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# Genetic insights into *S*-haplotype determination in sour cherry cultivars released at the Fruit Research Institute, Čačak

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**Abstract**. Sour cherry (*Prunus cerasus* L.) possesses considerable potential to maintain a prominent position in the fruit-growing structure of the Republic of Serbia, owing to both its economic importance and long-standing cultivation tradition. Breeding activities on this tetraploid species at the Fruit Research Institute, Čačak (FRI) have a decades-long background, resulting in five cultivars released to date – 'Čačanski Rubin', 'Iskra', 'Nevena', 'Sofija' and 'Šumadinka'. Identification of *S*-haplotypes in released and parental cultivars represents a crucial step in their molecular characterisation and is highly relevant for both breeders and growers, as sour cherry also exhibits a gametophytic self-incompatibility system controlled by two multi-allelic genes located at the *S*-locus. The aim of this study was to compile existing knowledge and present new data on *S*-haplotypes (*S-RNase* and *SFB* alleles) of parental and released cultivars from the FRI sour cherry breeding programme. *S*-haplotype identification was carried out using the polymerase chain reaction method, employing three consensus primer pairs specific to the first and second *S-RNase* introns, allele-specific primers for both *S-RNase* and *SFB* genes, and genetic tests to detect stylar-part and pollen-part mutants. This study determined the complete *S*-haplotype of 'Čačanski Rubin' ($S_1S_4S_{13}'S_{36a}$) and, for the first time, identified three *S*-haplotypes in 'Heimanns Konserven Weichsel' ($S_9S_{13}S_{36b2}$). Furthermore, the pedigrees of FRI-released cultivars were analysed based on *S*-locus polymorphism.

**Key words**: *Prunus cerasus* L., *S-RNase*, *SFB*, *S*-genotyping, gametophytic self-incompatibility, sour cherry breeding

## Introduction

Sour cherry (*Prunus cerasus* L.) is one of the leading temperate zone fruit species in the Republic of Serbia (RS), with an average annual production of 124,264 tonnes (2014–2023; FAOSTAT, 2025), ranking third in importance after plum and apple. The fruit of this species is appreciated for its distinctive sweet-acidic taste and excellent suitability for processing into various products. Additionally, sour cherry, together with raspberry, is among the most important fruits in terms of export from the RS.

Since its initiation, the decades-long sour cherry breeding programme at the Fruit Research Institute, Čačak (FRI) can be divided into two phases, both aimed at developing self-fertile, high-yielding cultivars with different ripening times, large, high-quality

fruits suitable for freezing, processing and fresh consumption, featuring a favourable flesh-to-stone ratio and dark red or colorless juice that remains intact when separated from the stalk (Milenković et al., 2006; Radičević et al., 2020). Particular attention was also given to developing genotypes that are tolerant or resistant to cherry leaf spot, brown rot and shot-hole (Cerović et al., 1998; Radičević et al., 2018). To date, five sour cherry cultivars have been released – 'Čačanski Rubin' and 'Šumadinka' in the first phase, and 'Iskra', 'Nevena' and 'Sofija' in the second phase (Radičević et al., 2020). The current breeding strategy, initiated in 2011, aligns with the requirements of modern sour cherry production, particularly in terms of breeding objectives (high cropping potential, improved fruit quality for both industrial processing and fresh consumption, tolerance to cherry leaf spot, as well as earlier ripening, self-fertility and suitability for mechanical harvesting) and the appropriate choice of parental genotypes to achieve these goals. The strategy is based on the genetic potential of well-adapted foreign cultivars, such as 'Heimanns Konserven Weichsel', or FRI-released cultivars ('Nevena', 'Sofija' and 'Šumadinka'), that have these cultivars in their pedigree, in combination with autochthonous genotypes of Serbian and Hungarian origin (Radičević et al., 2020). Priority has also been given to sour cherry germplasm from the West Serbia region, which represents a rich source of diversity for clonal selection, with genotypes that can be used as commercial cultivars or as parents in breeding programmes. Radičević et al. (2019) reported that many of these genotypes have been evaluated, some were selected as elite, and 'GV-6' and 'GV-10' are currently undergoing the recognition procedure due to their early ripening, large fruits and disease resistance.

