SZENT ISTVÁN UNIVERSITY
FACULTY OF HORTICULTURAL SCIENCE
DEPARTMENT OF GENETICS AND PLANT BREEDING

CHARACTERIZATION OF PLUM CULTIVARS USING SSR AND *S*-LOCUS MARKERS

DOCTORAL (Ph.D.) DISSERTATION THESES

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The applicant met the requirement of the Ph.D regulations of the Szent István University and the thesis is accepted for the defense process.

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1. INTRODUCTION AND OBJECTIVES

European plum crops have a significant economic importance in our country and molecular genetics might be useful in solving many problems that arise during commercial production. European plums belong to the genus *Prunus* of the family Rosaceae and thereby all species show gametophytic self-incompatibility system. Plums may have been the first species among all the fruits to attract human interest. This group contains 20-40 species that are distributed in different parts of the world. European, Asian and American plums can be distinguished by their origin. Domestic (or European) plum (*Prunus domestica* L.) cultivars are the most widespread in the world. *P. domestica* is a hexaploid species ($2n=6\times=48$), therefore several theories of its origin have emerged. It is assumed that domestic plum is a naturally formed hybrid that originated about 2000 years ago from the parents of the diploid myrobalan (*P. cerasifera*) and tetraploid sloe (*P. spinosa*) (RYBIN 1936).

Plums in Hungary are highly variable due to the spontaneous hybridization among different species. Furthermore, the regional genotypes were propagated by both vegetatively and seeds during centuries which contributed the high diversity available in the country. However, until now there has been no available information about the genetic structure of Hungarian plums.

Crop reliability of fruit species depends on external factors and genetically controlled mechanisms. Fertilization of rosaceous fruit tree species is governed by GSI which mainly determines the commercial fruit production by effecting fruit set proportion. Cultivars sharing different *S*-alleles (belonging to different cross- incompatibility groups) must be interplanted in orchards; therefore, the information of *S*-genotypes is necessary for fruit growing and breeding. GSI is controlled by the single polymorphic *S*-locus (named after the term, sterility), which located on the chromosome 6 and containing two genes, the *S-ribonuclease* (*S-RNase*) and *S-haplotype-specific F-box* (*SFB*) (HEGEDŰS et al. 2012). The physical distance between the tightly linked *SFB* and *S-RNase* varies from 380 bp to 30 kb in different *S*-haplotypes. These two genes regulate the formation of the self-compatible (SC) or self-incompatible (SI) phenotype. The incompatibility reactions take place between the pistil *S-RNase* (MCCLURE et al. 1989) and the pollen-expressed F-box proteins (USHIJIMA et al. 2003) within the pollen tubes. Both genes exhibit high polymorphism and high sequence diversity.

Studies of the Rosaceous fruit tree *S*-locus are primarily focused on diploid species (e.g., apricots, peaches, almonds, sweet cherries), while information is hardly available for polyploid fruit trees despite the fact that polyploidy is a prominent feature of plant genomes. Tetraploid sour cherry and hexaploid European plum crops have great economic importance in our country. It is well known that both polyploid species includes self-compatible and self-incompatible accessions. In the case of sour cherries, Japanese and American researchers have clarified the genetic basis of the phenomenon and the *S*-genotype of the most important cultivated accessions was recorded. Until now, only a few works are available regarding the *S*-locus of European plum cultivars, due to the complex genome structure.

2. The overall objectives of this research were as follows:

- Characterization of the genetic variability of Hungarian landscape plum cultivars using microsatellite (SSR) markers.
- Comparison of the obtained results with the economically important international widespread cultivars.
- Knowledge of the *S*-allele system of the polyploid plum species.
- Characterization of *S*-locus in hexaploid plum cultivars and assessment of its variability.
- Identification of additional *S*-alleles beyond the three known plum *S*-alleles.
- Determination of *S*-genotype of plum cultivars.
- Molecular data to assess the genetic polymorphism of plums.

3. MATERIALS AND METHODS

3.1. Plant materials

A total of 55 plum genotypes [*P. domestica* L., *Prunus italica* Borkh., *Prunus insititia* L., *Prunus cerasifera* Ehrh., *Prunus syriaca* (Borkh.) Karp., *Prunus cocomilia* Ten., and a *Prunus besseyi* Bail. *Prunus salicina* Lindl. hybrid] originated in different geographical regions were evaluated in the experiments. The Hungarian and foreign plum cultivars are kept in the germplasm collection of the Szent István University, Faculty of Horticultural Science in Budapest (Soroksár) and National Agricultural Research and Innovation Center, Fruitculture Research Institute, Research Station of Cegléd. The samples include 19 modern foreign cultivars, 13 traditional cultivars, 20 landrace cultivars, and three rootstock cultivars.

3.2. DNA-based analysis

Genomic DNA was extracted from fully expanded young leaves and buds using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA concentrations and purification parameters were measured using a NanoDrop ND-1000 spectrophotometer (Bio-Science, Budapest, Hungary).

For microsatellite analysis a set of seven SSR primer pairs was selected on the basis of previous reports on different *Prunus* species, covering different linkage groups: CPSCT021 (MNEJJA et al. 2004), CPDCT044 (MNEJJA et al. 2005), BPPCT007, BPPCT025, BPPCT037, BPPCT039 (DIRLEWANGER et al. 2002). The forward primers were labelled with 6-FAM fluorescent dye for detection in a capillary genetic analyzer.

For *S*-genotype analysis PCRs with PaConsII consensus primers were conducted according SONNEVELD et al. (2001). For *F-box* gene analysis PCRs with 62F and 1010R consensus primers were conducted according NUNES et al. (2006).

We designed allele-specific reverse primers in some cases (PdomAR-R, PdomB-R, PdomE-R, PdomH-R, PdTf-R, PdEm-R and PdHag-R).

