrian, unknown origin.
Opal	Early Favourite × Oullins.
Páczelt szilvája	Bred by Páczelt János in Nagykárolyban.
Sanctus Hubertus	Belgian cultivar (1963).
Szo1	Soviet seedling.
Szo2	Soviet seedling.
Szo3	Soviet seedling.
Tragedy	Californian seedling.
Tuleu timpuriu	Tuleu gras × Sermina.
Vengerka virla	Russian cultivar.
Vörös nagygyümölcsű	Hungarian landrace.
Vörös szilva	Hungarian landrace (Közép- and Dél-Alföld).
Wagenheim	Old German cultivar.
Zöld ringló	Italian cultivar with unknown origin.

Source: Surányi, 2019

Erika Brigitte Chinarro Jiménez

6.2 DNA isolation

Buds of the twenty-nine cultivars after collection were kept frozen at -20°C. The total genomic DNA was isolated using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. The evaluation of DNA quantity and quality was assessed by spectrophotometry using a Nanodrop ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE, USA). Isolated DNA samples were also kept at -20°C.

6.3 PCR amplification and gel electrophoresis

Three different primer pairs combinations that span the first and second introns were used for the amplification of the *S-RNase* gene of 29 *P. domestica* accessions. For the second intron two set of primers were used: PaConsII-F and PaConsII-R designed by Sonneveld et al. (2003), EM-PC2consFD and EM-PC3consRD designed by Sutherland et al. (2004). For the first intron, consensus primers were used to detect variability PaConsI-F and EM-PC1consRD designed by Sonneveld et al. (2003) and Ortega et al. (2005). Primer sequences are listed in **Table 3**.

For the PCR reaction, 40–60 ng of genomic DNA was used for a volume of 12.5 µL approximately containing 10 x DreamTaq™ Green buffer (Thermo Fisher Scientific, Waltham, MA, United States) with final concentrations of 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 0.4µM of each primer, and 0.625 U of DreamTaq™ DNA polymerase (Thermo Fisher Scientific). For the PCR amplification a thermal cycler model 2720 (Applied Biosystems, Foster City, CA, United States) was used.

Table 3 *Primer pairs used for amplification S-RNase flanking first and second intron regions.*

Primer pair	Primer sequence 5'-3'	Reference
PaConsI-F	F: MCT TGT TCT TGS TTT YGC TTT CTT C	Sonneveld et al. (2003) and Ortega et al. (2005)
EM-PC1consRD	R: GCC AYT GTT GMA CAA AYT GAA	
PaConsII-F	F: GGC CAA GTA ATT ATT CAA ACC	Sonneveld et al. (2003))
PaConsII-R	R: CAW AAC AAA RTA CCA CTT CAT GTA AC	
EM-PC2consFD	F: TCA CMA TYC ATG GCC TAT GG	Sutherland et al. (2004).
EM-PC3consRD	R: AWS TRC CRT GYT TGT TCC ATT C	

Amplified products from PCR were separated by electrophoresis in ethidium bromide (EtBr) stained agarose gel at 1% for about 45 min at 90 V using TBE 1x as running buffer. DNA bands were visualized by UV light. The evaluation of the precise size of amplicons of the first intron region was based on the fluorescently labelled (FAM) forward primer, in an automated capillary sequencer model ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) the amplicons were run, and the resulting data of nucleotide sequences was analyzed using the

Erika Brigitte Cromata Jiménez

software ABI Peak Scanner 1.0 and internal size standards were used GS500 LIZ (Applied Biosystems). In the case of the second intron the estimated fragment sized was based on the comparison to the GeneRuler™ 1 kb DNA Ladder (Thermo Fisher Scientific).

6.4 Cloning and DNA sequencing

PCR products of important European plum Hungarian landraces amplified by the PaConsII primer pair were selected based on the different size of the fragments aimed to identify the higher number of alleles.

For this purpose, a pTZ57R/T vector (Thermo Fisher Scientific) was used to perform a direct cloning of the PCR fragments. The ligated plasmid vectors were transformed into JM109 *Escherichia coli* competent cells (Zymo Research Corp., Irvine, CA, United States). The transformed cells were selected according to the blue-white visual screening technique. Using M13 sequencing primers, the nucleotides of each fragment were determined in both directions. Fragments from different plasmid size were purified with an EZ-10 Spin Column Plasmid DNA kit (Bio Basic Inc., Markham, Canada) and sequenced in an automated sequencer model ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

6.4 DNA sequence analysis

The analysis of DNA and deduced amino acid sequences from the alleles obtained were performed at NCBI using BLASTN 2.11.0 software (Altschul et al., 1990).

MEGA program (Kumar et al., 2018) was used to create a phylogenetic tree using the "Minimum Evolution" technique (Rzhetsky & Nei, 1992). Next to the branches were the percentages of duplicate trees in which the related *S-RNase* allele sequences grouped together in the bootstrap test (1,000 replicates) (Felsenstein, 1985). The phylogenetic tree is scaled, in the way that branch lengths are measured in the same units as the evolutionary distances used to estimate the phylogenetic tree. The evolutionary distances were calculated using the JTT matrix-based approach (Jones et al., 1992) and are measured in amino acid substitutions per site. A gamma distribution (shape parameter = 0.8) was used to model rate variance among sites. Close-Neighbor-Interchange (CNI) algorithm (Nei & Kumar, 2000) was used to search the ME tree at a level of 1. The initial tree was generated using the Neighbor joining algorithm (Saitou & Nei, 1987). The analyses included 35 amino acid sequences presented in **Table 4**. Ambiguous positions were removed completely for each sequence pair (pairwise deletion option). There was a total of 144 positions in the final dataset.

Erika Brigitte Chonata Imre-PhD

Table 4 *Accession numbers of amino acids used for phylogenetic tree for each S-allele from NCBI data base.*

S_{fs1}	S_{fs2}	S_{ps6}	S_{vn4}	S_{vn16}	S_{zr15}
QNJ34523.1	BAF56247.1	MN069630.1	MN052898.1	QNJ34532.1	BAF42764.1
AEB96584.1	AFJ20702.1	MN069631.1	MG595258.1	ABV71999.1	PQP94592.1
AWB51947.1	AAT69244.1	AFJ20684.1	ABG76012.1	ABV72003.1	CAN90137.1
AWB51943.1	ABG76213.1		BAC75461.1		BAK19918.1
AAP92437.1	ABE27180.1		AFH56918.1		
CBI68345.1			AZP02735.1		
ACJ22520.1			CBI68343.1		
AZP02732.1			ABO34169.1		
BAA95157.1			ABW86860.1		

6. RESULTS AND DISCUSSION

7.1 Primer test for the *S-RNase* gene of *Prunus domestica* L

The characterization of *S*-locus was carried out based on the intron length polymorphism of the two introns present in the *S-RNase* gene of *Prunus domestica* L. Three different primer pairs were used, in the case of the second intron PaConsII-F and PaConsII-R designed by Sonneveld et al. (2003) and EM-PC2consFD and EM-PC3consRD designed by Sutherland et al. (2004) were used. For the first intron the combination of PaConsI-F primer designed by Sonneveld et al. (2003) with the EM-PC1consRD designed by Ortega et al. (2005). Studies focused on the genetics of self-incompatibility in other *Prunus* species have used these *S-RNase* molecular markers. The three assayed primer pairs demonstrated a successful PCR amplification of *S-RNase* alleles in the entirely 29 cultivars studied, obtaining several amplification fragments in each sample as presented in **Table 5**. Due to the hexaploidy genome of European plum the maximum number of alleles expected was six.

Table 5 *Number of alleles amplified with the three primers spanning the second and first intron in a S-RNase gene of Prunus domestica L.*

Sample name	**Number of amplified alleles**		
	Second intron		**First intron**
	EM-PC2consFD EM-PC3consRD Sutherland et al. (2004)	PaConsII-F PaConsII-R Sonneveld et al. (2003)	PaConsI-F EM-PC1consRD Sonneveld et al. (2003) and Ortega et al. (2005)
Besztercei	4	2	5
Kirke szilvája	5	4	4
Duránci	3	3	4

Erika Brigitte Obenata Jiménez

Vengerka virla	4	4	5
Wagenheim	4	3	4
Sanctus Hubertus	5	2	4
Fehérszilva	5	4	4
Bühli korai	3	3	3
Gömöri nyakas	5	3	4
Mirabelle de Nancy	4	4	5
Centenar	6	4	4
Anna Spath	4	3	4
Vörösszilva	6	3	5
Tragedy	3	3	5
Páczelt szilvája	3	4	5
California blue	5	3	4
Ageni 707	5	5	4
Szo3	4	3	5
Vörös nagygyümölcsű	6	3	6
Tuleu timpulia	4	3	6
Czar	3	2	4
Szo2	4	3	5
Opal	3	4	3
Szo1	4	4	5
Zöld ringló	4	2	5
Bódi szilva	6	4	4
Bellamira	4	4	6
Grand prize	5	3	5
Giant	6	4	5
Total number amplified fragments	***127***	***96***	***132***
Average number of fragments	***4.38***	***3.31***	***4.55***

Note: The total amplicons obtained through PCR in the case of the second intron is the result of the counting of the separated fragments in an electrophoresis gel, while in the case of the second intron the number of fragments are the result of precise fragment length determination

In the case of the primers used to detect variability in the second intron, the number of bands amplified varied from two to six, the EM-PC2consFD and EM-PC3consRD detected higher number of bands, 127 total in the 29 samples with an average of 4.38 bands per cultivar **Figure 7**, while PaConsII-F and PaConsII-R detected 96 bands, with an average 3.31 bands among the 29 cultivars as displayed in **Figure 8**. These results are in accordance with the study performed by Kota-Dombrovska & Lācis (2013) using six different primer combinations determined that the primer pair EM-PC2consFD and EM-PC3consRD detected the highest number of amplified fragments per sample (4.0 in average).

In regard to length size of PCR products, the bands varied significantly ranging from 300 bp to 1500 bp, these data compared to a similar study carried out by Halász et al. (2014) to characterize self-incompatibility genotypes

of European plum cultivars, the results of the second intron amplification using EM-PC2consFD and EM-PC3consRD primers were 4 to 6 bands for each of the cultivars with bands ranging from 300 bp to 2500 bp in length. From our results, the intensity of the bands exhibited among cultivars was variable, which can explain that different alleles could have been in the same band but due to slight proximity in fragment size they could not be differentiated.

Six amplification fragments which represent a complete *S*-genotype were obtained for five cultivars: 'Centenar', 'Vörösszilva', 'Bódi szilva', 'Vörös nagygyümölcsű' and 'Giant' using the primer pair EM-PC2consFD and EM-PC3consRD and partial genotypes for the rest of cultivars (3-5 fragments). In the case of amplification using the primers PaConsII-F and PaConsII-R only partial S-genotypes were identified (2–5 fragments).

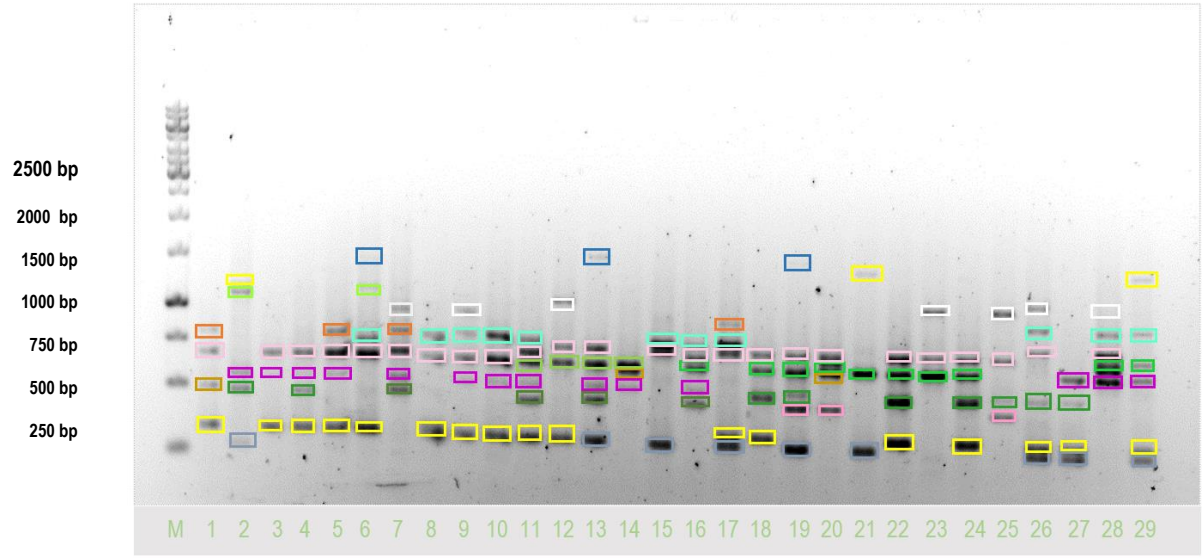

Figure 7 *Gel electrophoresis of European plum cultivars with PCR products amplifying the second intron region of the Prunus S-RNase gene [EM-PC2consFD - EM-PC3consRD* Sutherland et al. (2004)*].*

Fragments marked with a frame of the same color indicate the same S-allele. Samples: 1. Besztercei, 2. Kirke szilvája, 3. Duránci, 4. Vengerka virla, 5. Wagenheim, 6. Sanctus Hubertus 7. Fehérszilva, 8. Bühli korai, 9. Gömöri nyakas, 10. Mirabelle de Nancy, 11. Centenar, 12. Anna Spath, 13. Vörösszilva, 14. Tragedy, 15. Páczelt szilvája, 16. California blue, 17. Ageni 707, 18. Szo3, 19. Vörös nagygyümölcsű, 20. Tuleu timpulia, 21. Czar, 22. Szo2, 23. Opal, 24. Szo1, 25. Zöld ringló, 26. Bódi szilva, 27. Bellamira, 28. Grand prize, 29. Giant, Marker: GeneRuler 1kb DNA Ladder (Thermo Fisher Scientific).