Sour cherry is an allotetraploid fruit species that exhibits gametophytic self-incompatibility (GSI), like other *Prunus* species. GSI is controlled by two multiallelic, closely linked genes at the *S*-locus, with the S-RNase produced in the style interacting in an allele-specific manner with the pollen SFB protein (Bošković & Tobutt, 1996; Yamane et al., 2003). The first DNA markers for assessing cross-(in)compatibility and self-compatibility in cherries, made available to breeders, were developed based on known sequence variations, including base pair substitutions and insertions/deletions, in both the *S-RNase* and *SFB* genes (Quero-García et al., 2019). To our knowledge, 12 functional ($S_1$, $S_4$, $S_6$, $S_9$, $S_{12}$, $S_{13}$, $S_{14}$, $S_{16}$, $S_{26}$, $S_{33}$, $S_{34}$ and $S_{35}$) and nine non-functional ($S_1'$, $S_{6m}$, $S_{6m2}$, $S_{13m}$, $S_{13}'$, $S_{36a}$, $S_{36b}$, $S_{36b2}$ and $S_{36b3}$) *S*-haplotypes have been identified in sour cherry (Sonneveld et al., 2001, 2003; Yamane et al., 2003; Ikeda et al., 2005; Hauck et al., 2006a, 2006b; Tsukamoto et al., 2006, 2008a, 2008b, 2010; Lisek et al., 2017; Sebolt et al., 2017; Halász et al., 2019; Marić et al., 2024).

The initiation of the CherrySeRB project (*Genetic potential of Serbian autochthonous cherry genotypes for temperature-adaptable reproductive behaviour and nutraceutical value*), supported by the Science Fund of the RS – Program IDEAS (2022–2025), has recently enabled the identification of *S*-haplotypes in tetraploid sour cherry genotypes. Accordingly, this study employed polymerase chain reaction (PCR)-based detection methods with a range of primers, including consensus, allele-specific, derived cleaved amplified polymorphic sequence (dCAPS) and insert-specific, to identify the *S*-haplotypes of previously uncharacterised parental cultivars utilized in FRI breeding efforts for developing sour cherry cultivars. The results also allowed verification of the reported parental combinations of five released sour cherry cultivars.

## Materials and Methods

*Plant material and DNA isolation*. Seven sour cherry cultivars (refer to Tables 1 and 2) were used in this study. Samples were collected from the cherry gene-bank collection and the field trial of FRI at the experimental sites 'Ljubić' and 'Čačak', respectively, as well as from the cherry gene-bank collection of the Faculty of Agriculture, University of Belgrade, at the experimental site 'Radmilovac'. Fresh young leaves of the two cultivars ('Čačanski Rubin' and 'Heimanns Konserven Weichsel'), whose *S*-haplotypes have not been published or uncompleted to date, were collected in spring 2023, frozen in liquid nitrogen and stored at -80°C. Total genomic DNA was then extracted from the frozen leaves following the method of Doyle & Doyle (1987), dissolved in TE buffer containing RNase A (Invitrogen, Groningen, the Netherlands), and stored at -20°C until PCR analysis.

Table 1. *S*-haplotypes of FRI-released sour cherry cultivars
*Tabela 1. S-haplotipovi sorti višnje stvorenih u Institutu za voćarstvo, Čačak*

| Released cultivar<br>*Priznata sorta* | Parental combination<br>*Roditeljska kombinacija* | *S*-haplotype<br>*S-haplotip* | Reference<br>*Referenca* |
|---|---|---|---|
| 'Čačanski Rubin' | 'Shasse Morello' × 'Köröser Weichsel' | $S_1S_4S_{13}'S_{36a}$ | This study/*Ovo istraživanje* |
| 'Iskra' | 'Köröser Weichsel' × 'Heimanns Rubin Weichsel' | $S_1S_{13}S_{36b}S_{36b2}$ | Marić et al., 2024 |
| 'Nevena' | 'Köröser Weichsel' × 'Heimanns Konserven Weichsel' | $S_4S_9S_{36b}S_{36b2}$ | Marić et al., 2024 |
| 'Sofija' | 'Čačanski Rubin' × 'Heimanns Konserven Weichsel' | $S_1S_{13}'S_{36b2}S_x$ | Marić et al., 2024 |
| 'Šumadinka' | 'Köröser Weichsel' × 'Heimanns Konserven Weichsel' | $S_1S_{13}S_{36b}S_{36b2}$ | Marić et al., 2024 |