PCRs were carried out in a PTC 2720 thermocycler (Applied Biosystems, Waltham, Massachusetts, USA) using the program described for the primers. About 40–60 ng of genomic DNA was used for PCR amplification in a 12,5-mL reaction volume containing 10·× DreamTaq Green buffer (Fermentas, Szeged, Hungary) as well as KCl and (NH4)2SO4 at a ratio optimized for robust performance of DreamTaq DNA polymerase in PCR with final concentrations of 4.5 mM of $MgCl_2$, 0.2 mM

of dNTPs, 0.2 mM of the adequate primers, and 0.75 U of DreamTaq DNA polymerase (Fermentas).

To control PCR and determine the approximate sizes of alleles, 4 µl of the PCR products were separated by electrophoresis in 1% TBE agarose gels for 50 min. at 100 V and DNA bands were visualized by ethidium bromide staining. Fragment lengths were estimated by comparison with a 1-kb DNA ladder (Promega, Madison, WI).

For SSR analysis to determine the exact size of the fragments, the fluorescently labelled products were run on an automated sequencer ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Budapest, Hungary).

After *S*-PCR analysis DNA fragments were purified, cloned and sequenced according to HALÁSZ et al. (2014).

Alignment of sequences was carried out using the BioEdit 7.2.0. program (HALL 1999), and homology was tested using BLASTN analysis (ALTSCHUL et al. 1990). Bioinformatic analyses were performed using MEGA6 (TAMURA et al. 2013).

3.3. Statistical analysis

For determination of fragment sizes (genotyping), GENOTYPER 3.7 software and the GS500 LIZ size standard (Applied Biosystems) were used. The neighbor-joining algorithm was used to construct a dendrogram based on Jaccard's index using the software PAST 2.17c (HAMMER et al. 2001). Numbers on major branches represent bootstrap supports from 2000 replicates. PCA was also carried out using PAST software. To further analyze the genetic composition of plum accessions, a Bayesian approach was used to estimate the number of clusters with STRUCTURE 2.3.4. software (PRITCHARD et al. 2000). Estimation of the best K value was conducted with STRUCTURE Harvester (EARL and VON HOLDT 2012) following the method of EVANNO et al. (2005). POLYSAT (CLARK and JASIENIUK 2011) was used to estimate pairwise F_{st} values according to NEI (1973).

4. RESULTS

4.1. Polymorphism of SSR loci

In 55 plum accessions, amplification of genomic DNA was successful in each of the seven SSR loci developed from different *Prunus* species (peach, almond, and plum). Altogether, the primer pairs produced a total of 135 alleles ranging from 6 to 27 alleles per locus. A wide range of fragment length was detected among the accessions, from 108 to 268 bp.

The mean value found was 19.3 alleles per locus, which is similar to other studies on European plum. The seven SSR markers in the present study displayed relatively high polymorphism levels: CPDCT044 had the largest number of alleles (27), whereas BPPCT037 amplified the smallest number of alleles (6).

4.2. Genetic structure of plums

The genetic relationships among plum cultivars and genotypes of different origin were depicted using neighbor-joining cluster analysis (**Figure 1**). The 55 plum cultivars were classified into three main groups of different sizes. It is interesting that these cultivars represent seven different plum species with various ploidy levels (*P. domestica*, *P. italica*, *P. insititia, P. cerasifera*, *P. syriaca*, *P. besseyi*, *P. salicina*, and *P. cocomilia*), but samples belonging to the same species did not group together.

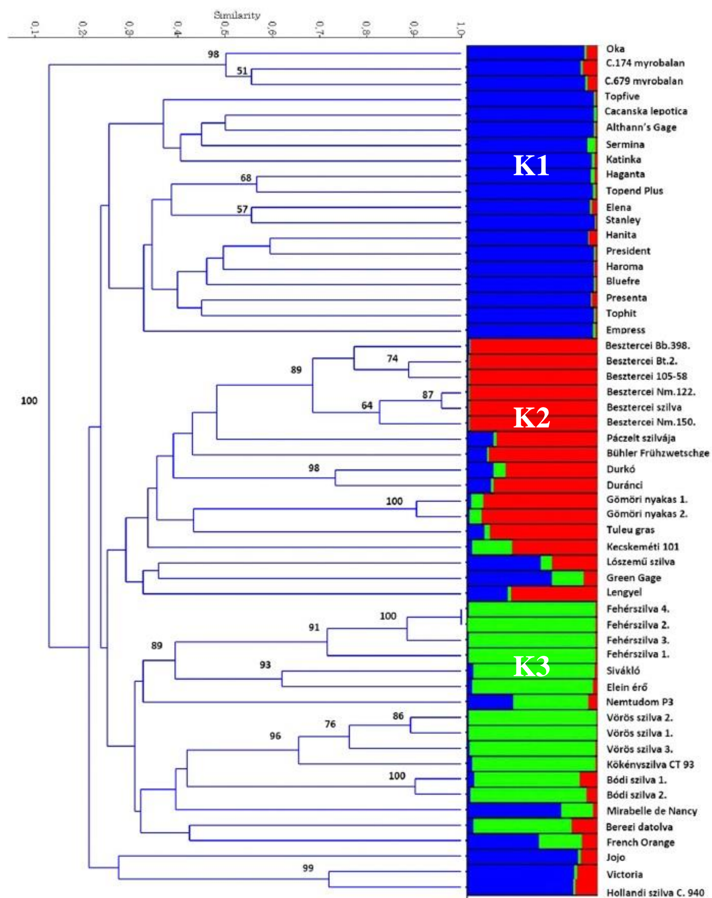


Figure 1. Neighbor-joining dendrogram of Jaccard's indices and genetic structure of 55 plum cultivars performed with seven simple sequence repeat markers. The genotyping results were used to classify the cultivars into subpopulations, only confident branches with bootstrap values ≥50 were assigned. Genetic structure was revealed by STRUCTURE program with K=3 as found by simulation and DK likelihood method. The division of Q-value bar plot into three reconstructed populations corresponds to the three major significant clusters in the dendrogram.