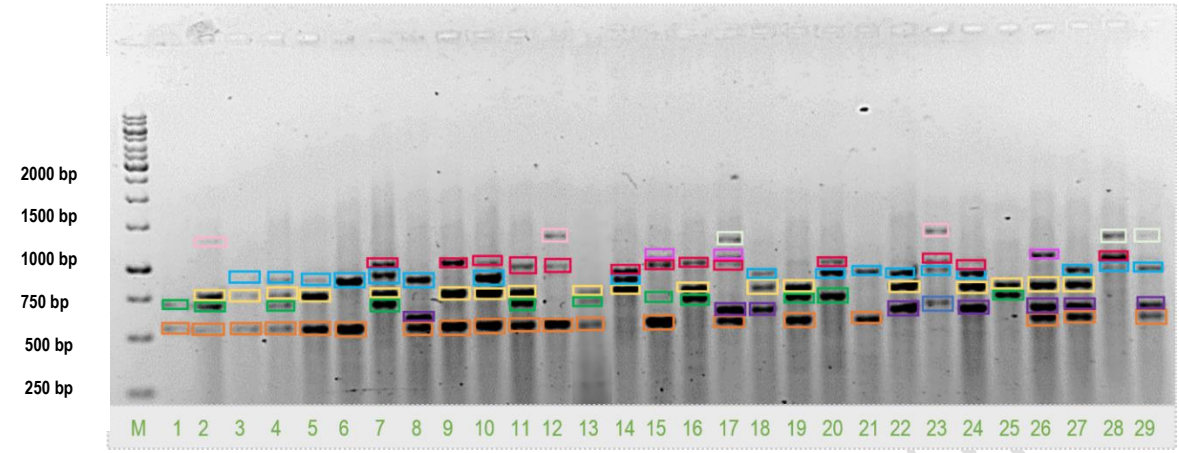

Figure 8 *Gel electrophoresis of European plum cultivars PCR products amplifying the second intron region of the Prunus S-RNase gene [PaConsII-F and PaConsII-R* Sonneveld et al. (2003)].

Fragments marked with a frame of the same color indicate the same S-allele. Samples: 1. Besztercei, 2. Kirke szilvája, 3. Duránci, 4. Vengerka virla, 5. Wagenheim, 6. Sanctus Hubertus 7. Fehérszilva, 8. Bühli korai, 9. Gömöri nyakas, 10. Mirabelle de Nancy, 11. Centenar, 12. Anna Spath, 13. Vörösszilva, 14. Tragedy, 15. Páczelt szilvája, 16. California blue, 17. Ageni 707, 18. Szo3, 19. Vörös nagygyümölcsű, 20. Tuleu timpulia, 21. Czar, 22. Szo2, 23. Opal, 24. Szo1, 25. Zöld ringló, 26. Bódi szilva, 27. Bellamira, 28. Grand prize, 29. Giant, Marker: Gene Ruler 1kb DNA Ladder (Thermo Fisher Scientific).

In the case of the primers used to detect variability in the first intron, due to the small size and limited variation in length, sequences were less than 500 bp according to the agarose gel presented in **Figure 9**, but the fragments could not be differentiated, therefore a fluorescently labelled primers were used to conduct a precise fragment length determination to improve discrimination between amplification products.

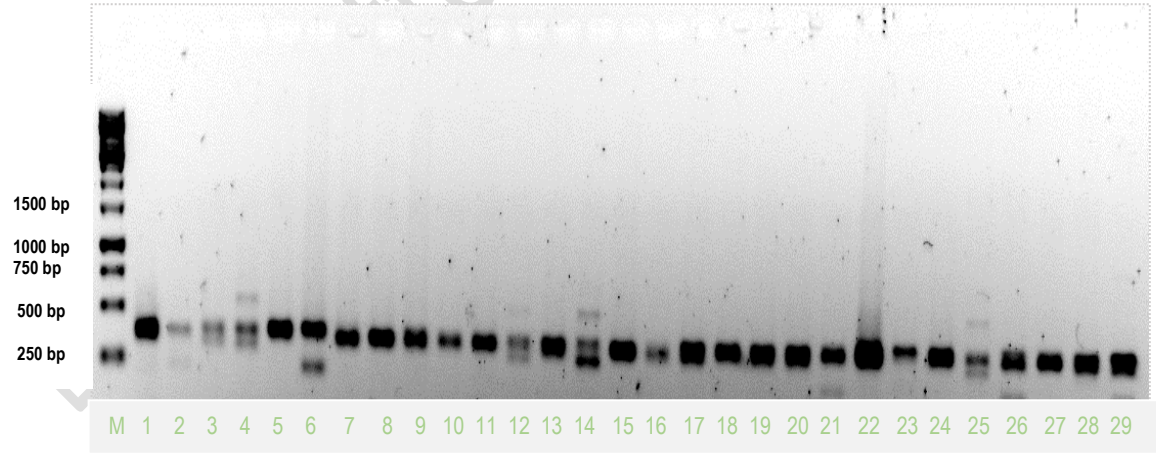

Figure 9 *Gel electrophoresis of European plum cultivars PCR products amplifying the first intron region of the Prunus S-RNase gene [PaConsI-F and EM-PC1consRD* Sonneveld et al. (2003)*,* Ortega et al. (2005)*].*

Samples: 1. Besztercei, 2. Kirke szilvája, 3. Duránci, 4. Vengerka virla, 5. Wagenheim, 6. Sanctus Hubertus 7. Fehérszilva, 8. Bühli korai, 9. Gömöri nyakas, 10. Mirabelle de Nancy, 11. Centenar, 12. Anna Spath, 13. Vörösszilva, 14. Tragedy, 15. Páczelt szilvája, 16. California blue, 17. Ageni 707, 18. Szo3, 19. Vörös nagygyümölcsű, 20. Tuleu timpulia, 21. Czar, 22. Szo2, 23. Opal, 24. Szo1, 25. Zöld ringló, 26. Bódi szilva, 27. Bellamira, 28. Grand prize, 29. Giant Marker: Gene Ruler 1kb DNA Ladder (Thermo Fisher Scientific).

The cases of sexual incompatibility presented in *P. domestica* controlled by the *S*-locus have high expectations of heterozygozity for its functioning, which, when combined with the polyploidy level of this species, predicted significant polymorphism in our findings. These results showed very high polymorphism, in fact among the three primers used in the present study, the PaConsI-F and EM-PC1consRD allowed the identification of the highest number of fragments in the total of 29 European plum cultivars, 132 fragments lengths exact sized were determined with an average number of 4.55 fragments per sample. As presented in **Table 6**, most cultivars presented between 4 and 5 alleles, the range of base pair number were from 229 to 427 bp. The cultivars 'Vörös nagygyümölcsű', 'Tuleu timpulia' and 'Bellamira' exhibited a complete *S*-genotype carrying *s*ix alleles according to the chromatograms (**Figure 10**). These results are consistent with the available study in *P. domestica* about the *S*-genotype determination using precise first intron length determination carried out by Halász et al. (2014). In the investigation in all the 16 European plums cultivars analyzed up to six fragments were amplified, mostly 4-5 per sample with sizes ranging between 232 and 416 bp.

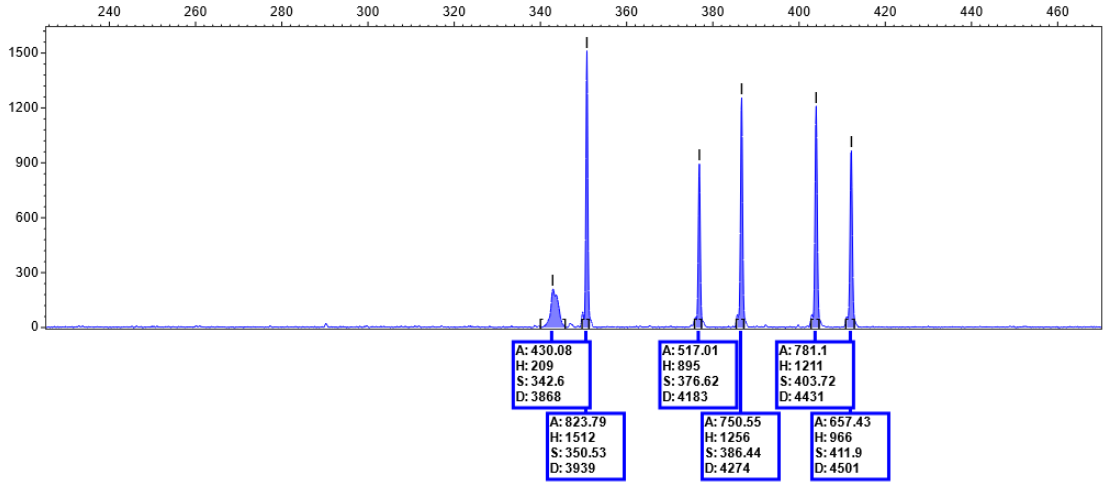

Figure 10 *Chromatogram of the fluorescently labelled first intron fragments of the S-RNase gene of the cultivar 'Vörös nagygyümölcsű' indicating a complete S-genotype with six alleles.*

Erika Jiménez

Table 6 *Precise sized S-RNase alleles obtained from PCR analysis using the PaConsI-F and EM-PC1consRD primers and automated DNA sequencing. Sizes marked the same color indicate the same S-allele.*

PaConsI-F & EM-PC1consRD Sonneveld et al. (2003), Ortega et al. (2005)	Number of peaks	Size					
Besztercei	5	351	373	388	390	412	
Kirke szilvája	4	229	322	377	385		
Duránci	4	327	377	388	412		
Vengerka virla	5	325	377	385	393	412	
Wagenheim	4	377	388	390	412		
Sanctus Hubertus	4	229	393	404	412		
Fehérszilva	4	351	360	377	427		
Bühli korai	3	373	390	393			
Gömöri nyakas	4	360	377	388	412		
Mirabelle de Nancy	5	373	377	383	393	412	
Centenar	4	377	385	395	412		
Anna Spath	4	325	373	404	412		
Vörösszilva	5	351	377	386	404	412	
Tragedy	5	325	327	330	377	412	
Páczelt szilvája	5	373	383	388	390	404	
California blue	4	351	365	377	412		
Ageni 707	4	351	383	404	416		
Szo3	5	365	374	377	404	416	
Vörös nagygyümölcsű	6	343	351	377	386	404	412
Tuleu timpulia	6	351	365	377	393	404	412
Czar	4	229	373	393	412		
Szo2	5	365	374	377	404	416	
Opal	3	368	404	412			
Szo1	5	365	374	377	404	416	
Zöld ringló	5	325	373	377	384	404	
Bódi szilva	4	229	360	377	416		
Bellamira	6	348	365	373	377	393	416
Grand prize	5	351	377	383	404	412	
Giant	5	229	351	393	404	416	

Using the same primer pair, a similar range in the length of amplified fragments was identified in sweet cherries (Vaughan et al., 2006) and Japanese plums (Guerra et al., 2009), demonstrating significant conservation of the primer region among *Prunus* species and primer pair transferability. Therefore, characterization of *S-RNase* alleles not only provides a solid foundation for its use in compatibility and self-incompatibility research, but also as molecular marker in genetic variability analyses. Because of its high degree of polymorphism and its codominant detection, *S*-locus markers have been used in conjunction with other codominant markers as simple sequence repeats to obtain an accurate genetic variability characterization. For instance, Baraket et al. (2019) in a characterization of plum genetic resources using SSR and *S*-locus markers claimed that SSR markers alone did not allow a level polyploidy identification, due to homozygosity, being *S*-genotyping a complement to identify cultivars with different polyploidy levels. These results were consistent with the study carried out by Halász et al. (2021a) using the same combination of markers for the characterization of the tetraploid *P. spinosa* and the hexaploid *P. domestica* subsp. *Insititia* who confirmed the successful use of both markers for diversity studies in polyploid *Prunus* species, however researchers also claimed that phylogenetic implications may skew results in the case of non-related cultivars sharing common *S*-alleles.

Erika Brigitte Chomicki Pérez

As discussed before, some cultivars may have incomplete *S*-genotypes due to a narrow base difference in the same alleles as can be observed in **Table 7** where the alleles have been ordered ascendingly, and some only have 1 or 2 bp number difference. Because of this homozygosity, the known cases of SC in this specie, and the evidenced and documented influence of polyploidy in SC Solanaceae and Scrophulariaceae (Vieira et al., 2008), the possibility that polyploidy can generate SC is still enigmatic, even with the findings that in *P. spinosa* or *P. insititia* polyploidy did not appear to cause SC (Nunes et al., 2006).