Table 2. *S*-haplotypes of parental sour cherry cultivars
*Tabela 2. S-haplotipovi roditeljskih sorti višnje*

| Parental cultivar/*Roditeljska sorta* | *S*-haplotype/*S-haplotip* | Reference/*Referenca* |
|---|---|---|
| 'Čačanski Rubin' | $S_1S_4S_{13}'S_{36a}$ | This study/*Ovo istraživanje* |
| 'Köröser Weichsel' ('Crisana') | $S_1S_4S_{35}S_{36b}$ | Yamane et al., 2001; Tsukamoto et al., 2008a, 2010 |
| 'Heimanns Konserven Weichsel' | $S_9S_{13}S_{36b2}S_x$ | This study/*Ovo istraživanje* |

*PCR analysis for S-RNase and SFB genotyping.* To identify *S-RNase* and *SFB* alleles, different PCR analyses were performed using three consensus primer pairs spanning the first (PaConsI-F/PaConsI-R; Sonneveld et al., 2003) and second (PaConsII-F/PaConsII-R, Sonneveld et al., 2003; Pru-C2/PCE-R, Tao et al., 1999, Yamane et al., 2001) introns of the *S-RNase* gene, in combination with various primers (allele-specific, dCAPS and insert-specific) designed for both genes of the *S*-locus. PCR reactions and amplification conditions for the consensus primers were identical to those used by Sonneveld et al. (2003), Sebolt et al. (2017) and Marić et al. (2024). Further identification of the *S-RNase* alleles originating from sweet cherry ($S_1$-, $S_4$-, $S_6$-, $S_9$-, $S_{12}$- and $S_{13}$-*RNase*; Sonneveld et al., 2001, 2003) and ground cherry ($S_{26}$- and $S_{36}$-*RNase*; Hauck et al., 2006b; Tsukamoto et al., 2010) was conducted following the protocols outlined by Sebolt et al. (2017) and Marić et al. (2024). Additionally, for 'Heimanns Konserven Weichsel', specific PCR reactions were performed for the $S_{14}$, $S_{16}$, $S_{33}$, $S_{34}$ and $S_{35}$ alleles (Sonneveld et al., 2003; Tsukamoto et al., 2008a), applying annealing temperatures and extension times recommended by Sebolt et al. (2017), with some modifications implemented in this study. In order to verify the *S*-haplotypes in the assessed sour cherry cultivars, allele-specific primers for $SFB_1$, $SFB_4$, $SFB_9$, $SFB_{13}$ and $SFB_{36}$ (Ikeda et al., 2005; Tsukamoto et al., 2006, 2010; Sebolt et al., 2017) were used, while the PCR reaction and amplification conditions were described by Sebolt et al. (2017) and Marić et al. (2024). In 'Čačanski Rubin', an additional primer pair reported by Tsukamoto et al. (2008b) and the PCR protocol described by Sebolt et al. (2017) were used to distinguish the $SFB_1$ allele from the pollen-part mutant $SFB_1'$.

To discriminate the mutant $S_{13}$- and $S_{36}$-haplotypes, dCAPS primers followed by digestion with restriction enzymes were used for both haplotypes (*Dde*I, *Mae*II and *Spe*I for the $S_{13}$-haplotype; *Ava*II, *Hind*III and *Rsa*I for the $S_{36}$-haplotype; Fermentas, Thermo Scientific, Waltham, MA, USA), whereas allele-specific and insert-specific primers were required to distinguish non-functional variants of the $S_{36}$-haplotype, as described by Sebolt et al. (2017) and Marić et al. (2024).