4.3. Structure analysis of the examined cultivars

STRUCTURE analysis was carried out to determine the genetic constitution of different groups. The most likely value of K was 3, indicating three genetically distinct reconstructed populations (RPs) within the studied genotypes. The first RP (RP1, blue) is the biggest group with 25 genotypes. It contains all foreign, modern, and traditional polyploid cultivars with two diploid *P. cerasifera* rootstock accessions. Hungarian landraces and Hungarian traditional cultivars formed two different groups, because the second RP (RP2, red) contains 16 and the third RP (RP3, green) includes other 14 Hungarian accessions. Differentiation, estimated as the F_{st} value, among the three groups of plum accessions was the highest (0.0431) in the case of RP1 including foreign cultivars. Lower genetic differentiation characterized the two other RPs (RP2 F_{st} = 0.0234 and RP3 F_{st} = 0.0294) containing the Hungarian accessions.

4.4. Principal component analysis of the examined cultivars

PCA (**Figure 2**) confirmed the information provided by the dendrogram and also supplied further details. The first two principal axes accounted for 12.03% and 10.20% of the total variation, respectively, together explaining 22.23% of the total variability. In the PCA scatter plot, clear separation occurred among 'Besztercei' accessions and 'Fehérszilva' accessions, which are at a significant genetic distance from other plum genotypes

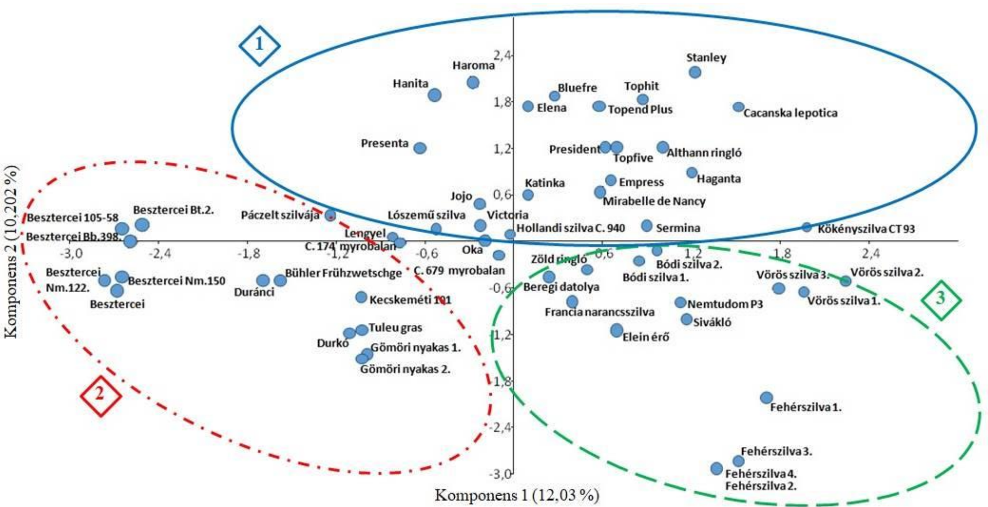


Figure 2. Distribution of 55 plum cultivars on the two first principal component analysis axes determined from simple sequence repeat genotyping.

4.5. Determination of *S*-genotypes by ILP-marker

Plums, like other species of the Rosaceae family, show a gametophytic incompatibility system. We analyzed the *S*-locus of 55 European plum accessions. Allelic variants of the locus can be represented by different sizes of 2nd intron region of *S-RNase* gene. We observed high variability with values ranging from 550 to 4000 bp (**Figure 3.**). Altogether 113 PCR amplicons of C2-C5 region were cloned resulting 339 DNA sequences. Twenty-four different fragments were detected: 11 alleles with partial (C2-C5) (**Figure 4.**) and 3 alleles (S_{12}, S_{13}, S_{17}) with complete (SP-C5) sequences (**Figure 5.**). To confirm the identity of the detected *S*-alleles, partial and total *S-RNase* alleles were screened by BLASTN homology in the GenBank database. Typical intron sizes of *S-RNase* alleles were determined.

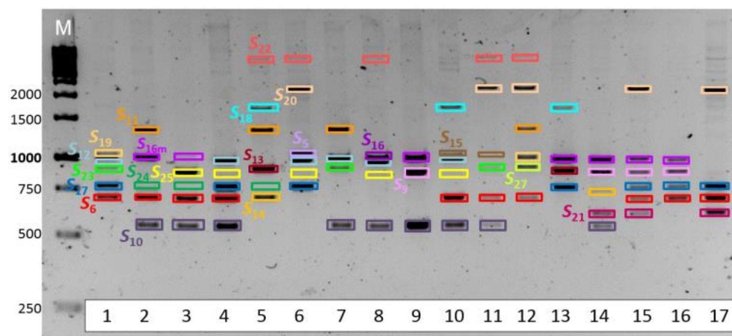
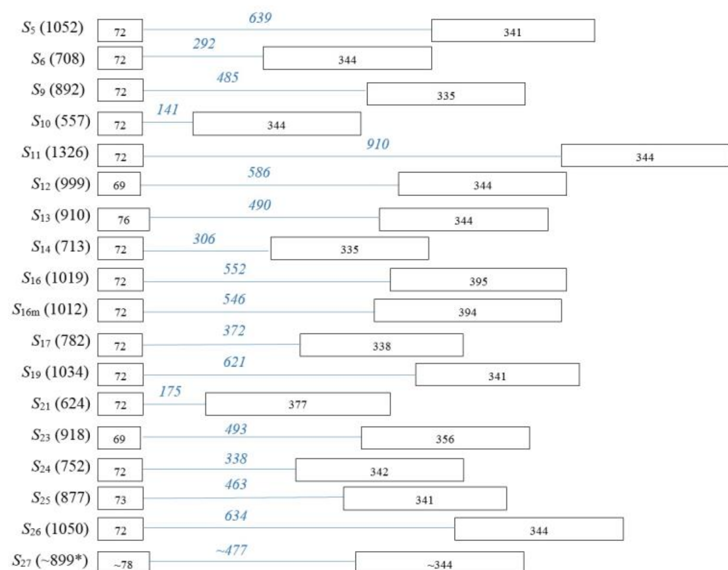