Table 7 *Presence (1) or absence (0) of precise sized RNase fragments obtained through an automated sequencer.*

	ALLELES																									
SAMPLES	229	322	325	327	330	343	348	351	360	365	368	373	374	377	383	384	385	386	388	390	393	395	404	412	416	427
Kirke szilvája	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0
Duránci	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0
Vengerka virla	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	0	0
Wagenheim	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	0	1	0	0
Sanctus Hubertus	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0
Fehérszilva	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Bühli korai	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	0	0
Gömöri nyakas	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0
Mirabelle de Nancy	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	0	1	0	0	1	0	0
Centenar	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	1	0	0
Anna Spath	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Vörösszilva	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0
Tragedy	0	0	1	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Páczelt szilvája	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0	1	0	0	0
California blue	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0
Ageni 707	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0
Szo3	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0
Vörös nagygyümölcsű	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	1	0	0
Tuleu timpulia	0	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0
Czar	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0
Szo2	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0
Opal	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Szo1	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0
Zöld ringló	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0
Bódi szilva	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
Bellamira	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0
Grand prize	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	0
Giant	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0
Besztercei	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	1	0	0
Total alleles	5	1	4	2	1	1	1	9	3	6	1	8	3	20	4	1	3	2	5	4	8	1	14	16	8	1

Erika Birlife Conrado Jiménez

7.2 Sequence analysis of *S-RNase* alleles

The Hungarian landraces selected for sequencing represents a valuable breeding material available at the GenBank at our university and it is critical to obtain information about its genetic background. Genomic DNA fragments of this cultivars amplified by the primer pair PaConsII-F and PaConsIIR (Sonneveld et al., 2003) were purified and sequenced: 'Duranci', 'Fehérszilva' (**Figure 11**), 'Páczelt szilvája', 'Vörös nagygyümölcsű', 'Bódi szilva' and 'Zöld ringló'. For each of these six varieties, between 3 to 6 different size allele fragments were sequenced obtaining a total of 25 DNA sequences.

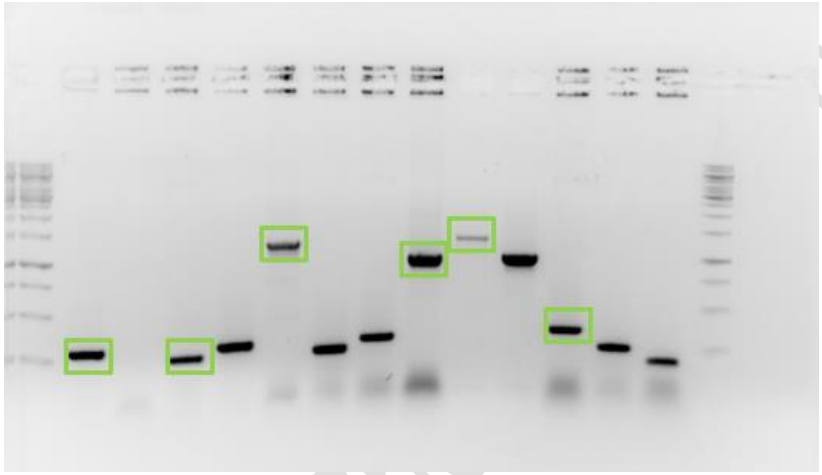

Figure 11 *PCR fragments of S-RNase allele amplified with the primer PaConsII-F and PaConsII-R selected for plasmid isolation in case of cultivar 'Fehérszilva'.*

A homology analysis was performed using the 25 newly obtained sequences at NCBI using the BLASTN search tool. The data obtained demonstrated high similarity with other already available *S-RNases Prunus* sequences, in the case of 'Duranci' the sequences obtained did not correspond to the *RNases and for* 'Bódi szilva' only a partial part of the protein was present in the sequence. For the 4 remaining cultivars, a total of 6 different *S-RNase* sequences were found, the rest of sequences analyzed corresponded to same alleles. Each of the new *S*-alleles was assigned a code identified as follow: S_{fs1}, S_{fs2}, S_{ps6}, S_{vn4}, S_{vn16}, S_{zr16} from 'Fehérszilva', 'Páczelt szilvája', 'Vörös nagygyümölcsű' and 'Zöld ringló', respectively (**Table 8**). The lengths of the *S-RNase* sequences range from 527 bp to 1050 base pair, the polymorphism length in the second intron is much higher, with base pair varying from 141 bp to 638 bp. The length of the deduced amino acid sequence did not alter significantly, yielding 128, 131, and 137 aa.

Table 8 *Results of the BLASTN analysis of sequenced P. domestica S-RNase alleles, containing the allele code, total and intron length, deduced amino acid length. Accession, specie, description, coverage, identity, and E-value of the closest homologs.*

Allele	Accession / Specie	Description	Coverage (%)	Identity (%)	E-value
S_{fs1} ***779 bp*** ***Intron: 638 bp*** **Protein: 137 aa**	MN069633.1 *Prunus domestica* subsp. insititia	*S_C-RNase* Genotype B3	98	99.48	0.0
	AY259114.1 *Prunus avium*	*S_{23}-RNase*	89	88.33	0.0
	MN069634.1 *Prunus domestica* subsp. insititia	*S_C-RNase* *Genotype L1*	88	99.42	0.0
	MF125899.1 *Prunus humilis*	*S_{10}-RNase*	82	97.95	0.0
	MF125903.1 *Prunus humilis*	*S_{14}-RNase*	82	95.97	0.0
S_{fs2} ***1050 bp*** ***Intron: 366 bp*** **Protein: 137 aa**	AB289863.1 *Prunus speciosa*	*S_{06}-RNase*	99	93.19	0.0
	JQ627810.1 *Prunus virginiana*	*S_{22}-RNase*	55	96.55	0.0
	MG601111.1 *Prunus laurocerasus*	*S_{16}-RNase*	50	88.98	8e-173
	DQ768219.1 *Prunus mume*	*S_{14}-RNase*	42	86.32	1e-126
	MG601110.1 *Prunus laurocerasus*	*S_{15}-RNase*	40	86.14	2e-124
S_{ps6} ***527 bp*** ***Intron: 141 bp*** **Protein: 128 aa**	MN069630.1 *Prunus spinosa*	*S_b-RNase* *Genotype D2*	100	99.62	0.0
	MN069631.1 *Prunus spinosa*	*S_b-RNase* *Genotype L1*	97	99.61	0.0
	MN069632.1 *Prunus spinosa*	*S_b-RNase* *Genotype Z3*	97	99.42	0.0
	JQ627792.1 *Prunus virginiana*	*S_4-RNase*	90	97.55	2e-175
	DQ118142.1 *Prunus webbii*	*S_1-RNase*	95	97.49	7e-171
S_{vn4} ***712 bp*** ***Intron: 306 bp*** **Protein: 131 aa**	MN052898.1 *Prunus spinosa*	*$S_{3\text{-}1}$-RNase* *Genotype D5*	100	99.02	0.0
	MG595258.1 *Prunus laurocerasus*	*S_8-RNase*	100	98.88	0.0
	DQ677584.1 *Prunus spinosa*	*$S_{3\text{-}1}$-RNase*	99	99.72	0.0
	DQ677585.1 *Prunus spinosa*	*$S_{3\text{-}2}$-RNase*	99	99.44	0.0
	MN052897.1 *Prunus spinosa*	*$S_{3\text{-}1}$-RNase* Genotype T1	98	99.57	0.0
S_{vn16}	DQ677584.1 *Prunus spinosa*	*$S_{3\text{-}1}$-RNase*	100	99.86	0.0
	MN052897.1 *Prunus spinosa*	*$S_{3\text{-}1}$-RNase* Genotype T1	99	99.71	0.0
	DQ677585.1 *Prunus spinosa*	*$S_{3\text{-}2}$-RNase*	100	99.57	0.0

Erika Brizuela Chonata Jiménez

701 bp ***Intron: 306 bp*** **Protein: 131 aa**	MN052898.1 *Prunus virginiana*	*$S_{3\text{-}1}$-RNase* *Genotype D5*	100	97.43	0.0
	MG595258.1 *Prunus laurocerasus*	*S_8-RNase*	100	99.00	0.0
S_{zr15} ***705 bp*** ***Intron: 292 bp*** **Protein: 137 aa**	AM746947.1 *Prunus domestica*	*S_6-RNase*	99	99.57	0.0
	AB252411.1 *Prunus salicina*	*S_{Sa}-RNase*	100	98.72	0.0
	AB084142.1 *Prunus salicina*	*S_{Sa}-RNase*	95	98.81	0.0
	AF433649.1 *Prunus salicina*	*S_{S1}-RNase*	80	98.95	0.0
	MW407937.1 *Prunus domestica*	*S_{S3}-RNase*	99	89.89	0.0

Homology comparisons revealed that all sequences were homologous to the *Prunus S-RNase* gene, 3 of the new alleles identified S_{ps6}, S_{vn4}, S_{vn16} presented the major percentage of identity to self-incompatibility genes found in *P. spinosa and* S_{fs1}, S_{fs2}, S_{zr15}, with the highest identity to *P. insititia, P. speciosa*, and *P domestica* groups, respectively. This mayor interspecific identities in comparison with the only one intraspecific observed (S_{zr15}) also has been mentioned in previous studies and has been related to the trans-specific evolution pattern, that describes a common origin present in the species prior to separation of the evolutionary lineages (Sutherland et al., 2008). For instance, Halász et al. (2021b) determined that almond presented the highest identity sequence to a cherry laurel sequence.

The degree of identity between the partial plum *S*-RNase alleles varied between 0.27 and 0.43 based on the amino acid similarity matrix (**Table 9**), which further confirms the uniqueness and novelty of the European plum alleles identified. To demonstrate the evolutionary history of these newly isolated 6 plum *S-RNase* alleles, an alignment of the deduced AA sequences was carried out by making a phylogenetic tree based on the Minimum Evolution method (**Figure 12**). New *P. domestica* alleles (marked by green color) clustered with *S*-RNases of other *Prunus* species supported by significant bootstraps. All isolated sequences widely dispersed on the tree according to the trans-specific pattern. We have confirmed that no evidence can be found about *P. domestica* sequences would be more closely located on the phylogenetic tree to its putative parents, *P. spinosa* and *P. cerasifera* than with any other characterized *Prunus* species (Fernandez i Marti et al., 2021).

Erika Brigitte Chonata Jiménez

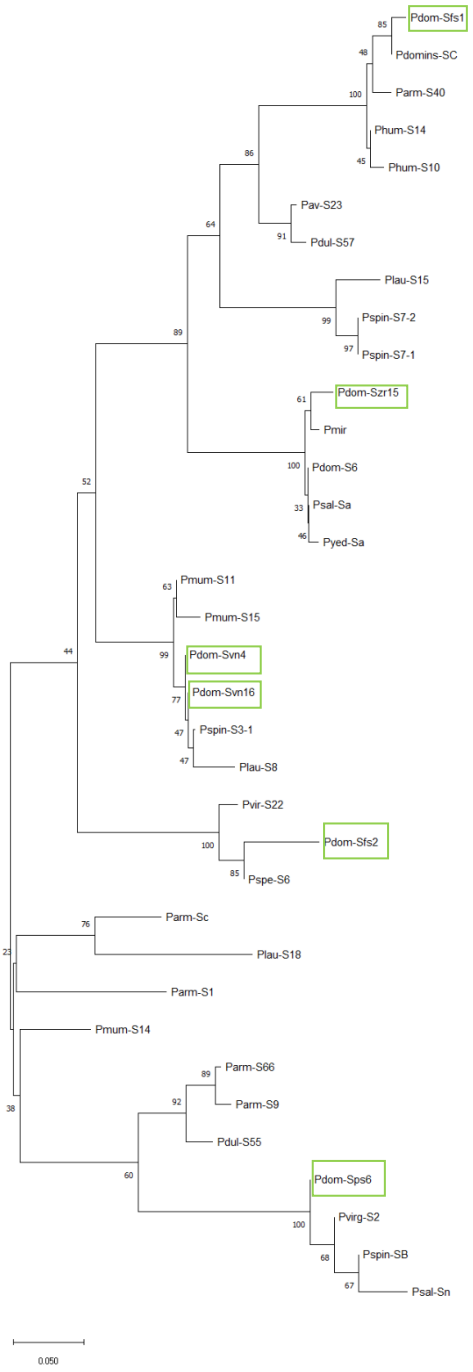

Figure 12 *Evolutionary relationships of taxa*

The Minimum Evolution method (Rzhetsky & Nei, 1992) was used to infer evolutionary history. The optimal tree with the sum of branch length = 1.64957398 is shown. Next to the branches were the percentages of replicate trees in which the related *S-RNase* allele sequences grouped together in the bootstrap test (1,000 replicates) (Felsenstein, 1985). The phylogenetic tree is scaled, in the way that branch lengths are measured in the same units as the evolutionary distances used to estimate the phylogenetic tree. The evolutionary distances were calculated using the JTT matrix-based approach (Jones et al., 1992) and are measured in amino acid substitutions per site. A gamma distribution (shape parameter = 0.8) was used to model rate variance among sites. Close-Neighbor-Interchange (CNI) algorithm (Nei & Kumar, 2000) was used to search the ME tree at a level of 1. The initial tree was generated using the Neighbor joining algorithm (Saitou & Nei, 1987). The analyses included 35 amino acid sequences. Ambiguous positions were removed completely for each sequence pair (pairwise deletion option). There was a total of 144 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). The *Prunus* species abbreviations are as follow: Pdom: *P. domestica* , Pdomins: *P. insititia*, Parm: P. armeniaca, Phum: *P. humilis*, Pav: *P. avium*, Pdul: *P dulcis*, Plau: *P. laurocerasus*, Pspin: *P.spinosa*, Pmir: *P.mira*, Psal: *P. salicina*, Pyed: *P. yedoensis*, Pvir: *P. virginiana*, Pspe: *P. speciosa*.