*Detection and visualization of DNA fragments*. PCR products obtained with the consensus primer pairs and restriction fragments were separated on a 2% agarose gel (70 V $cm^{-1}$ for 4 h), whereas products of the allele-specific and insert-specific PCRs for both genes of the *S*-locus were separated on a 1.5% agarose gel (70 V $cm^{-1}$ for 2–3 h) using the Biometra Horizon 11.14 system (Analytik Jena GmbH, Jena, Germany). In addition to Agarose Serva Premium (Serva Electrophoresis GmbH, Heidelberg, Germany), Agarose Serva for PCR (Serva Electrophoresis GmbH) was used for electrophoretic separation of fragments obtained from restriction analyses. Visualization of DNA bands was performed by ethidium bromide staining and UV light

using the BIO-PRINT-1500/26M imaging system (Vilber Lourmat, Collégien, France). A 1 Kb plus DNA ladder (Invitrogen, Groningen, the Netherlands) and the FastLoad 50 bp DNA Ladder (Serva Electrophoresis GmbH) were used to determine the size of the DNA fragments.

## Results and Discussion

*Identification of S-haplotypes in 'Čačanski Rubin' and 'Heimanns Konserven Weichsel'*. The *S*-haplotypes in these two sour cherry cultivars were identified in the following steps: first, by amplification with consensus primers specific for both *S-RNase* introns, then using primers specific to *S-RNase* and *SFB* alleles, combined with additional genetic tests to identify the pollen-part and stylar-part mutants.

The PCR products amplified with primers specific to the first *S-RNase* intron (PaConsI-F/R; Table 3) ranged in length from ~420 bp ($S_9/S_{12}$-*RNase*) to ~520 bp ($S_4/S_6$-*RNase*). In this study, primers for the first intron failed to amplify $S_{13}$-*RNase*, consistent with results reported by Sonneveld et al. (2003) and Schuster et al. (2007) for sweet cherry, as well as Lisek et al. (2017) and Marić et al. (2024) for sour cherry genotypes. The lengths of the PCR products amplified with the two primer pairs specific to the second *S-RNase* intron (PaConsII-F/R and Pru-C2/PCE-R) are shown in Table 3. Using these primers, three fragments were obtained in both cultivars. The PCR products amplified with PaConsII-F/R primers ranged from ~790 bp ($S_9$-*RNase*) to ~1,060 bp ($S_4$-*RNase*), and those amplified with Pru-C2/PCE-R primers varied from ~550 bp ($S_9/S_{26}$-*RNase*) to ~820 bp ($S_4$-*RNase*). In both cultivars, besides the ~880 bp band ($S_1/S_{13}$-*RNase*) obtained with the PaConsII-F/R, two additional bands of approximately 490 bp and 330 bp were amplified, indicating the presence of $S_{13}$-*RNase*. Sebolt et al. (2017) and Marić et al. (2024) reported that Pru-C2/PCE-R successfully amplified all alleles identified in sour cherry to date, except for $S_{35}$-*RNase*. Therefore, $S_{35}$-specific primers for both *S*-locus genes ($S_{35}$-*RNase* and $SFB_{35}$) were used; however, neither cultivar yielded an amplification product, indicating the absence of the $S_{35}$-haplotype.

The aforementioned results were further confirmed using *S-RNase* and *SFB* allele-specific primers, combined with additional genetic tests for the $S_1$, $S_{13}$ and $S_{36}$ alleles. In 'Čačanski Rubin', PCR products of 820 bp and 768 bp, corresponding to $S_1$-*RNase* and $SFB_1$, respectively, were detected (Tables 4 and 5). The presence of the functional $SFB_1$ variant in this cultivar was validated by the absence of amplification with PcSFB1'-F/PaSFB1-R primer pairs, specifically designed to anneal within the 615 bp insertion and to amplify the expected 826 bp fragment of $SFB_1'$. In this released cultivar, which was also the parental cultivar in the pedigree of 'Sofija', DNA fragments of 820 bp and 780 bp, corresponding to $S_4$-*RNase* and $SFB_4$, respectively, were obtained. The $S_9$ allele was identified in 'Heimanns Konserven Weichsel' through the amplification of PCR products of 495 bp and 250 bp, confirming the presence of $S_9$-*RNase* and $SFB_9$ (Tables 4 and 5).