Figure 3. *S*-locus of 17 European plum cultivars was characterized using consensus PCR primers [PaConsII-F and PaConsII-R; SONNEVELD et al. (2003)] for *S-RNase* amplification. Fragments with the same color frame indicate the same *S*-allele. 1.'Althann ringló', 2. 'Bluefre', 3. 'Cacanska lepotica', 4. 'Elena', 5. 'Empress', 6. 'Haganta', 7. 'Hanita', 8. 'Haroma', 9. 'Jojo', 10. 'Katinka', 11. 'Presenta', 12. 'President', 13. 'Sermina', 14.'Stanley', 15. 'Topend Plus', 16. 'Topfive', 17. 'Tophit'. Marker: GeneRuler 1kb DNA Ladder (Thermo Fisher Scientific).

In contrast to modern plums, under the same name of traditional cultivars such as the Besztercei clones and landscape genotypes (the various Fehérszilva, Vörös szilva, Gömöri nyakas and Bódi szilva cultivars) are found a completely uniform pattern in their *S*-genotypes.



* estimated size based on similarity obtained

Figure 4. Schematic structures of European plum [previously published (S_5, S_6; S_9) and new S_{10}-S_{27} alleles] *RNase* genes. Boxes and lines sign exons and introns, respectively (not to scale). Numbers represent values of the region in boxes and above the lines, while parentheses indicate the size (bp) of the sequenced partial (C2-C5) *S-RNase*.

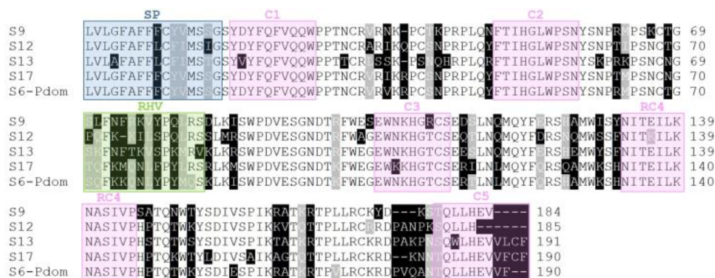


Figure 5. Amino acid sequence alignment of known (S_6-Pdom) and new (S_9, S_{12}, S_{13}, S_{17}) European plum *S-RNase* alleles. The signal peptide (SP, blue), the five conservative regions (C1, C2, C3, RC4, C5; pink) and the hypervariable (RHV, green) region are shown (USHIJIMA et al. 1998). The black border represents the variable amino acids. NCBI Database ID of Sequence Matching: AM746947 (S_6).

4.6. Phylogenetic analysis of new European plum *S-RNase* alleles

To identify trans-specific alleles, protein sequences of the plum *S*-RNases were aligned with those of other *Prunus* species that are most similar to them. In addition, the sequence alignment was determined in percent value. Identified plum *S*-RNase sequences and homologous sequences from the NCBI GenBank database showed very high identity in both modern (nucleotide level on average: 96.8%; protein level on average: 93.2%) and landscape cultivars (nucleotide level on average: 97.2%; protein level on average: 96.7%).

We identified six trans-specific alleles using GeneBank database that showed remarkable identity in their intron region (95.9–99.6%): S_{11} - *Psal* S_{26}, S_{12} - *Psal* S_{23}, S_{14} - *Pspin* $S_{3\text{-}1}$, S_{14} - *Pspin* $S_{3\text{-}2}$, S_{14} - *Psal* S_{25}, S_{23} - *Pspin* S_8, S_{24} - *Pavi* S_{30}. These data can be imprints of relatively recent introgression events, and could support theory of complex origin of European plum.

4.7. Determination of *S*-genotype of plum cultivars

In summary, we report a full or partial *S*-genotype of 55 plum cultivars of different origins: 20 modern, 12 traditional, 20 landscape and 3 rootstock cultivars (**Table 1**.). Identification of tested individuals based on their unique *S*-genotype was successful.

Table 1. Origin, type of fertilization and defined *S*-genotype of the examined European plum cultivars.