Erika Bri ménez

Table 9 Estimates of Evolutionary Divergence between Sequences

	Pdom-Sfs1	Pdom-Sfs2	Pdom-Sps6	Pdom-Svn4	Pdom-Svn16	Pdom-Szr15	Pdomins-SC	Parm-S40	Phum-S14	Phum-S10	Pav-S23	Pdul-S57	Pspin-S7-2	Plau-S15	Pspe-S6	Pwr-S22	Parm-S1	Pspin-S7-1	Parm-Sc	Pspin-SB	Pwrg-S2	Pspin-S3-1	Plau-S8	Pmum-S14	Psal-Sn	Parm-S66	Plau-S18	Pdul-S55	Parm-S9	Pmum-S11	Pmum-S15	Psal-Sa	Pyed-Sa	Pdom-S6
Pdom-Sfs1																																	
Pdom-Sfs2	0,43026725																																
Pdom-Sps6	0,49428677	0,41169818																															
Pdom-Svn4	0,27342871	0,25323124	0,31638940																														
Pdom-Svn1	0,28118496	0,24626177	0,31016824	0,00000100																													
Pdom-Szr1	0,27497739	0,35995422	0,44238646	0,22161235	0,22738459																												
Pdomins-S	0,00749646	0,40500212	0,45916451	0,25421161	0,26804762	0,24452389																											
Parm-S40	0,03884320	0,40499483	0,47071005	0,27427979	0,26762136	0,26373036	0,02311323																										
Phum-S14	0,03068539	0,38810089	0,43770541	0,24191806	0,25544874	0,23407834	0,02280205	0,01519093																									
Phum-S10	0,03862133	0,40223577	0,45318848	0,25392946	0,26771122	0,24568915	0,03061136	0,02294423	0,00743577																								
Pav-S23	0,12401990	0,32949005	0,42913041	0,20306377	0,19793386	0,20187707	0,11608485	0,12194985	0,12504507	0,13495087																							
Pdul-S57	0,13090395	0,34856863	0,43085371	0,19588280	0,21020548	0,19478851	0,12174022	0,11295606	0,11146494	0,12091569	0,01521446																						
Pspin-S7-2	0,21663682	0,30407757	0,48220411	0,26034520	0,26772658	0,23350592	0,21483824	0,20176119	0,19156969	0,20235365	0,18499215	0,17558185																					
Plau-S15	0,23304399	0,30881958	0,49229914	0,28861051	0,28404070	0,19332285	0,22004960	0,22702044	0,21828739	0,22937639	0,20072409	0,21246812	0,04744847																				
Pspe-S6	0,34280687	0,05247295	0,38609540	0,23308393	0,23476227	0,31560199	0,33940835	0,33576639	0,32446975	0,33751339	0,26167391	0,28773575	0,22653616	0,25233395																			
Pwr-S22	0,32977338	0,07794118	0,39406109	0,21934517	0,22090062	0,30138134	0,32639459	0,32496855	0,31375767	0,32672672	0,24914104	0,27716651	0,24126560	0,26681819	0,03747344																		
Parm-S1	0,37551245	0,26047250	0,27850895	0,26233182	0,25574019	0,39336383	0,36945063	0,37640487	0,35478011	0,36842979	0,28516092	0,30220222	0,30491166	0,32677077	0,20555984	0,21850632																	
Pspin-S7-1	0,21663682	0,30407757	0,48220411	0,26034520	0,26772658	0,23350592	0,21483824	0,20176119	0,19156969	0,20235365	0,18499215	0,17558185	0,00000100	0,04744847	0,22653616	0,24126560	0,30491166																
Parm-Sc	0,35426318	0,30239210	0,32052042	0,28642953	0,26794060	0,29532169	0,33469615	0,33975840	0,32096760	0,33398565	0,30480349	0,32222765	0,33312698	0,32537583	0,24841669	0,23639268	0,22093700	0,33312698															
Pspin-SB	0,52533308	0,45397596	0,01536738	0,31656427	0,31287581	0,47460318	0,50723753	0,49978572	0,48416010	0,50082522	0,45523855	0,46472254	0,51189016	0,53966287	0,40477570	0,42517303	0,29326869	0,51189016	0,35207617														
Pwrg-S2	0,50917204	0,45564067	0,03903351	0,30677276	0,30916320	0,47579030	0,49604826	0,50418284	0,47345418	0,48976453	0,44123299	0,45051927	0,49660370	0,53992520	0,39283585	0,41256137	0,28476096	0,49660370	0,35528010	0,01529709													
Pspin-S3-1	0,29233697	0,25721882	0,32184016	0,00770597	0,00763395	0,23782266	0,27904828	0,27855150	0,26627804	0,27867049	0,20851416	0,22083830	0,27883064	0,29526049	0,24555782	0,23153646	0,26674627	0,27883064	0,25663644	0,32463610	0,32085242												
Plau-S8	0,32260920	0,28780469	0,35333306	0,02308304	0,02342162	0,26203695	0,30329854	0,32334794	0,29009400	0,30282830	0,23838342	0,24221429	0,30914992	0,33877468	0,26724024	0,25294230	0,29697282	0,30914992	0,32106229	0,35484345	0,34402051	0,03131874											
Pmum-S14	0,33260974	0,26866065	0,23379887	0,17743006	0,17475714	0,24469903	0,31297253	0,30653567	0,30929794	0,32788671	0,26918309	0,30209155	0,33190329	0,33202087	0,20322378	0,20676858	0,32821501	0,33190329	0,23182012	0,23660428	0,23236958	0,18877336	0,20737981										
Psal-Sn	0,60996308	0,52290836	0,03484341	0,36162545	0,35688393	0,54818347	0,58798536	0,57869405	0,56016865	0,58034271	0,52605498	0,53683033	0,56796685	0,60081004	0,46433997	0,48887967	0,33238266	0,56796685	0,38589298	0,01690838	0,01688312	0,37045198	0,40609985	0,27549783									
Parm-S66	0,45780456	0,35558792	0,21079249	0,26466539	0,24518369	0,34983391	0,43737849	0,43511197	0,41634420	0,43152211	0,30556984	0,31874843	0,40815447	0,42592949	0,31499848	0,32411341	0,28921661	0,40815447	0,28118250	0,20150214	0,19744096	0,25642048	0,30033671	0,24788751	0,21600708								
Plau-S18	0,45904591	0,42297999	0,28024024	0,30851352	0,31556825	0,38956700	0,43905332	0,45446402	0,41681588	0,43212181	0,36968329	0,37077450	0,48736307	0,51600433	0,37219468	0,36127044	0,26470077	0,48736307	0,15983605	0,28935628	0,28002894	0,30325363	0,34528606	0,23547092	0,32863994	0,35525783							
Pdul-S55	0,44577155	0,38838429	0,20122783	0,25372340	0,25562914	0,35031574	0,43300184	0,44118945	0,41173551	0,42708188	0,29107849	0,29993685	0,39518378	0,44390476	0,31333480	0,32263159	0,29134395	0,39518378	0,31663952	0,18919125	0,17449932	0,26688036	0,28936754	0,21241493	0,20202222	0,04820235	0,31750060						
Parm-S9	0,46318333	0,37397499	0,22425683	0,25453859	0,25645851	0,36776807	0,45050390	0,45855615	0,42918771	0,44456636	0,30837644	0,31709731	0,41263728	0,46145329	0,31332557	0,32240130	0,29186913	0,41263728	0,30964238	0,21182124	0,19634482	0,26775740	0,29033736	0,26317357	0,22765122	0,01536389	0,34519218	0,04792243					
Pmum-S11	0,26127735	0,26105490	0,32400653	0,01521253	0,00765518	0,23265609	0,25398362	0,26214914	0,24171857	0,25370292	0,18142937	0,19577051	0,24828713	0,27618619	0,22933839	0,21582269	0,25887182	0,24828713	0,27443045	0,31125827	0,30172033	0,01535459	0,03861402	0,16928347	0,35489770	0,24821439	0,32117110	0,24950327	0,25029056				
Pmum-S15	0,27189716	0,28452407	0,34910111	0,03081924	0,02327370	0,25485809	0,26471817	0,27274790	0,25228352	0,26439722	0,20331587	0,21815599	0,27148798	0,29994758	0,25224276	0,23833514	0,28218333	0,27148798	0,29775400	0,33699380	0,32676259	0,03111950	0,05474882	0,16928347	0,36921597	0,27194835	0,34643302	0,27343172	0,27432198	0,01508694			
Psal-Sa	0,24064800	0,37370040	0,45757685	0,21064402	0,21627117	0,02221656	0,23338735	0,24288389	0,22304424	0,23449568	0,17085536	0,17388968	0,20141332	0,18364167	0,29074618	0,27693291	0,35441003	0,20141332	0,28408405	0,45958320	0,44568902	0,22661439	0,25042378	0,22853051	0,53028038	0,33735419	0,37604051	0,32316928	0,34067268	0,19961518	0,22107359		
Pyed-Sa	0,24432700	0,38611326	0,47137758	0,21392812	0,21971728	0,02973126	0,23702269	0,24661262	0,22653803	0,23816130	0,17347550	0,17659210	0,20457066	0,18664850	0,30230092	0,28831318	0,35969917	0,20457066	0,29518884	0,47387296	0,45955354	0,23020203	0,25431454	0,24410485	0,54727806	0,34971238	0,38851809	0,33575477	0,35321771	0,20273555	0,22449617	0,00728168	
Pdom-S6	0,24380085	0,36604891	0,45042733	0,20226633	0,21627117	0,01483455	0,23338735	0,24608860	0,22304424	0,23449568	0,17289649	0,17388968	0,20389343	0,18580288	0,29074618	0,27693291	0,35959135	0,20389343	0,28796144	0,45958320	0,44568902	0,22661439	0,24210393	0,22853051	0,53028038	0,34245277	0,36837835	0,32316928	0,34067268	0,20214710	0,22393263	0,00000100	0,00734549	
Pmir	0,25642169	0,36773905	0,45291277	0,20479050	0,21750076	0,02250815	0,24413905	0,24526813	0,23370310	0,24530169	0,18390839	0,18494731	0,21545900	0,18626915	0,30460186	0,29047805	0,37475495	0,21545900	0,28904324	0,46240799	0,45223619	0,22790069	0,24522482	0,21328939	0,53393782	0,35833977	0,39856515	0,35303695	0,37057893	0,20466553	0,22675027	0,01492168	0,02246750	0,01492168

Note: Number of amino acid substitutions per site from between sequences are shown. The JTT matrix-based model (Jones et al., 1992) was used for the analyses. A gamma distribution (shape parameter = 0.8) was used to model rate variation among sites. The analyses included 35 amino acid sequences. Ambiguous positions were removed completely for each sequence pair (pairwise deletion option). There was a total of 144 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Erika Brigitte Chávez Jiménez

7. CONCLUSIONS

- The present study describes for the first time the European plum (*P. domestica* L.*) S*-allele diversity of the GenBank collection at the university. Three consensus primer pairs spanning the first and second intron of the *S-RNase* gene were compared to evaluate the robustness of *S*-genotyping in hexaploid cultivars. The results revealed high genetic diversity among the 29 genotypes, different allelic composition was found in the 29 accessions evaluated. Among the three primers used in this study, the PaConsI-F and EM-PC1consRD allowed the identification of the greatest number of alleles, 132 exact sized fragments, with an average of 4.55 fragments per sample.
- The purification and cloning of *P. domestica* genomic DNA amplified with the primers PaConsII allowed us to obtain six new European plum *S-RNase* sequences, which were temporarily marked with the cultivar's name abbreviation until publication: S_{fs1}, S_{fs2}, S_{ps6}, S_{vn4}, S_{vn16}, S_{zr16}. From these sequences a deduced amino acid sequences and typical intron length were determined, which allowed to carry out a phylogenetic analysis confirming that the new *S*-allele sequences are distributed across the phylogeny tree showing trans-specific origin.
- This research has allowed us to continue the *S*-locus analysis of polyploid fruit tree species that has been ongoing for a time at the Plant Biotechnology Department. It provides an additional basis for understanding the functioning of this complex reproductive system and novel *S-RNase* sequences useful for germplasm characterization and evolutionary studies.

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8. SUMMARY

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Variability of the S-RNase alleles in the hexaploid European plum (Prunus domestica L.)

Agriculture Biotechnology MSc.

The gametophytic self-incompatibility system (GSI) that promotes outcrossing and avoids inbreeding is present in the hexaploid European plum (*Prunus domestica* L.) and is controlled by two tightly linked genes that constitute the *S*-locus. One of them, a *S*-RNase protein with highly polymorphism in its two introns. This study is the first to describe the variability of the *S-RNase* alleles in the hexaploid plum of the GenBank collection at the university. For this purpose, three consensus primer pairs targeting the first and second intron were compared to assess the robustness of *S*-genotyping in hexaploid cultivars. The effectivity of the three primers was demonstrated in the 29 accessions, obtaining different allelic composition for all the cultivars, which exhibits a high genetic diversity. Among the three primers used in this study, the primer targeting the first intron (PaConsI-F and EM-PC1consRD) allowed the identification of the highest number of alleles, 132 exact sized fragments, with an average of 4.55 fragments per sample. In the case of the primers used to detect variability in the second intron, the EM-PC2consFD and EM-PC3consRD detected 127 bands with an average of 4.38 bands per cultivar, while PaConsII-F and PaConsII-R detected 96 bands, with an average 3.31 bands among the 29 cultivars.