Table 3. Identification of *S-RNase* alleles with three different consensus primer pairs in the released and parental sour cherry cultivars
*Tabela 3. Identifikacija alela S-RNaze pomoću tri različita para konsenzus prajmera kod priznatih i roditeljskih sorti višnje*

| Cultivar *Sorta* | Results with PaConsI-F/R primers *Rezultati sa PaConsI-F/R prajmerima* | | | | Results with PaConsII-F/R primers *Rezultati sa PaConsII-F/R prajmerima* | | | | Results with Pru-C2/PCE-R primers *Rezultati sa Pru-C2/PCE-R prajmerima* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Allele 1 *Alel 1* | Allele 2 *Alel 2* | Allele 3 *Alel 3* | Allele 4 *Alel 4* | Allele 1 *Alel 1* | Allele 2 *Alel 2* | Allele 3 *Alel 3* | Allele 4 *Alel 4* | Allele 1 *Alel 1* | Allele 2 *Alel 2* | Allele 3 *Alel 3* | Allele 4 *Alel 4* |
| 'Čačanski Rubin' | $S_1$ (~450 bp) | $S_4/S_6$ (~520 bp) | /* / | / / | $S_1/S_{13}$ (~880 + 490 + 330 bp) | $S_4$ (~1,060 bp) | $S_{36}$ (~980 bp) | / / | $S_1/S_{13}$ (~630 bp) | $S_4$ (~820 bp) | $S_{36}$ (~720 bp) | / / |
| 'Heimans Konserven Weichsel' | $S_9/S_{12}$ (~420 bp) | / / | / / | / / | $S_9$ (~790 bp) | $S_{13}$ (~880+ 490 + 330 bp) | $S_{36}$ (~980 bp) | / / | $S_1/S_{13}$ (~630 bp) | $S_9/S_{26}$ (~550 bp) | $S_{36}$ (~720 bp) | / / |

*/ – Not amplified allele / *Neamplifikovani alel*.

Table 4. Identification of *S-RNase* alleles with the specific primers in the released and parental sour cherry cultivars
*Tabela 4. Identifikacija alela S-RNaze pomoću specifičnih prajmera kod priznatih i roditeljskih sorti višnje*

| Cultivar<br>*Sorta* | *S-RNase*-allele results based on specific primers<br>*Rezultati sa S-RNaze alel-specifičnim prajmerima* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $S_1$ (820 bp) | $S_4$ (820 bp) | $S_6$ (470 bp) | $S_9$ (495 bp) | $S_{12}$ (562 bp) | $S_{13}$ (306 bp) | $S_{26}$ (773 bp) | $S_{36}$ (760 bp) |
| 'Čačanski Rubin' | +* | + | – | | | + | | + |
| 'Heimanns Konserven Weichsel' | | | + | – | + | – | + | |

*+ (PCR product obtained using *S-RNase* allele-specific primers); – (no amplification using *S-RNase* allele-specific primers); empty cell (PCR with *S-RNase* allele-specific primers was not conducted based on the results obtained using consensus primers) / *+ (PCR proizvod dobijen korišćenjem S-RNaza alel-specifičnih prajmera); – (odsustvo amplifikacije korišćenjem S-RNaza alel-specifičnih prajmera); prazna ćelija (PCR sa S-RNaza alel-specifičnim prajmerima nije sproveden na osnovu rezultata dobijenih korišćenjem konsenzus prajmera).*

Table 5. Identification of *SFB* alleles with the specific primers in the released and parental sour cherry cultivars
*Tabela 5. Identifikacija SFB alela pomoću specifičnih prajmera kod priznatih i roditeljskih sorti višnje*

| *Culti*var<br>*Sorta* | *SFB*-allele results based on specific primers<br>*Rezultati sa SFB alel-specifičnim prajmerima* | | | | | |
|---|---|---|---|---|---|---|
| | $SFB_1$ (768 bp – PaSFB1-F/R) (No – PcSFB1'-F/PaSFB1-R) | $SFB_1'$ (1,383 bp – PaSFB1-F/R) (826 bp – PcSFB1'-F/PaSFB1-R) | $SFB_4$ (780 bp) | $SFB_9$ (250 bp) | $SFB_{13}$ (439 bp) | $SFB_{36}$ (721 bp) |
| 'Čačanski Rubin' | +*/No | – / – | + | | + | + |
| 'Heimanns Konserven Weichsel' | | | | + | + | + |