	Plum cultivars	Species	Fertility properties[1]	*S*-genotype
Modern cultivars	Althann's Gauge	*Prunus italica*	SI	$S_6S_{12}S_{17}S_{19}S_{23}$
	Bluefre	*P. domestica*	PSC	$S_6S_{10}S_{11}S_{16m}S_{24}$
	Cacanska lepotica	*P. domestica*	PSC	$S_6S_{10}S_{16m}S_{24}S_{25}$
	Elena	*P. domestica*	SC	$S_6S_{10}S_{12}S_{17}S_{25}$
	Empress	*P. domestica*	SI	$S_{11}S_{13}S_{14}S_{18}S_{22}S_{24}$
	Haganta	*P. domestica*	PSC	$S_5S_{12}S_{17}S_{20}S_{22}S_{25}$
	Hanita	*P. domestica*	SC	$S_{10}S_{11}S_{12}S_{23}$
	Haroma	*P. domestica*	SC	$S_{10}S_{12}S_{16}S_{22}S_{25}$
	Hollandi szilva C. 940	*P. italica*	SC	$S_6S_{10}S_{17}S_{26}$
	Jojo	*P. domestica*	SC	$S_9S_{10}S_{16m}$
	Lengyel	*P. domestica*	SC	$S_{10}S_{14}S_{17}S_{21}S_{26}$
	Katinka	*P. domestica*	SC	$S_6S_{10}S_{12}S_{15}S_{18}$
	Oka	*P. besseyi* × *P. salicina*	SI	$S_{10}S_{21}$
	Presenta	*P. domestica*	SC	$S_6S_{10}S_{15}S_{20}S_{22}S_{23}$
	President	*P. domestica*	SI	$S_6S_{11}S_{19}S_{20}S_{22}S_{27}$
	Sermina	*P. cocomilia*	SI	$S_{13}S_{16m}S_{17}S_{18}$
	Stanley	*P. domestica*	PSC	$S_9S_{10}S_{14}S_{16m}S_{21}$
	Topend Plus	*P. domestica*	SC	$S_6S_9S_{16m}S_{17}S_{20}S_{21}$
	Topfive	*P. domestica*	PSC	$S_6S_9S_{16m}S_{17}$
	Tophit	*P. domestica*	PSC	$S_6S_{17}S_{20}S_{21}$
Landscape cultivars	Fehérszilva 3.	*P. domestica*	PSC	S_{10}
	Fehérszilva 4.	*P. insititia*	SC	$S_{10}S_{14}S_{17}S_{21}S_{26}$
	Gömöri nyakas 1.	*P. insititia*	SC	$S_{10}S_{14}S_{17}S_{21}S_{26}$
	Gömöri nyakas 2.	*P. domestica*	PSC	S_{10}
	Kecskeméti 101	*P. domestica*	PSC	$S_6S_{10}S_{13}S_{17}$
	Elein érő	*P. domestica*	SC	$S_6S_{10}S_{11}S_{17}S_{28}$
	Fehérszilva 1.	*P. domestica*	SC	$S_6S_{10}S_{13}S_{17}S_{26}$
	Fehérszilva 2.	*P. domestica*	SC	$S_6S_{10}S_{13}S_{17}S_{26}$

Table 1. (Continuation).

	Plum cultivars	Species	Fertility properties[1]	*S*-genotype
Landscape cultivars	Fehérszilva 3.	*P. domestica*	SC	$S_6S_{10}S_{13}S_{17}S_{26}$
	Fehérszilva 4.	*P. domestica*	SC	$S_6S_{10}S_{13}S_{17}S_{26}$
	Gömöri nyakas 1.	*P. domestica*	PSC	$S_{10}S_{14}S_{17}S_{26}$
	Gömöri nyakas 2.	*P. domestica*	PSC	$S_{10}S_{14}S_{17}S_{26}$
	Kecskeméti 101	*P. domestica*	SC	$S_6S_{10}S_{17}S_{26}$
	Lószemű szilva	*P. domestica*	SC	$S_6S_{10}S_{17}S_{29}$
	Nemtudom P3	*P. insititia*	SC	$S_{10}S_{11}S_{17}S_{26}S_{28}$
	Páczelt szilvája	*P. domestica*	SC	$S_6S_{10}S_{17}S_{26}$
	Siválkó	*P. domestica*	SC	$S_6S_{10}S_{11}S_{17}S_{28}$
	Vörös szilva 1.	*P. domestica*	SC	$S_6S_{10}S_{17}$
	Vörös szilva 2.	*P. domestica*	SC	$S_6S_{10}S_{17}$
	Vörös szilva 3.	*P. domestica*	SC	$S_6S_{10}S_{17}$
Traditional cultivars	Besztercei 105-58	*P. domestica*	SC	$S_{10}S_{14}S_{17}S_{20}$
	Besztercei Bb.398	*P. domestica*	SC	$S_{10}S_{14}S_{17}S_{20}$
	Besztercei Bt. 2	*P. domestica*	SC	$S_{10}S_{14}S_{17}S_{20}$
	Besztercei Nm.122.	*P. domestica*	SC	$S_{10}S_{14}S_{17}S_{20}$
	Besztercei Nm.150.	*P. domestica*	SC	$S_{10}S_{14}S_{17}S_{20}$
	Besztercei szilva	*P. domestica*	SC	$S_{10}S_{14}S_{17}S_{20}$
	Bühler Frühzwetschge	*P. domestica*	SC	S_{10}
	French Orange	*P. domestica*	SC	S_{10}
	Green Gauge	*P. italica*	SI	S_{10}
	Mirabelle de Nancy	*P. syriaca*	PSC	$S_{10}S_{14}$
	Tuleu gras	*P. domestica*	MS	S_6S_{10}
	Victoria	*P. domestica*	SC	$S_6S_{10}S_{17}S_{26}$
Rootstock cultivars	C. 174 myrobalan	*P. cerasifera*	SC	$S_{10}S_{21}$
	C. 679 myrobalan	*P. cerasifera*	SC	$S_{10}S_{17}$
	Kökényszilva CT 93	*P. insititia*	SC	S_6S_{10}

[1]SI: Self-incompatibility; SC: Self-compatibility; PSC: Partially self-compatibility; MS: Male sterile (HARSÁNYI 1979, SZABÓ 2001, SURÁNYI 2006a, KISSNÉ and MAROSI 2006, SURÁNYI 2014)

4.8. Polymorphism of the European plum *F-box* gene

The pollen component of *S*-allele system, the *S*-haplotype F-box (*SFB*) locus was also characterized. Size of the fragments was detected between 900 and 1000 bp. A total of 10 novel sequences were isolated in 17 *Prunus domestica* cultivars. The new alleles were labelled alphabetically (SFB_A-SFB_J) since the sequences could not be linked to the identified *RNase* alleles. Phylogenetic analysis was performed using other *Prunus*, *Malus*, and *Pyrus SFB* sequences. This further confirmed the differences between the *S*-haplotype F-box proteins of the *Prunoideae* and *Maloideae* subfamilies.