Six new *S-RNase* sequences were obtained from important Hungarian landraces such as, 'Fehérszilva', 'Páczelt szilvája', 'Vörös nagygyümölcsű', and 'Zöld ringló', which were result from the PCR amplification, cloning and sequencing of the second intron. A homology analysis of these new *S-RNase* alleles revealed high similarity with available *S-RNases Prunus* sequences. A phylogenetic analysis was performed using the obtained deduced amino acid sequences and typical intron length, confirming that the new *S*-allele sequences are distributed across the phylogeny tree, indicating a trans-specific origin. The data and new sequences identified will provide valuable information for germplasm characterization, evolutionary studies and for pollination compatibility analyses in plum breeding programs.

Erika Brigitte Chonata Jiménez

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All my achievements are dedicated to my angel in heaven, mi mami Celita, who was my example and inspiration, every day she gave me her unconditional love, support, and patience, who inspires me to make my dreams come true and to be better day by day.

Erika Brigitte Chonata Jiménez

10. BIBLIOGRAPHY

Abdallah, D., Baraket, G., Perez, V., Ben Mustapha, S., Salhi-Hannachi, A., & Hormaza, J. I. (2019). Analysis of self-incompatibility and genetic diversity in diploid and hexaploid plum genotypes. *Frontiers in Plant Science*, *10*. https://doi.org/10.3389/fpls.2019.00896

Ahmad, M. H., Rao, M. J., Hu, J., Xu, Q., Liu, C., Cao, Z., Larkin, R. M., Deng, X., Bosch, M., & Chai, L. (2022). Systems and breakdown of self-incompatibility. *Critical Reviews in Plant Sciences*, *41*(3), 209–239. https://doi.org/10.1080/07352689.2022.2093085

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Bahrin, A. A., Moshawih, S., Dhaliwal, J. S., Kanakal, M. M., Khan, A., Lee, K. S., Goh, B. H., Goh, H. P., Kifli, N., & Ming, L. C. (2022). Cancer protective effects of plums: A systematic review. *Biomedicine & Pharmacotherapy*, *146*, 112568. https://doi.org/10.1016/J.BIOPHA.2021.112568

Baraket, G., Abdallah, D., Ben Mustapha, S., Ben Tamarzizt, H., & Salhi-Hannachi, A. (2019). Combination of Simple Sequence Repeat, *S*-Locus Polymorphism and Phenotypic Data for Identification of Tunisian Plum Species (*Prunus* spp.). *Biochemical Genetics*, *57*(5), 673–694. https://doi.org/10.1007/S10528-019-09922-4

Bošković, R., & Tobutt, K. R. (1996). Correlation of stylar ribonuclease zymograms with incompatibility alleles in sweet cherry. *Euphytica*, *90*(2), 245–250. https://doi.org/10.1007/BF00023865/METRICS

Brace, J., Ockendon, D. J., & King, G. J. (1993). Development of a method for the identification of *S* alleles in *Brassica oleracea* based on digestion of PCR-amplified DNA with restriction endonucleases. *Sexual Plant Reproduction*, *6*(2), 133–138. https://doi.org/10.1007/BF00227658/METRICS

Butac, M., Bozhkova, V., Zhivondov, A., Milosevic, N., Bellini, E., Nencetti, V., Blazek, J., Balsemin, E., Lafarque, B., Kaufmane, E., Gravite, I., Vasiljeva, M., Pintea, M., Juraveli, A., Webster, T., Hjalmarsson, I., Trajkovski, V., & Hjeltnes, S. H. (2012). Overview of plum breeding in Europe. *Acta Horticulturae*, *981*(1), 91–98. https://doi.org/10.17660/ACTAHORTIC.2013.981.9

Büyükköroğlu, G., Dora, D. D., Özdemir, F., & Hizel, C. (2018). Techniques for Protein Analysis. *Omics Technologies and Bio-Engineering: Towards Improving Quality of Life*, *1*, 317–351. https://doi.org/10.1016/B978-0-12-804659-3.00015-4

Cappadocia, M. (2003). FLOWERING AND REPRODUCTION | Gametophytic Self-Incompatibility. *Encyclopedia of Applied Plant Sciences*, 333–342. https://doi.org/10.1016/B0-12-227050-9/00019-3

Casselman, A. L., Vrebalov, J., Conner, J. A., Singhal, A., Giovannoni, J., Nasrallah, M. E., & Nasrallah, J. B. (2000). Determining the physical limits of the *Brassica S* locus by recombinational analysis. *The Plant Cell*, *12*(1), 23–33. https://doi.org/10.1105/TPC.12.1.23

Claessen, H., Keulemans, W., Van de Poel, B., & De Storme, N. (2019). Finding a compatible partner: Self-incompatibility in european pear (*Pyrus communis*); molecular control, genetic determination, and impact on fertilization and fruit set. *Frontiers in Plant Science*, *10*, 407. https://doi.org/10.3389/FPLS.2019.00407/BIBTEX

DeNettancourt, D. (1977). Incompatibility in angiosperms . In *Monographs on theoretical and applied genetics* (Springer, Vol. 3).

Erika Brigitte Coronata Jiménez

FAO (2019). Plums and sloes. Production indices. https://www.fao.org/faostat/en/#data/QI

Faust, M., Timon, B., Surányi, D., Nyujtó, F., & Gradziel, T. M. (2011). Origin and dissemination of *Prunus* crops: peach, cherry, apricot, plum and almond. International Society for Horticultural Science (ISHS).

Felsenstein, J. (1985). Confidence Limits on Phylogenies: An Approach Using the Bootstrap. *Evolution*, *39*(4), 783. https://doi.org/10.2307/2408678

Fernandez i Marti, A. F., Castro, S., DeJong, T. M., & Dodd, R. S. (2021). Evaluation of the *S*-locus in *Prunus domestica*, characterization, phylogeny and 3D modelling. *PLoS ONE*, *16*(5). https://doi.org/10.1371/journal.pone.0251305

Furstenau, T. N., & Cartwright, R. A. (2017). The impact of self-incompatibility systems on the prevention of biparental inbreeding. *PeerJ*, *2017*(11). https://doi.org/10.7717/PEERJ.4085/SUPP-1

Gago, D., Sánchez, C., Aldrey, A., Christie, C. B., Bernal, M. Á., & Vidal, N. (2022). Micropropagation of Plum (*Prunus domestica* L.) in Bioreactors Using Photomixotrophic and Photoautotrophic Conditions. *Horticulturae 2022, Vol. 8, Page 286*, *8*(4), 286. https://doi.org/10.3390/HORTICULTURAE8040286

Guerra, M. E., & Rodrigo, J. (2015). Japanese plum pollination: A review. *Scientia Horticulturae*, *197*, 674–686. https://doi.org/https://doi.org/10.1016/j.scienta.2015.10.032

Guerra, M. E., Rodrigo, J., López-Corrales, M., & Wünsch, A. (2009). *S-RNase* genotyping and incompatibility group assignment by PCR and pollination experiments in Japanese plum. *Plant Breeding*, *128*(3), 304–311. https://doi.org/10.1111/J.1439-0523.2008.01552.X

Guerra, M. E., Wünsch, A., Ĺopez-Corrales, M., & Rodrigo, J. (2010). Flower Emasculation as the Cause for Lack of Fruit Set in Japanese Plum Crosses. *Journal of the American Society for Horticultural Science*, *135*(6), 556–562. https://doi.org/10.21273/JASHS.135.6.556

Halász, J., & Hegedűs, A. (2006). A critical evaluation of methods used for *S*-genotyping: from trees to DNA level. *International Journal of Horticultural Science*, *12*(2), 19–22.

Halász, J., Kurilla, A. &, & Hegedûs, A. (2014). Preliminary characterization of the self-incompatibility genotypes of European plum (*Prunus domestica* L.) cultivars. *International Journal of Horticultural Science*, *20*(4), 23–26.

Halász, J., Makovics-Zsohár, N., Szőke, F., Ercisli, S., & Hegedűs, A. (2021a). Simple Sequence Repeat and *S*-Locus Genotyping to Assist the Genetic Characterization and Breeding of Polyploid *Prunus* Species, *P. spinosa* and *P. domestica* subsp. *insititia*. *Biochemical Genetics*, *59*(4), 1065–1087. https://doi.org/10.1007/s10528-021-10090-7

Halász, J., Molnár, A. B., Ilhan, G., Ercisli, S., & Hegedűs, A. (2021b). Identification and Molecular Analysis of Putative Self-Incompatibility Ribonuclease Alleles in an Extreme Polyploid Species, *Prunus laurocerasus* L. *Frontiers in Plant Science*, *12*. https://doi.org/10.3389/fpls.2021.715414

Herrera, S., Rodrigo, J., Hormaza, J. I., & Lora, J. (2018). Identification of Self-Incompatibility Alleles by Specific PCR Analysis and S-RNase Sequencing in Apricot. *International Journal of Molecular Sciences*, *19*(11). https://doi.org/10.3390/IJMS19113612

Igic, B., Lande, R., & Kohn, J. R. (2008). Loss of self-incompatibility and its evolutionary consequences. *International Journal of Plant Sciences*, *169*(1), 93–104. https://doi.org/10.1086/523362/0

Erika Brigitte Gonzalez Jiménez

Igwe, E. O., & Charlton, K. E. (2016). A Systematic Review on the Health Effects of Plums (*Prunus domestica* and *Prunus salicina*). *Phytotherapy Research : PTR*, *30*(5), 701–731. https://doi.org/10.1002/PTR.5581

Jones, D. T., Taylor, W. R., & Thornton, J. M. (1992). The rapid generation of mutation data matrices from protein sequences. *Computer Applications in the Biosciences : CABIOS*, *8*(3), 275–282. https://doi.org/10.1093/BIOINFORMATICS/8.3.275

Kardile, H. B., Yilma, S., & Sathuvalli, V. (2022). Molecular Approaches to Overcome Self-Incompatibility in Diploid Potatoes. *Plants 2022, Vol. 11, Page 1328*, *11*(10), 1328. https://doi.org/10.3390/PLANTS11101328

Kota-Dombrovska, I., & Lācis, G. (2013). Evaluation of Self-Incompatibility Locus Diversity of Domestic Plum (*Prunus Domestica* L.) using DNA-Based S-Genotyping. *Proceedings of the Latvian Academy of Sciences. Section B. Natural, Exact, and Applied Sciences.*, *67*(2), 109–115. https://doi.org/doi:10.2478/prolas-2013-0017

Kozma, P., Nyéki, J., Soltész, M., & Szabó, Z. (2003). Floral biology, pollination and fertilization in temperate zone fruit species and grape. *Floral Biology, Pollination and Fertilization in Temperate Zone Fruit Species and Grape.*

Kučera, V., Chytilová, V., Vyvadilová, M., & Klíma, M. (2006). Hybrid breeding of cauliflower using self-incompatibility and cytoplasmic male sterility. *Http://Agriculturejournals.Cz/Doi/10.17221/3754-HORTSCI.Html*, *33*(4), 148–152. https://doi.org/10.17221/3754-HORTSCI

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/MOLBEV/MSY096

Larsen, B., Ørgaard, M., Toldam-Andersen, T. B., & Pedersen, C. (2016). A high-throughput method for genotyping *S-RNase* alleles in apple. *Molecular Breeding*, *36*(3), 1–10. https://doi.org/10.1007/S11032-016-0448-0

Lee, H. S., Huang, S., & Kao, T. H. (1994). S proteins control rejection of incompatible pollen in *Petunia inflata*. *Nature*, *367*(6463), 560–563. https://doi.org/10.1038/367560A0

Lewis, D., & Crowe, L. K. (1954). Structure of the incompatibility gene. *Heredity 1954 8:3*, *8*(3), 357–363. https://doi.org/10.1038/hdy.1954.38

Makovicsné Zsohár, N., & Halász, J. (2019). *Characterization of plum cultivars using SSR And S-Locus Markers.* [Doctoral dissertation theses]. Szent István University.

McClure, B. A., Haring, V., Ebert, P. R., Anderson, M. A., Simpson, R. J., Sakiyama, F., & Clarke, A. E. (1989). Style self-incompatibility gene products of *Nicotlana alata* are ribonucleases. *Nature 1989 342:6252*, *342*(6252), 955–957. https://doi.org/10.1038/342955a0

Muñoz-Sanz, J. V., Zuriaga, E., Cruz-García, F., McClure, B., & Romero, C. (2020). Self-(In)compatibility Systems: Target Traits for Crop-Production, Plant Breeding, and Biotechnology. *Frontiers in Plant Science*, *11*, 195. https://doi.org/10.3389/FPLS.2020.00195/BIBTEX

Nei, M., & Kumar, S. (2000). Evolutionary Change of DNA Sequences. *Molecular Evolution and Phylogenetics*, 33–50. https://global.oup.com/academic/product/molecular-evolution-and-phylogenetics-9780195135855

Erika Brigitte Chorda Jiménez

Neumuller, M., Dittrich, F., & Holzapfel, C. (2021). Recent achievements and future challenges in breeding for European plum cultivars: Finding the suitable genetic resources for important traits. *Acta Horticulturae*, *1322*, 1–5. https://doi.org/10.17660/ACTAHORTIC.2021.1322.1

Nikolić, D., & Milatović, D. (2010). Examining self-compatibility in plum (*Prunus domestica* L.) by fluorescence microscopy. *Genetika*, *42*(2), 387–396. https://doi.org/10.2298/GENSR1002387N

Nunes, M. D. S., Santos, R. A. M., Ferreira, S. M., Vieira, J., & Vieira, C. P. (2006). Variability patterns and positively selected sites at the gametophytic self-incompatibility pollen SFB gene in a wild self-incompatible *Prunus spinosa* (Rosaceae) population. *New Phytologist*, *172*(3), 577–587. https://doi.org/10.1111/J.1469-8137.2006.01838.X

OCDE. (2002). *Consensus Document on the Biology of Prunus spp. (Stone fruits). https://bch.cbd.int/en/database/41870*

O'Farrell, P., Goodman, H., & O'Farrell, P. (1977). High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell, 12(4)*, 1133-1142. https://www.sciencedirect.com/science/article/pii/0092867477901763

Ortega, B.G. Sutherland, F. Dicenta, R. Boškovic, & K.R. Tobutt. (2005). Determination of incompatibility genotypes in almond using first and second intron consensus primers: Detection of new *S*-alleles and correlation of reported *S* genotypes. *Plant Breed.*, *124*, 188–196.