*+ (PCR product obtained with *SFB* allele-specific primers); No (the allele was not amplified with certain primer pairs specific to pollen-part mutant); – (no amplification with *SFB* allele-specific primers); empty cell (PCR with *SFB* allele-specific primers was not conducted since the cultivar does not possess the specific allele) / *+ (PCR proizvod dobijen korišćenjem SFB alel-specifičnih prajmera); No (alel nije amplifikovan korišćenjem određenog para prajmera specifičnih za nefunkcionalnu SFB alelnu varijantu); – (odsustvo amplifikacije korišćenjem SFB alel-specifičnih prajmera); prazna ćelija (PCR sa SFB alel-specifičnim prajmerima nije sproveden jer sorta ne poseduje dati alel).*

The primers specific to the $S_{13}$-haplotype enabled the amplification of 306 bp (*$S_{13}$-RNase*) and 439 bp (*$SFB_{13}$*) fragments in both cultivars (Tables 4 and 5). In order to distinguish the functional *$S_{13}$-RNase* from the stylar-part mutant *$S_{13m}$-RNase*, amplification with $MaeS_{13m}$-F + $S_{13}$-RNase-spR, followed by digestion with the *Mae*II restriction enzyme, was applied. The functional *$S_{13}$-RNase* variant in these cultivars was identified based on the obtained PCR product of 161 bp and the absence of *Mae*II digestion (Table 6). As Marić et al. (2024) reported, the bands tended to fade quickly due to the small product size. The $S_{13}$-haplotype is an example in which three variants of a single ancestral haplotype need to be distinguished (Sebolt et al., 2017). Therefore, the next step was to discriminate between the functional *$SFB_{13}$* and the pollen-part mutant *$SFB_{13}'$*, differing by a 1-bp substitution in their coding region (the guanine in *$SFB_{13}$* is replaced by a thymine in *$SFB_{13}'$*; Tsukamoto et al., 2006). After applying the dCAPS marker test, which included *Dde*I and *Spe*I digestion (Table 6), only 'Čačanski Rubin' produced bands of 278 bp and 280 bp, resulting in restriction fragments of 254/24 bp and 260/20 bp, respectively, thus revealing the *$SFB_{13}'$*. However, the dCAPS primers (refer to Table 6) did not amplify in 'Heimanns Konserven Weichsel', which was consistent with the results reported by Marić et al. (2024) for 'Iskra' and 'Šumadinka'. Furthermore, sequencing of the *$SFB_{13}$* PCR product (439 bp) in 'Heimanns Konserven Weichsel' revealed functional *$SFB_{13}$* (data not shown), as previously reported for 'Iskra' and 'Šumadinka'.

Table 6. Identification of $S_{13}$-haplotypes in the released and parental sour cherry cultivars
*Tabela 6. Identifikacija $S_{13}$-haplotipova kod priznatih i roditeljskih sorti višnje*

| Cultivar<br>*Sorta* | Discrimination $S_{13}$-*RNase*/$S_{13m}$-*RNase*<br>*Razlikovanje* $S_{13}$-*RNaze*/$S_{13m}$-*RNaze* | Discrimination $SFB_{13}$/$SFB_{13}'$<br>*Razlikovanje* $SFB_{13}$/$SFB_{13}'$ | |
|---|---|---|---|
| | MaeS$_{13m}$-F + S$_{13}$-RNase-spR | DdeS13-F + SFB13-spR | SpeS13'-F + SFB13-spR |
| 'Čačanski Rubin' | 161 bp | 278 bp | 280 bp (weak band/*slaba traka*) |
| 'Heimanns Konserven Weichsel' | 161 bp | No amplification<br>*Bez amplifikacije* | No amplification<br>*Bez amplifikacije* |
| | *Mae*II digestion<br>*Digestija enzimom MaeII* | *Dde*I digestion<br>*Digestija enzimom DdeI* | *Spe*I digestion<br>*Digestija enzimom SpeI* |
| 'Čačanski Rubin' | 161 bp | 254/24 bp | 260/20 bp (weak band/*slaba traka*) |
| 'Heimanns Konserven Weichsel' | 161 bp | /* | / |

*/ – Digestion of PCR product was not conducted because the allele was not amplified with specific primer pair / *Digestija PCR proizvoda nije sprovedena jer alel nije amplifikovan određenim parom specifičnih prajmera.*