4.9. Mutation events resulting loss of function

In the *Prunoideae* subfamily, mutations affecting the *S*-locus are more than typical for the corresponding pollen component, but there are also style component mutation events. Moreover, we identified a putative non-functional *S-RNase* and *F-box* allele (S_{16m} and SFB_J'). The 2-nucleotide substitution in the sequence of the S_{16} allele precedes the intron region, thereby causing a nonsense mutation in which the GTA triplet appearing in S_{16} was changed to TAA, or stop codon, in S_{16m}. Several single-nucleotide substitutions occurred in the SFB_J allele sequence, the first led to a nonsense mutation, since in *P.dom.* S_9 *SFB*, the TTG triplet in SFB_J changed to TAG, or stop codon.

4.10. NEW SCIENTIFIC ACHIEVEMENTS

1. Using microsatellite markers (SSR) we proved that Hungarian plum landraces show a high degree of genetic polymorphism. Our data provides directly usable information for breeding and cultivation programs utilizing these landraces.

2. In many cases, most genotypes kept under identical name (‘Vörös szilva’, ‘Gömöri nyakas’ and ‘Bódi szilva’ accessions originated from different regions) showed dissimilar DNA fingerprints.

3. Our data clarified the origin of ‘Fehérszilva’ which supposed to be a variant of ‘Vörös szilva’ and not ‘Besztercei’ plum as previously reported.

4. A total of 24 *S-ribonuclease* alleles were identified in 55 hexaploid plum cultivars, 11 alleles with partial (C2-C5) and 3 alleles (S_{12}, S_{13}, S_{17}) with complete (SP-C5) sequences. Specific intron sizes of the *S-RNase* alleles were determined, and therefore, alleles can be detected using ILP-markers.

5. A total of 10 novel *SFB* sequences were isolated in 17 *Prunus domestica* cultivars. The new alleles were labelled alphabetically (SFB_A-SFB_J).

6. We identified a putative non-functional *S-RNase* and *F-box* allele (S_{16m} and SFB_J’).

7. We report a full or partial *S*-genotype of 55 plum cultivars of different origins: 20 modern, 12 traditional, 20 landscape and 3 rootstock cultivars.

5. DISCUSSION OF RESULTS

Genetic variability of Hungarian traditional and landscape European hexaploid plum (*Prunus domestica* L.) cultivars was compared to economically important international cultivars. Moreover, we carried out a comprehensive molecular analysis of self- and mutual compatibility relationships.

5.1. Genetic characterization of *Prunus domestica* species using microsatellite markers (SSR)

Genetic variability, diversity and phylogenetic relationship analysis of 20 economically significant, 12 traditional, 20 landscape and 3 rootstock cultivars were carried out with 7 microsatellite markers. The primer pairs amplified a total of 135 alleles ranging from six to 27 alleles per locus, displaying high polymorphism. The mean value found was 19.3 alleles per locus, which is similar to other studies on European plum. SEHIC et al. (2015) found 22.7 alleles per locus in 76 plum genotypes, KAZIJA et al. (2014) reported 18.7 alleles per locus on 62 plum accessions, whereas XUAN et al. (2011) registered 20 alleles per locus in 45 cultivars. The highest number of alleles was observed with markers CPSCT021 and CPDCT044 (26 and 27, respectively) and only 6 alleles occurred in BPPCT037 loci. Our results are attributed to the great genetic diversity of tested accessions which can be a consequence of the complex polyploid genome structure. All genotypes were clearly distinguished with the seven SSRs used in this study, indicating that microsatellite analysis is an appropriate tool for the identification and fingerprinting of plum cultivars. MERKOUROPOULOS et al. (2017) also used a total of seven microsatellite markers that were enough to discriminate 54 plum cultivars.

The 55 plum cultivars were classified into three main groups of different sizes. It is interesting that these cultivars represent seven different plum species with various ploidy levels (*P. domestica*, *P. italica*, *P. insititia, P. cerasifera*, *P. syriaca*, *P. besseyi*, *P. salicina*, and *P. cocomilia*), but samples belonging to the same species did not group together. Because all species are able to hybridize, intermediate forms may exist between each of the species (NEUMÜLLER 2011), resulting in the absence of a strong discriminating line around the genotypes supposed to represent different species. The only exception to the mixed arrangement was the out-group formed by the diploid species ‘Oka’ and two myrobalan (‘C. 174 myrobalan’, ‘C. 679 myrobalan’) accessions. Most international modern

cultivars grouped together, whereas Hungarian traditional cultivars and landraces appear to be more diversified with some distantly related accessions (e.g., 'Besztercei' clones and 'Beregi datolya'). The foreign cultivars positioned distantly from the Hungarian accessions in the dendrogram similarly to the position of 'Stanley' among east Anatolian traditional plums (ÖZ et al. 2013).