Qiao, H., Wang, H., Zhao, L., Zhou, J., Huang, J., Zhang, Y., & Xue, Y. (2004). The F-box protein AhSLF-S2 physically interacts with S-RNases that may be inhibited by the ubiquitin/26S proteasome pathway of protein degradation during compatible pollination in Antirrhinum. *The Plant Cell*, *16*(3), 582–595. https://doi.org/10.1105/TPC.017673

Reales, A., Sargent, D. J., Tobutt, K. R., & Rivera, D. (2010). Phylogenetics of Eurasian plums, *Prunus* L. section *Prunus* (Rosaceae), according to coding and non-coding chloroplast DNA sequences. *Tree Genetics and Genomes*, *6*(1), 37–45. https://doi.org/10.1007/S11295-009-0226-9

Ricci, A., Sabbadini, S., Prieto, H., Padilla, I. M. G., Dardick, C., Li, Z., Scorza, R., Limera, C., Mezzetti, B., Perez-Jimenez, M., Burgos, L., & Petri, C. (2020). Genetic Transformation in Peach (*Prunus persica* L.): Challenges and Ways Forward. *Plants 2020, Vol. 9, Page 971*, *9*(8), 971. https://doi.org/10.3390/PLANTS9080971

Romero, C., Vilanova, S., Burgos, L., Martínez-Calvo, J., Vicente, M., Llácer, G., & Badenes, M. L. (2004). Analysis of the S-locus structure in Prunus armeniaca L. Identification of S-haplotype specific S-RNase and F-box genes. *Plant Molecular Biology*, *56*(1), 145–157. https://doi.org/10.1007/S11103-004-2651-3/METRICS

Rybin, W. (1936). Spontane und experimentell erzeugte Bas tarde zwischen Schwarz dom und Kirschpflaume und das Abstammungsproblem der Kulturpfl alj'!le. *Pianta* , *25*, 22–58.

Ryugo, Kay. (1988). *Fruit culture : its science and art*. 344.

Rzhetsky, A., & Nei, M. (1992). A Simple Method for Estimating and Testing Minimum-Evolution Trees. *Molecular Biology and Evolution*, *9*(5), 945–945. https://doi.org/10.1093/OXFORDJOURNALS.MOLBEV.A040771

Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, *4*(4), 406–425. https://doi.org/10.1093/OXFORDJOURNALS.MOLBEV.A040454

Erika Brigitte Chávez Jiménez

Sassa, H. (2016). Molecular mechanism of the *S-RNase*-based gametophytic self-incompatibility in fruit trees of Rosaceae. *Breeding Science*, *66*(1), 116–121. https://doi.org/10.1270/JSBBS.66.116

Sassa, H., Hirano, H., & Ikehashi, H. (1993). Identification and characterization of stylar glycoproteins associated with self-incompatibility genes of Japanese pear, Pyrus serotina Rehd. *MGG Molecular & General Genetics*, *241*(1–2), 17–25. https://doi.org/10.1007/BF00280196/METRICS

Sattler, M. C., Carvalho, C. R., & Clarindo, W. R. (2016). The polyploidy and its key role in plant breeding. *Planta*, *243*(2), 281–296. https://doi.org/10.1007/S00425-015-2450-X

Shukla, R. K., & Kishan. (2021). A review on European plum (*Prunus domestica*) for its pharmacological activities and phytochemicals. *Research Journal of Pharmacy and Technology*, *14*(2), 1155–1162. https://doi.org/10.5958/0974-360X.2021.00207.9

Silva, N. F., & Goring, D. R. (2001). Mechanisms of self-incompatibility in flowering plants. *Cellular and Molecular Life Sciences*, *58*(14), 1988–2007. https://doi.org/10.1007/PL00000832/METRICS

Sonneveld, T., Tobutt, K. R., & Robbins, T. P. (2003a). Allele-specific PCR detection of sweet cherry self-incompatibility (S) alleles S1 to S16 using consensus and allele-specific primers. *Theoretical and Applied Genetics*, *107*(6), 1059–1070. https://doi.org/10.1007/s00122-003-1274-4

Sottile, F., Caltagirone, C., Giacalone, G., Peano, C., & Barone, E. (2022). Unlocking Plum Genetic Potential: Where Are We At? *Horticulturae*, *8*(2). https://doi.org/10.3390/HORTICULTURAE8020128

Sun, L., Williams, J., Li, S., Wu, L., Khatri, W., Stone, P., Keebaugh, M., & Kaoa, T. H. (2018). *S*-Locus F-Box Proteins Are Solely Responsible for *S-RNase*-Based Self-Incompatibility of *Petunia* Pollen. *The Plant Cell*, *30*(12), 2959–2972. https://doi.org/10.1105/TPC.18.00615

Sun, P., Li, S., Lu, D., Williams, J. S., & Kao, T.-H. (2015). Pollen *S*-locus F-box proteins of *Petunia* involved in *S-RNase*-based self-incompatibility are themselves subject to ubiquitin-mediated degradation. *The Plant journal : for cell and molecular biology*, *83*(2), 213–223. https://doi.org/10.1111/tpj.12880

Surányi, D. 1947-. (2019). *A házi szilva : Prunus domestica LINNÉ és rokonfajai*.

Sutherland, B. G., Tobutt, K. R., & Robbins, T. P. (2004). Molecular genetics of self-incompatibility in plums. *Acta Horticulturae*, *663*, 557–562. https://doi.org/10.17660/ACTAHORTIC.2004.663.97

Sutherland, B. G., Tobutt, K. R., & Robbins, T. P. (2008). Trans-specific S-RNase and SFB alleles in *Prunus* self-incompatibility haplotypes. *Molecular genetics and genomics : MGG*, *279*(1), 95–106. https://doi.org/10.1007/s00438-007-0300-7

Takayama, S., & Isogai, A. (2005). Self-incompatibility in plants. *Annual Review of Plant Biology*, *56*, 467–489. https://doi.org/10.1146/ANNUREV.ARPLANT.56.032604.144249

Tao, R., Yamane, H., Sassa, H., Mori, H., Gradziel, T. M., Dandekar, A. M., & Sugiura, A. (1997). Identification of stylar RNases associated with gametophytic self-incompatibility in almond (*Prunus dulcis). Plant & Cell Physiology*, *38*(3), 304–311. https://doi.org/10.1093/OXFORDJOURNALS.PCP.A029167

Tao, R., Yamane, H., Sugiura, A., Murayama, H., Sassa, H., & Mori, H. (1999). Molecular Typing of *S*-alleles through Identification, Characterization and cDNA Cloning for S-RNases in Sweet Cherry. *Journal of the American Society for Horticultural Science*, *124*(3), 224–233. https://doi.org/10.21273/JASHS.124.3.224

Thiele, I., & Strydom, D. (1964). Incompatibility studies in some Japanese plum Cultivars (*Prunus salicina* Lindl.) grown in South Africa. *South African Journal Agricultural Science*, *7*, 165–168.

Erika Brigitta Orosz-Jánossy

Topp, B. L., Russell, D. M., Neumüller M., Dalbó, M., & Liu, W. (2012). Plum. In *Fruit breeding, Handbook of Plant Breeding* (pp. 571–622). Springer.

Trojak-Goluch, A., Kawka-Lipińska, M., Wielgusz, K., & Praczyk, M. (2021). Polyploidy in Industrial Crops: Applications and Perspectives in Plant Breeding. *Agronomy 2021, Vol. 11, Page 2574*, *11*(12), 2574. https://doi.org/10.3390/AGRONOMY11122574

Ushijima, K., Sassa, H., Dandekar, A. M., Gradziel, T. M., Tao, R., & Hirano, H. (2003). Structural and Transcriptional Analysis of the Self-Incompatibility Locus of Almond: Identification of a Pollen-Expressed F-Box Gene with Haplotype-Specific Polymorphism. *The Plant Cell*, *15*(3), 771. https://doi.org/10.1105/TPC.009290

Vaughan, S. P., Russell, K., Sargent, D. J., & Tobutt, K. R. (2006). Isolation of *S-locus F-box* alleles in *Prunus avium* and their application in a novel method to determine self-incompatibility genotype. *TAG. Theoretical and Applied Genetics. Theoretische Und Angewandte Genetik*, *112*(5), 856–866. https://doi.org/10.1007/S00122-005-0187-9

Vieira, C. P., Charlesworth, D., & Vieira, J. (2003). Evidence for rare recombination at the gametophytic self-incompatibility locus. *Heredity*, *91*(3), 262–267. https://doi.org/10.1038/SJ.HDY.6800326

Vieira, J., Santos, R. A. M., Ferreira, S. M., & Vieira, C. P. (2008). Inferences on the number and frequency of S-pollen gene (SFB) specificities in the polyploid *Prunus spinosa*. *Heredity 2008 101:4*, *101*(4), 351–358. https://doi.org/10.1038/hdy.2008.60

Wallace, T. C. (2017). Dried plums, prunes and bone health: A comprehensive review. *Nutrients*, *9*(4). https://doi.org/10.3390/NU9040401

Watanabe, M., Suwabe, K., & Suzuki, G. (2012). Molecular genetics, physiology and biology of self-incompatibility in Brassicaceae. *Proceedings of the Japan Academy. Series B, Physical and Biological Sciences*, *88*(10), 519. https://doi.org/10.2183/PJAB.88.519

Weise, S., Oppermann, M., Maggioni, L., Van Hintum, T., & Knupffer, H. (2017). EURISCO: The European search catalogue for plant genetic resources. *Nucleic Acids Research*, *45*(Database issue), D1003. https://doi.org/10.1093/NAR/GKW755

Williams, R. (1970). Techniques used in fruit set experiments. In *Towards regulating cropping*.

Wolf, J., Gottingerova, M., Kaplan, J., Kiss, T., Venuta, R., & Necas, T. (2020). Determination of the pomological and nutritional properties of selected plum cultivars and minor fruit species. *Horticultural Science*, *47*(4), 181–193. https://hortsci.agriculturejournals.cz/artkey/hor-202004-0001.php

Yaegaki, H., Shimada, T., Moriguchi, T., Hayama, H., Haji, T., & Yamaguchi, M. (2001). Molecular characterization of *S-RNase* genes and *S*-genotypes in the Japanese apricot (*Prunus mume* Sieb. et Zucc.). *Sexual Plant Reproduction*, *13*(5), 251–257. https://doi.org/10.1007/S004970100064/METRICS

Yamane, H., Tao, R., & Sugiura, A. (1999). Identification and cDNA cloning for S-RNases in self-incompatible Japanese plum (*Prunus salicina* Lindl. cv. Sordum). *Plant Biotechnology*, *16*(5), 389-396.