Differentiation among the four non-functional $S_{36}$ variants ($S_{36a}$, $S_{36b}$, $S_{36b2}$ and S$_{36b3}$) was performed using various primers, including restriction analysis for specific variants. In both cultivars, primers specific to $S_{36}$-*RNase* and $SFB_{36}$ amplified fragments of 760 bp and 721 bp (Tables 4 and 5), respectively, with the *SFB* fragment additionally digested using *Rsa*I restriction enzyme (Table 7). The 898 bp PCR product obtained with $S_{36a}$-specific primers, along with the absence of amplification using PcS36ab-F and PcS36b/b2/b3-R primers, which amplify all other variants, indicated that 'Čačanski Rubin' possesses the $S_{36a}$ variant. Additional fragments of 1,042 bp (distinguishing $S_{36a}$/$S_{36b}$/$S_{36b3}$ from $S_{36b2}$) and 270 bp (which was not digested with *Ava*II), together with the absence of amplification using $S_{36b2}$ insert-specific primers, final-

Table 7. Identification of $S_{36}$-haplotypes in the released and parental sour cherry cultivars
*Tabela 7. Identifikacija $S_{36}$-haplotipova kod priznatih i roditeljskih sorti višnje*

| Cultivar<br>*Sorta* | $S_{36}$-*RNase* | $S_{36a}$ | $S_{36b}$/$S_{36b2}$/$S_{36b3}$ | $S_{36b2}$ *vs.*<br>$S_{36a}$/$S_{36b}$/$S_{36b3}$ | $S_{36b2}$ | $S_{36b}$ | $SFB_{36}$ |
|---|---|---|---|---|---|---|---|
| | PcS36ab-F1 +<br>PcS36ab-R1 | PcS36ab-F +<br>PcS36a-spR | PcS36ab-F +<br>PcS36b/b2/b3-R | PcS36ab-F2 +<br>PcS36ab-R | PcS36ab-F2 +<br>PcS36b2-spR | AvaS36b2-F +<br>PcS36abspRNase-R2 | PcSFB36ab-F+<br>+ PcSFB36ab-R |
| 'Čačanski Rubin' | 760 bp | 898 bp | No* | 1,042 bp | No | 270 bp | 721 bp |
| 'Heimanns Konserven Weichsel' | 760 bp | No | 760 bp | 1,348 bp | 513 bp | 270 bp | 721 bp |
| | | | *Hind*III digestion<br>*Digestija enzimom HindIII* | | | *Ava*II digestion<br>*Digestija enzimom AvaII* | *Rsa*I digestion<br>*Digestija enzimom RsaI* |
| 'Čačanski Rubin' | | | / | | | 270 bp | 326/262/133 bp |
| 'Heimanns Konserven Weichsel' | | | 760 bp | | | 250/20 bp | 326/284/111 bp |

*No – the allele was not amplified using a certain primer pair specific to some non-functional variant; / – digestion of the PCR product was not conducted because the allele was not amplified with the specific primer pair; empty cell – allele identified based on the PCR reaction without restriction analysis / *No – alel nije amplifikovan korišćenjem određenog para prajmera specifičnih za neku nefunkcionalnu varijantu; / – digestija PCR proizvoda nije sprovedena jer alel nije amplifikovan određenim parom specifičnih prajmera; prazna ćelija – alel je identifikovan na osnovu PCR reakcije bez restrikcione analize.*

ly confirmed the presence of $S_{36a}$ in 'Čačanski Rubin' (Table 7). However, in 'Heimanns Konserven Weichsel', the absence of amplification with $S_{36a}$-specific primers, along with the amplification of a 760 bp fragment using $S_{36b}$/$S_{36b2}$/$S_{36b3}$-specific primers that was not digested with *Hind*III, indicated the presence of $S_{36b}$ and/or $S_{36b2}$. Furthermore, primers PcS36ab-F2 and PcS36ab-R, used to distinguish $S_{36b2}$ from the other two $S_{36}$ variants, enabled amplification of a 1,348 bp fragment (corresponding to $S_{36b2}$). This fragment, together with the amplified $S_{36b2}$ insert-specific fragment (513 bp) and a 270 bp fragment digested with *Ava*II (250/20 bp), confirmed that 'Heimanns Konserven Weichsel' carries the $S_{36b2}$ variant (Table 7).