STRUCTURE analysis indicated three genetically distinct groups of the studied genotypes (K=3). F_{st} values, the neighbor-joining cluster analysis and principal component analysis (PCA) concluded that modern cultivars clustered together, and Hungarian landraces positioned distantly from those. On the basis of molecular data, our results from Bayesian clustering analysis confirmed the groupings we detected in the neighbor-joining dendrogram (**Figure 1**). Parent–offspring relationships among some cultivars ('Bluefre', 'Stanley', 'President', 'Hanita', 'Haroma', and 'Presenta') such cultivars clustered closely on the dendrogram according to their pedigree. Accessions within the Hungarian landrace cultivar groups ('Besztercei', 'Fehérszilva', 'Vörös szilva', 'Bódi szilva', and 'Gömöri nyakas') grouped together with strong bootstrap support (89% to 100%). From several accessions known under the same name, only Fehérszilva 4 and Fehérszilva 2 proved to be identical. Other genotypes labelled by a common name were found to be different from each other in some of the assayed loci. Using seven SSRs, differences occurred among 'Vörös szilva', 'Gömöri nyakas', and 'Bódi szilva' accessions collected in different geographical locations. Traditionally, landraces showing similar morphological characters were labelled with the same name by village people, and they have been propagating those landraces by seeds for decades, which resulted in a certain level of genetic variations within specific landrace cultivar groups (SURÁNYI 2006a, 2013; TÓTH and SZANI 2004). 'Besztercei' plums were considered the first and most important landrace cultivar in Hungary (RAPAICS 1940). Because of the previously described reasons, genetic variations were described among 'Besztercei' plum trees and several forms were selected to combat genetic erosion experienced after some time in cultivation. First reports on the selected clones reflected differences in ripening time, size of fruits, and productivity (HARSÁNYI 1997), whereas later productivity was confirmed to be the only characteristic significantly improved by this strategy (SURÁNYI 2006a). Analysis of 50 'Besztercei' clones from a germplasm collection in Cegléd revealed diversity in 10 morphological traits and Plum pox virus (PPV) resistance (SURÁNYI 2006b). Each of the six 'Besztercei' plums used in this study showed a unique SSR fingerprint different from

others, and the clones grouped together in two small subgroups, both containing three accessions.

Each accession was characterized by unique microsatellite fingerprint. This could serve as a basis of molecular identification of Hungarian landscape accessions. Most genotypes kept under identical name (‘Vörös szilva’, ‘Gömöri nyakas’ and ‘Bódi szilva’ accessions from different regions) showed different DNA fingerprints. Our data clarified the origin of ‘Fehérszilva’ which supposed to be a variant of ‘Vörös szilva’ and not ‘Besztercei’ plum as previously reported.

In the PCA scatter plot, clear separation occurred among ‘Fehérszilva’ accessions. Two hypotheses have been put forward for the origin of ‘Fehérszilva’: 1.) ‘Fehérszilva’ is a mutant form of ‘Vörös szilva’ (SURÁNYI 2013) and 2.) ‘Fehérszilva’ was developed from ‘Besztercei’ and it is also known as ‘Fehér Besztercei’ (NAGY 1980). ‘Fehérszilva’ accessions were closer to ‘Vörös szilva’ genotypes and hence our SSR analysis seems to support the first theory.

The substantial dispersion of Hungarian traditional cultivars and landraces suggests a high level of genetic diversity present within the analyzed germplasm. Indeed, unique alleles were registered among the landraces in five SSR loci. It would be important to use this plant material in breeding programs to increase genetic variability that has been narrowed over the last decades because of the frequent application of some popular foreign cultivars. ‘Gömöri nyakas’, ‘Nemtudom P3’, ‘Fehérszilva’, ‘Beregi datolya’, and ‘Vörös szilva’ proved to be PPV tolerant in field experiments, which further demonstrates their value in future breeding or cultivation. In addition, ‘Bódi szilva’ and ‘Nemtudom szilva’ also proved to show elevated abiotic stress resistance (PETHŐ 2011, SURÁNYI 2013). Local plum cultivars and primitive landraces were reported to be perspective donors of resistance to frost and drought (PAUNOVIC 1988). Moreover, some of the Hungarian plums analyzed are useful as rich genetic resources to improve fruit quality and increase the nutritional content and health benefits of future cultivars. Large differences have been reported between the Hungarian landraces and the modern foreign cultivars with regard to carbohydrate profiles. The fruits of ‘Vörös szilva’ and ‘Lószemű szilva’ are rich in sugar alcohol (sorbitol), whereas ‘Bódi szilva’, ‘Vörös szilva’, and ‘Besztercei szilva’ produce monosaccharide-dominated fruits (TÓTH 2013). TÓTH (1957) detected 22% total sugar in the fruit juice of ‘Duránci’. The laxative effect of plum and plum juice was attributed to the presence of phenolics (mainly chlorogenic and neochlorogenic acids) and sorbitol coupled with its high fibre content in fruits (STACEWICZ-

SAPUNTZAKIS et al. 2001). This composition might also be the explanation why plums that are a good source of energy in the form of simple sugars do not mediate a rapid rise in blood sugar concentration. In addition, phenolic compounds and microelements accumulated in plums may serve as preventive agents against severe chronic diseases including cardiovascular disease, cancer, and osteoporosis (IGWE and CHARLTON 2016). 'Pożegača' (syn. 'Besztercei szilva') had the 13th highest total phenolics content among 178 cultivars of plum (SAHAMISHIRAZI et al. 2017), indicating that some genotypes with outstanding fruit composition might be found among the genetically diverse local landrace cultivars, as it was found in the Hungarian sour cherry breeding program (PAPP et al. 2010). Our data provided valuable information on local European plum germplasm in Hungary by detecting considerable genetic diversity that might be exploited in future breeding programs. Molecular marker strategies will undoubtedly contribute to reinforced breeding activity in plum by helping in the conservation, valorization, and the most efficient use of landrace cultivars to combine their outstanding fruit properties with other beneficial characteristics (e.g., disease resistance) provided by foreign cultivars.

Each accession was characterized by unique microsatellite fingerprint. This could serve as a basis of molecular identification of Hungarian landscape accessions.

5.2. Locus variability determining fertility of hexaploid plum cultivars

Plums, like other species of the Rosaceae family, show a gametophytic incompatibility system. We analyzed the *S*-locus of 55 European plum accessions. Allelic variants of the locus can be represented by different sizes of 2nd intron region of *S-RNase* gene. We observed high variability. Altogether, 24 different alleles were identified, since domestic plum has a hexaploid genom, maximum of six PCR products could be found in one sample. Altogether 3 to 6 bands for each of the cultivars were scored. The number of alleles detected at the *S*-locus is consistent with our SSR results, the number of SSR alleles detected at the loci.