Yamane, H., & Tao, R. (2009). Molecular Basis of Self(in)compatibility and Current Status of S-genotyping in Rosaceous Fruit Trees. *Journal of The Japanese Society for Horticultural Science - J JPN SOC HORT SCI*, *78*, 137–157. https://doi.org/10.2503/jjshs1.78.137

Erika Bright chonata jimenez

Ye, M., Peng, Z., Tang, D., Yang, Z., Li, D., Xu, Y., Zhang, C., & Huang, S. (2018). Generation of self-compatible diploid potato by knockout of *S-RNase*. *Nature Plants*, *4*(9), 651–654. https://doi.org/10.1038/S41477-018-0218-6

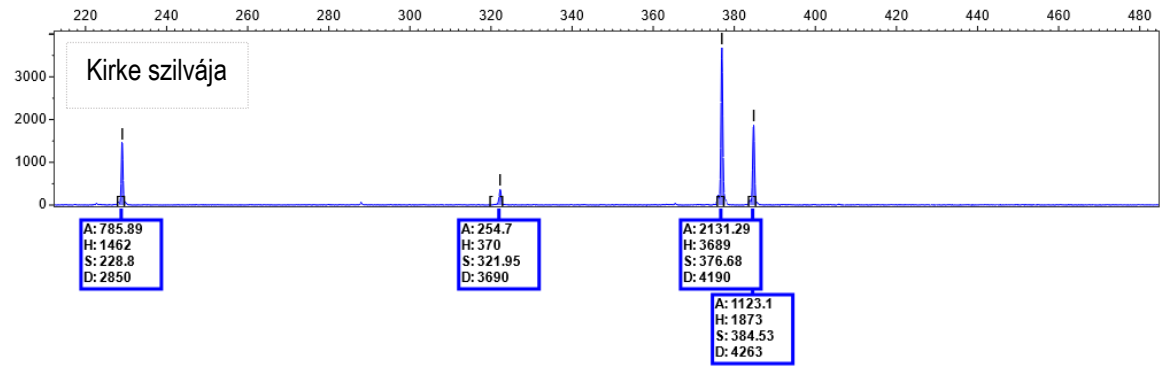
Zhebentyayeva, T., Shankar, V., Scorza, R., Callahan, A., Ravelonandro, M., Castro, S., DeJong, T., Saski, C. A., & Dardick, C. (2019). Genetic characterization of worldwide *Prunus domestica* (plum) germplasm using sequence-based genotyping. *Horticulture Research*, *6*(1). https://doi.org/10.1038/S41438-018-0090-6/41973059/41438_2018_ARTICLE_90.PDF

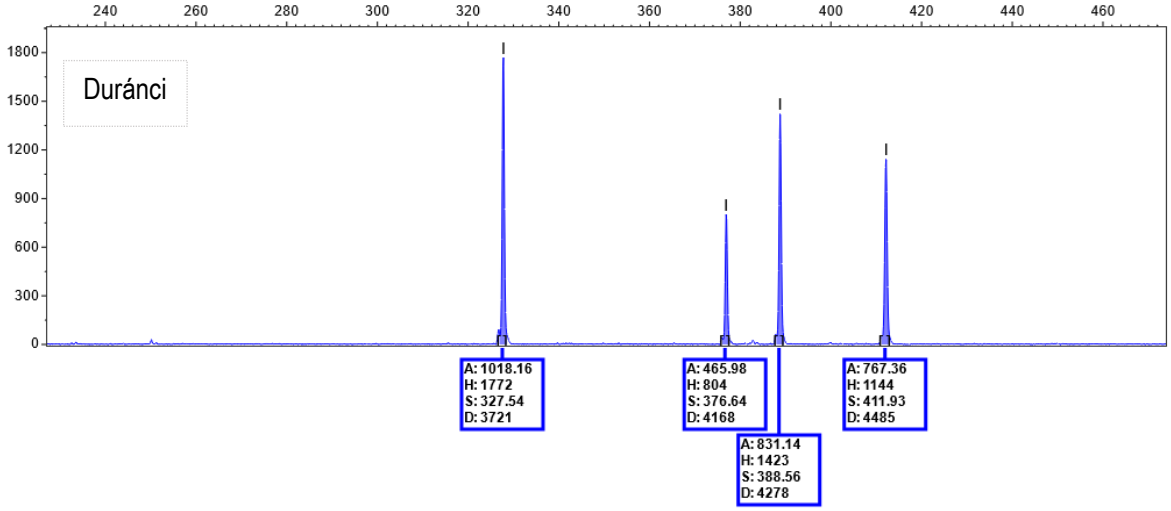
Zohary, D. (1992). Domestication of the Neolithic Near Eastern crop assemblage. Pages 81-86 in Préhistoire de l'agriculture: nouvelles approches expérimentales et ethnographiques. Monographie du CRA No. 6, Centre de Recherches Archéologiques (PC. Anderson, ed.). . *CNRS*.

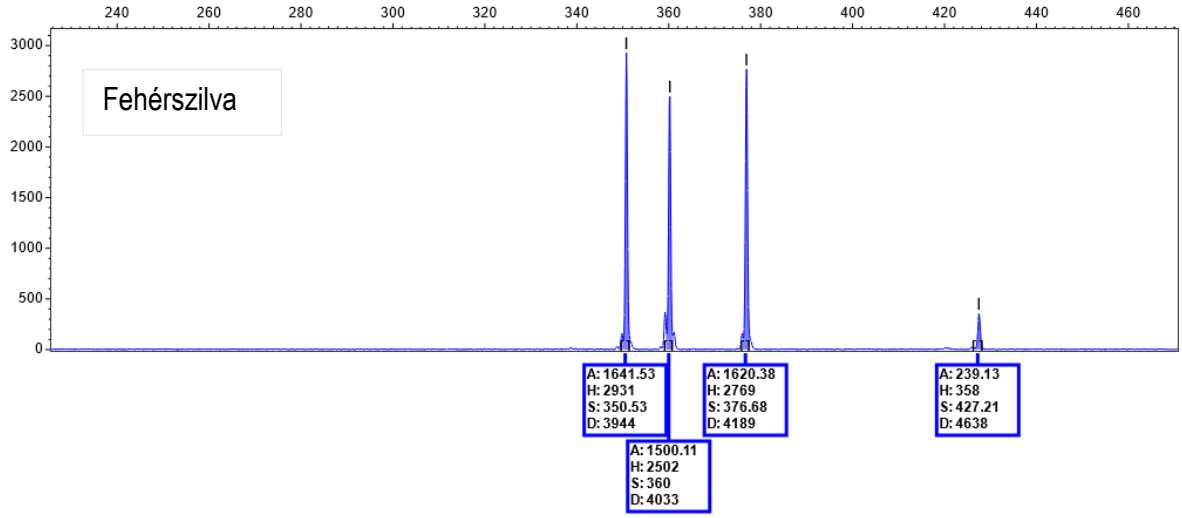
Erika Brigitte Chonata Jiménez

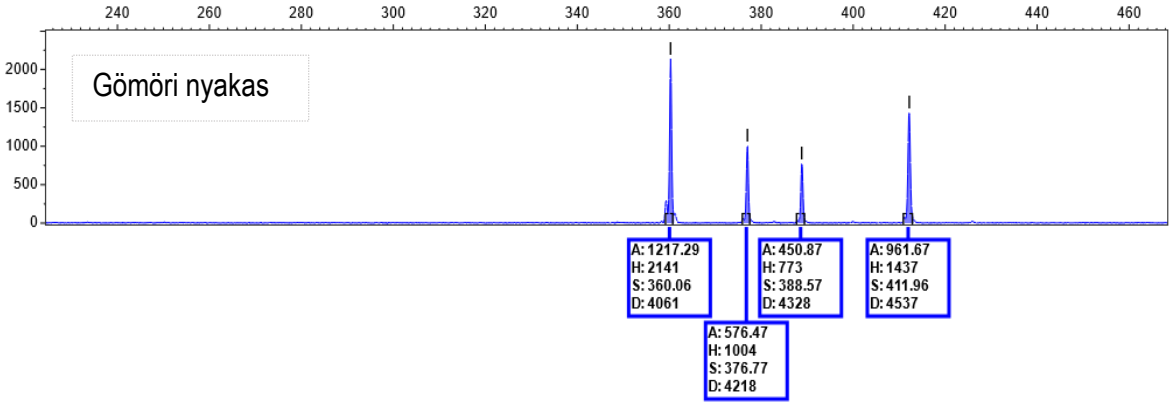
11. ATTACHMENTS

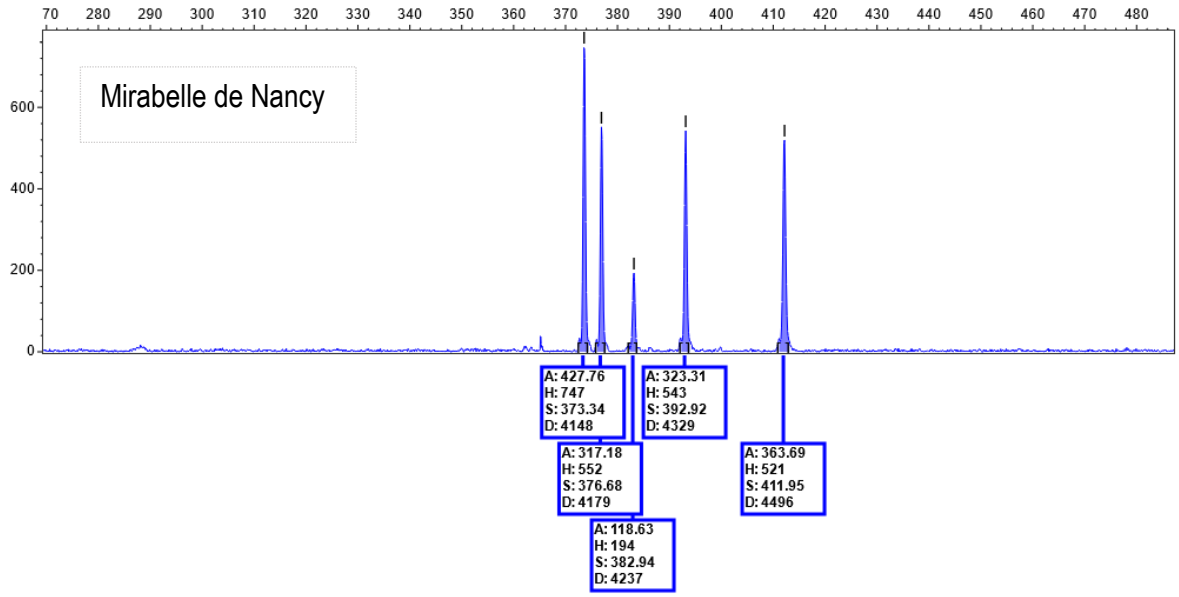
11.1 Chromatograms of the fluorescently labelled first intron fragments of the *S-RNase* gene of some cultivars in study

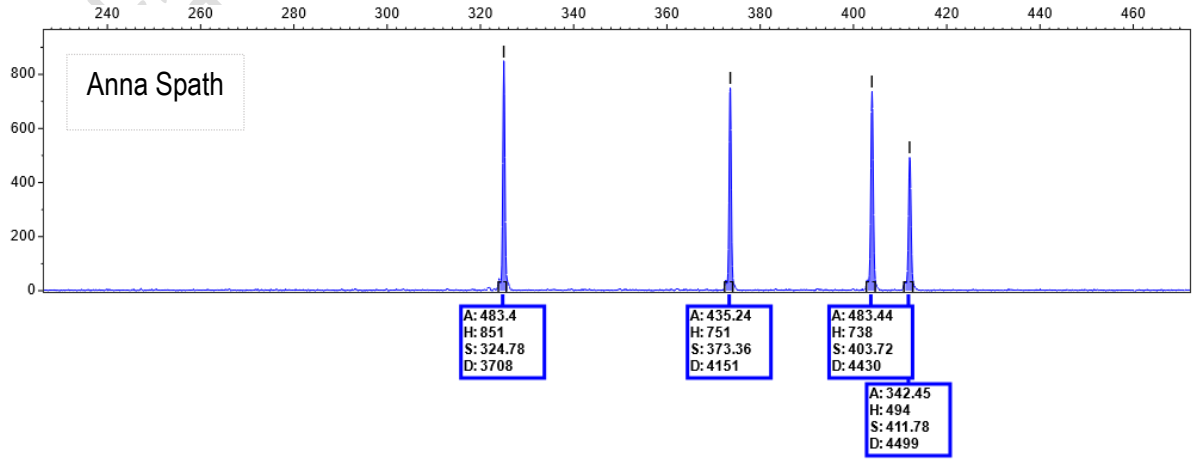


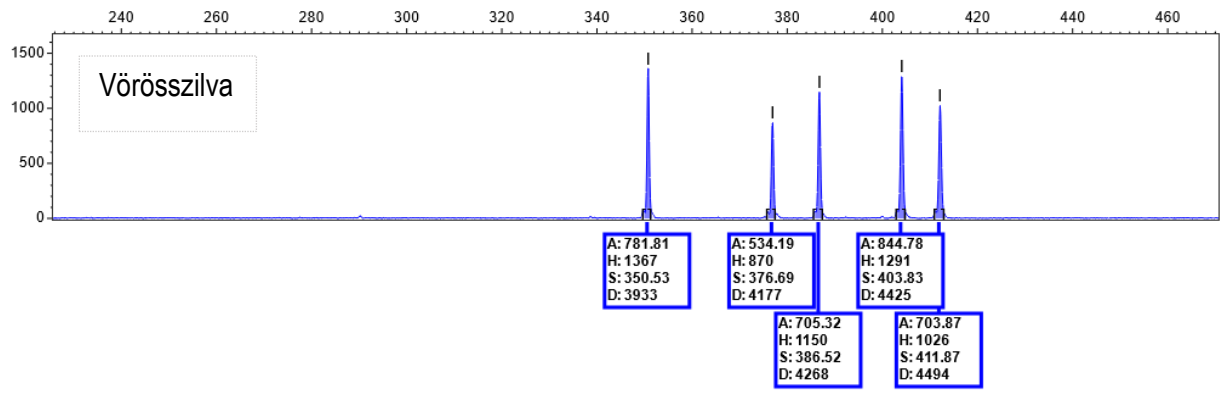


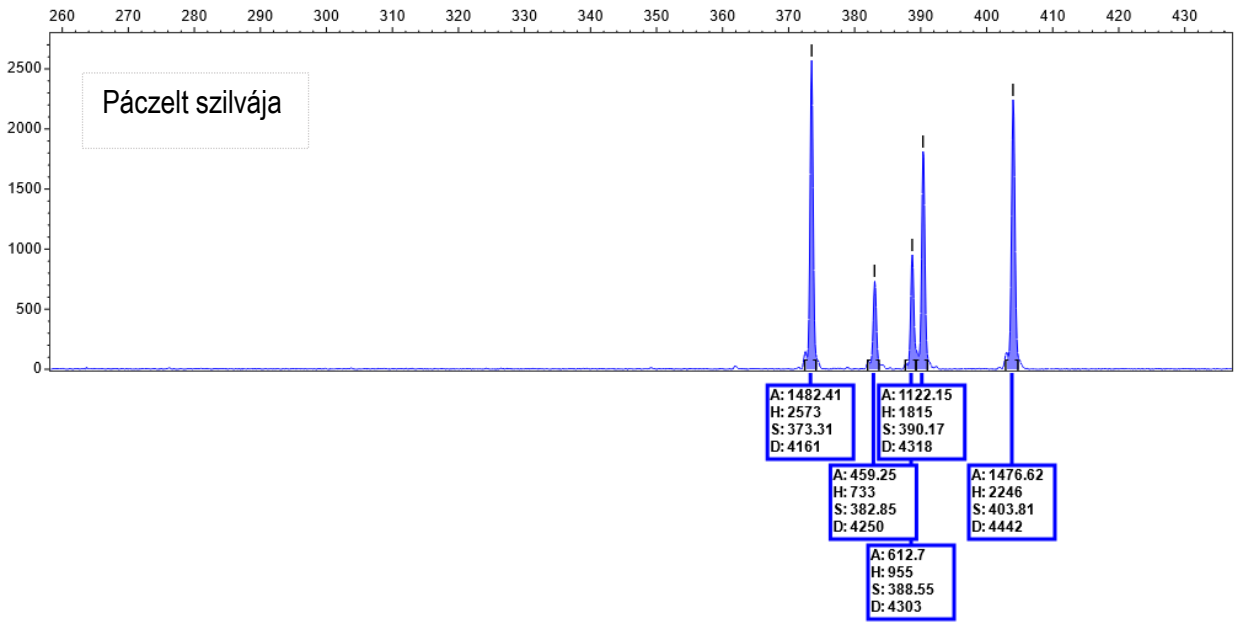


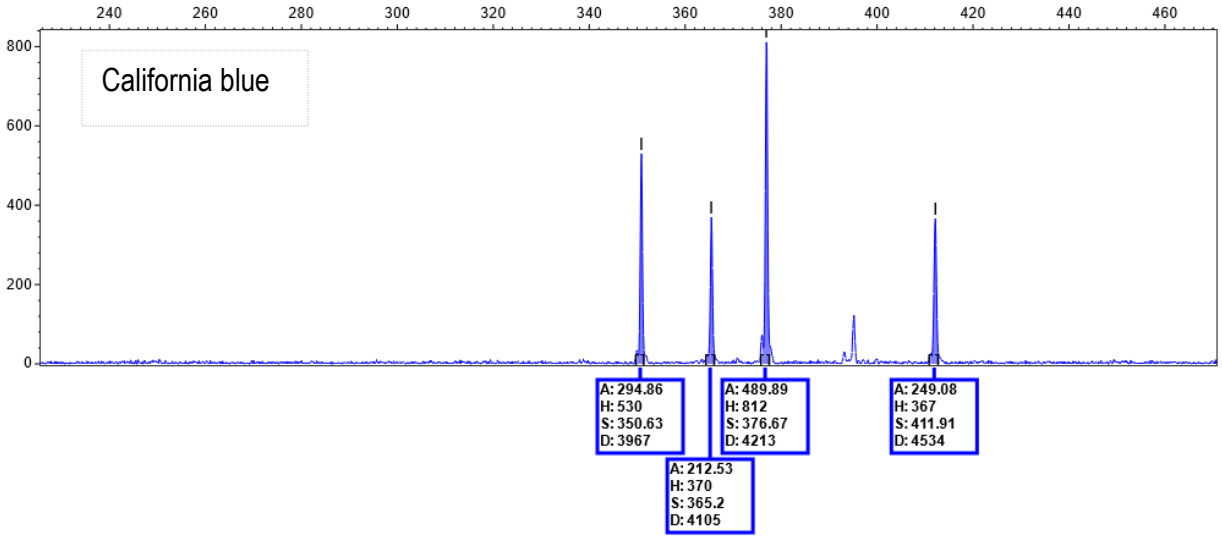

Gömöri nyakas
A: 1217.29
H: 2141
S: 360.06
D: 4061
A: 576.47
H: 1004
S: 376.77
D: 4218
A: 450.87
H: 773
S: 388.57
D: 4328
A: 961.67
H: 1437
S: 411.96
D: 4537

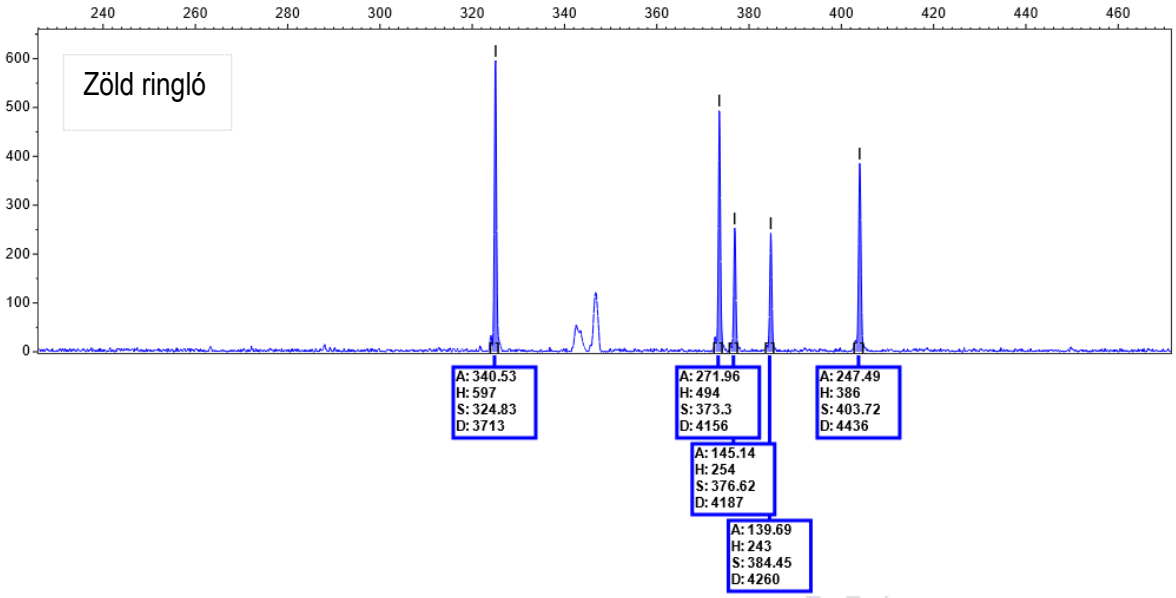

Mirabelle de Nancy
A: 427.76
H: 747
S: 373.34
D: 4148
A: 317.18
H: 552
S: 376.68
D: 4179
A: 118.63
H: 194
S: 382.94
D: 4237
A: 323.31
H: 543
S: 392.92
D: 4329
A: 363.69
H: 521
S: 411.95
D: 4496

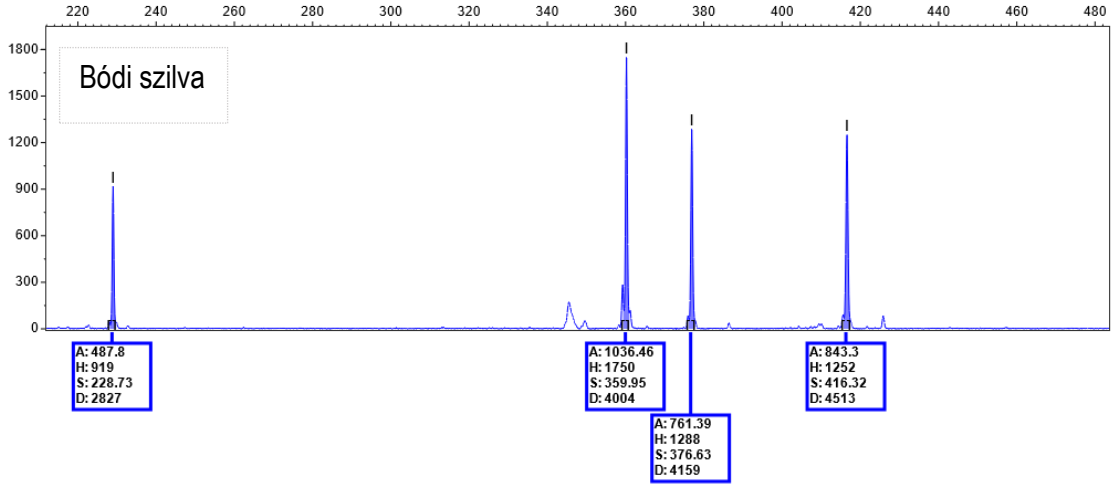

Anna Spath
A: 483.4
H: 851
S: 324.78
D: 3708
A: 435.24
H: 751
S: 373.36
D: 4151
A: 483.44
H: 738
S: 403.72
D: 4430
A: 342.45
H: 494
S: 411.78
D: 4499

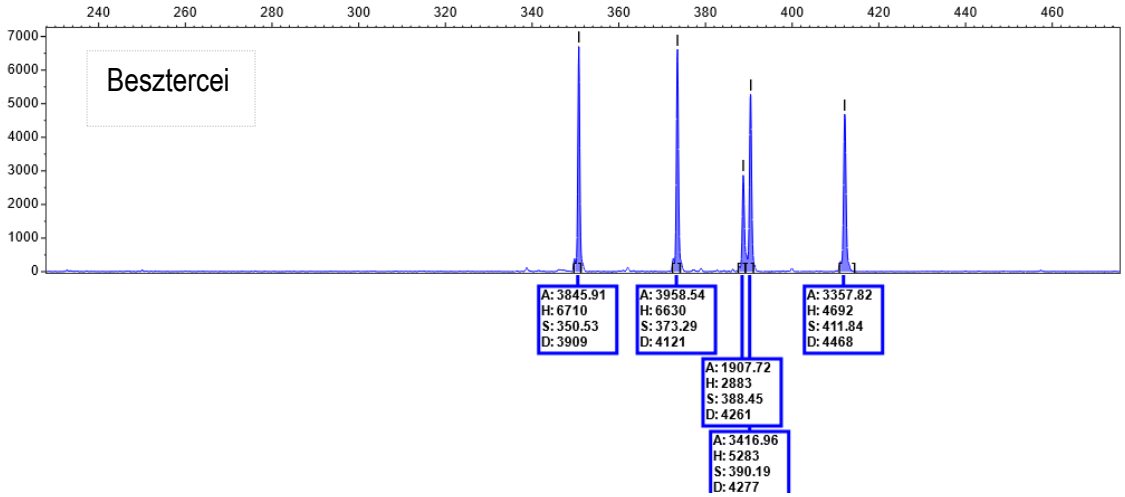

Vörösszilva
A: 781.81
H: 1367
S: 350.53
D: 3933
A: 534.19
H: 870
S: 376.69
D: 4177
A: 705.32
H: 1150
S: 386.52
D: 4268
A: 844.78
H: 1291
S: 403.83
D: 4425
A: 703.87
H: 1026
S: 411.87
D: 4494


Páczelt szilvája
A: 1482.41
H: 2573
S: 373.31
D: 4161
A: 1122.15
H: 1815
S: 390.17
D: 4318
A: 459.25
H: 733
S: 382.85
D: 4250
A: 1476.62
H: 2246
S: 403.81
D: 4442
A: 612.7
H: 955
S: 388.55
D: 4303


California blue
A: 294.86
H: 530
S: 350.63
D: 3967
A: 212.53
H: 370
S: 365.2
D: 4105
A: 489.89
H: 812
S: 376.67
D: 4213
A: 249.08
H: 367
S: 411.91
D: 4534


Zöld ringló
A: 340.53
H: 597
S: 324.83
D: 3713
A: 271.96
H: 494
S: 373.3
D: 4156
A: 145.14
H: 254
S: 376.62
D: 4187
A: 139.69
H: 243
S: 384.45
D: 4260
A: 247.49
H: 386
S: 403.72
D: 4436


Bódi szilva
A: 487.8
H: 919
S: 228.73
D: 2827
A: 1036.46
H: 1750
S: 359.95
D: 4004
A: 761.39
H: 1288
S: 376.63
D: 4159
A: 843.3
H: 1252
S: 416.32
D: 4513


Besztercei
A: 3845.91
H: 6710
S: 350.53
D: 3909
A: 3958.54
H: 6630
S: 373.29
D: 4121
A: 1907.72
H: 2883
S: 388.45
D: 4261
A: 3416.96
H: 5283
S: 390.19
D: 4277
A: 3357.82
H: 4692
S: 411.84
D: 4468

DECLARATION

on authenticity and public assess of final master's thesis/portfolio

Student's name:	Erika Brigitte Chonata Jiménez
Student's Neptun ID:	PL6J6Y
Title of the document:	Variability of the *S-RNase* alleles in the hexaploid European plum (*Prunus domestica* L.)
Year of publication:	2023
Department:	Plant Biotechnology

I declare that the submitted final master's thesis is my own, original individual creation. Any parts taken from another author's work are clearly marked, and listed in the table of contents.

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Erika Brigitte Chonata Jiménez

STATEMENT ON CONSULTATION PRACTICES

As a supervisor of Erika Brigitte Chonata Jiménez, (PL6J6Y), I here declare that the final master's thesis has been reviewed by me, the student was informed about the requirements of literary sources management and its legal and ethical rules.

I recommend/don't recommend the final master's thesis to be defended in a final exam.

The document contains state secrets or professional secrets: yes / no

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Dr. Júlia Halász Ph.D.
Internal supervisor

Erika Brigitte Chonata J.