The first study regarding the molecular genetics of self-incompatibility in domestic plum was published by SUTHERLAND et al. (2004a,b). They have identified three *S-RNase* alleles (S_5, S_6, S_9) in European plum (SUTHERLAND et al. 2008), which we identified in our samples also. To confirm the identity of the detected *S*-alleles, partial and total *S-RNase* alleles were screened by BLASTN homology in the GenBank database.

Typical intron sizes of *S-RNase* alleles were determined. Generally, molecular techniques detected that sizes of the second intron were higher than the size of the first intron in *Prunus S-RNases*. Altogether 21 full-length *S*-alleles were identified in cultivated sweet cherry cultivars and wild cherry populations. However, in case of an allele, the first intron was larger than the second (SONNEVELD et al. 2003, WÜNSCH and HORMAZA 2004, De CUYPER et al. 2005).

In all cases, the *S-RNase* allele sequences detected showed homology exclusively with other *Prunus* sequences, which seems to be confirmed by the fact that RNase based GSI in various plant families has a common evolutionary origin (IGIC and KOHN 2001). Isolated groups were discernible in the second intron comparisons by SUTHERLAND et al. 2008: introns which showed very large divergence with introns of other trans-specific alleles (<57,9%); and those which showed high similarity with one or more introns (74.1-99.9%). Our results were similar to those of SUTHERLAND et al. (2008). We identified six trans-specific alleles using GenBank database that showed remarkable identity in their intron region (95.9–99.6%): S_{11} - *Psal* S_{26}, S_{12} - *Psal* S_{23}, S_{14} - *Pspin* $S_{3\text{-}1}$, S_{14} - *Pspin* $S_{3\text{-}2}$, S_{14} - *Psal* S_{25}, S_{23} - *Pspin* S_{8}, S_{24} - *Pavi* S_{30}. These data can be imprints of relatively recent introgression events, and could support theory of complex origin of European plum.

The pollen component of *S*-allele system, the *S*-haplotype F-box (*SFB*) locus was also characterized. Size of the fragments was very similar with the locus of other species published earlier (NUNES et al. 2006). A total of 10 novel sequences were isolated in 17 *Prunus domestica* cultivars and the new alleles were labelled alphabetically (SFB_{A}-SFB_{J}). SUTHERLAND et al. (2008) studied *P. domestica* 'Verity' cultivar, which produced three *SFB* alleles and their deduced polypeptides showed exceptionally close identity with other *Prunus SFB* alleles: *P. domestica* S_{5} had 97.0% identity with *P. dulcis* S_{10} from 'Gabaix'; *P. domestica* S_{6} had 97.6% identity with *P. salicina* S_{a} from 'Burmosa' and *P. domestica* S_{9} showed a lower identity of 93.3% with *P. avium* S_{4} *SFB*. During *SFB*-sequence analysis, we also obtained values similar to Sutherland et al. (2008). Phylogenetic analysis was performed using other *Prunus*, *Malus*, and *Pyrus SFB* sequences. This further confirmed the differences between the *S*-haplotype F-box proteins of the *Prunoideae* and *Maloideae* subfamilies.

In the *Prunoideae*, most haplotypes are pollen-part mutants (HEGEDŰS et al. 2012), but stigma-part mutation events also occur. Moreover, we identified a putative non-functional *S-RNase* and *F-box* allele (S_{16m} and SFB_{J}'). In both cases, a mutation caused by a nucleotide

substitution in the protein coding region of the gene was detected, leading to a nonsense mutation that resulted in loss of allele function. In case of sour cherry similar change occurred. Sequence alignment highlighted a 1 bp deletion and 2 bp substitutions in the S_{6m2}-*RNase* compared to the wild type S_6. Deletion of a guanine at the nucleotide position +555 resulted in a frame shift that led to a premature stop codon (TSUKAMOTO et al. 2006). Later, similar event was identified: The S_{36b2}-*RNase* variant differed from the S_{36b}-*RNase* by a 1-bp substitution in the conserved region C2, causing a premature stop codon (TSUKAMOTO et al. 2010).

Fertilization of *Prunus* fruit tree species is governed by GSI, which mainly determines the commercial fruit production by effecting fruit set proportion. Cultivars sharing different *S*-alleles must be interplanted in orchards; therefore the information of *S*-genotypes necessary for fruit growing and breeding. Molecular *S*-genotyping and marker-assisted selection of SC offspring are now being successfully incorporated in *Prunus* breeding programs worldwide (TAO and IEZZOMI 2010). However, genetic polymorphism was quite high, so discrimination of all plum cultivars was successful based in their unique *S*-genotypes.

Currently there is no evidence of the self-compatibility of the European plum due to the lack of the complete gene sequences. In case of tetraploid sour cherry, (HAUCK et al. 2006) "one-allele-match" model of self-incompatibility is reported. If the hexaploid European plum cultivars harbour a similar system, then self-compatibility system requires the loss-of-function for a minimum of three *S*-haplotype-specificity components (MAKOVICS-ZSOHÁR and HALÁSZ 2016).

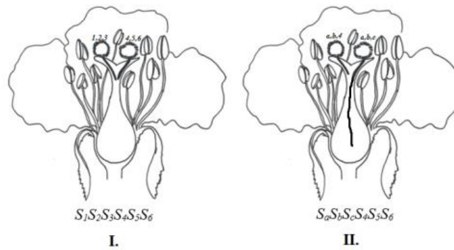


Fig.6. Putative function of gametophytic self- incompatibility for the hexaploid *Prunus domestica*. SC requires the loss-of-function for a minimum of three *S*-haplotype-specificity components (a,b,c).

Identification of *S*-genotypes of European plum cultivars and characterization of the traditional-, landscape and the economically significant cultivars of *P. domestica* by microsatellite markers are considered and proved a valuable information both practical and cultural aspects of evolution and these data could form an important basis for further investigation.

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