Seven *S*-haplotypes ($S_1$, $S_4$, $S_9$, $S_{13}$, $S_{13}'$, $S_{36a}$ and $S_{36b2}$) were identified in these two sour cherry cultivars. We determined all four *S*-haplotypes in 'Čačanski Rubin' ($S_1S_4S_{13}'S_{36a}$) and three in 'Heimanns Konserven Weichsel' ($S_9S_{13}S_{36b2}S_x$). This study revealed the complete *S*-haplotype of 'Čačanski Rubin', which is consistent with the result previously reported by Bošković et al. (2006), who noted, based on *S-RNase* alleles, that this cultivar could have the $S_1S_4S_{13}S_B$ genotype. Regarding 'Heimanns Konserven Weichsel', whose three *S*-haplotypes were identified for the first time, it is likely that the fourth *S*-haplotype, designated as $S_x$, represents a duplication of one of the three identified. This assumption is also supported by the fact that, in addition to the aforementioned, no amplification was obtained using primers specific to $S_{14}$-, $S_{16}$-, $S_{33}$- and $S_{34}$-*RNase.*

*Distribution of S-alleles in FRI-released sour cherry cultivars*. Although the number of released sour cherry cultivars is quite small from the aspect of allele frequency discussion, relative occurrences were calculated, excluding the cultivar 'Sofija', for which the fourth *S*-haplotype was not determined. Therefore, the most frequent *S*-haplotypes in this material were $S_1$, $S_{36b}$ and $S_{36b2}$, all occurring at a frequency of 18.75%, followed by $S_4$ and $S_{13}$ (12.5%), and $S_9$, $S_{13}'$ and $S_{36a}$ (6.25%). Collectively considered, the $S_{36}$ variants were the most frequent (together 43.75%), which is consistent with previously reported data indicating that the four non-functional $S_{36}$ variants are widespread *S*-haplotypes in sour cherry, since all genotypes analysed to date possess at least one or two variants of this haplotype (Tsukamoto et al., 2010; Sebolt et al., 2017; Halász et al., 2019). According to Lisek et al. (2017), among sour cherry cultivars originating from various European countries (predominantly Poland and Germany, as well as Romania, Russia and Ukraine), $S_{13}'$ (61.9%) was the most common, followed by $S_{36a}$ (57.1%) and $S_{26}$ (47.6%). However, Marić et al. (2024) noted that a more comprehensive understanding of *S*-haplotype frequency in sour cherry requires haplotyping a larger number of genotypes, along with comparative analyses across different regions and breeding programmes.

*S-haplotypes of FRI-released sour cherry cultivars and their parental origins*. *S*-haplotypes of released sour cherry cultivars and their parental cultivars are presented in Table 1 and Table 2, respectively. In Table 2, data for 'Shasse Morello' and 'Heimanns Rubin Weichsel' are missing because the trees of these two parental cultivars no longer exist in the FRI sour cherry collection. Consequently, no DNA was available, and these parental cultivars could not be analysed.

Among the five cultivars released within the FRI breeding programme, the pedigrees of 'Nevena' ($S_4S_9S_{36b}S_{36b2}$) and 'Šumadinka' ($S_1S_{13}S_{36b}S_{36b2}$) agreed with their respective parental haplotypes ['Köröser Weichsel' ($S_1S_4S_{35}S_{36b}$) × 'Heimanns Konserven Weichsel' ($S_9S_{13}S_{36b2}S_x$)]. Although the fourth *S*-haplotype in 'Heimanns Konserven Weichsel' was not determined, it is evident that 'Nevena' and 'Šumadinka' inherited $S_9$ and $S_{36b2}$, and $S_{13}$ and $S_{36b2}$, respectively, from this parental cultivar.

The incomplete *S*-haplotype of 'Sofija' ($S_1S_{13}'S_{36b2}S_x$), with the fourth unidentified *S*-haplotype, supports the reported parental combination ['Čačanski Rubin' ($S_1S_4S_{13}'S_{36a}$) × 'Heimanns Konserven Weichsel' ($S_9S_{13}S_{36b2}S_x$)] and indicates that $S_{36b2}$ and $S_x$ were inherited from 'Heimanns Konserven Weichsel'. While a progeny test is required to determine which *S*-haplotype is duplicated (Sebolt et al., 2017), these three cultivars, raised from crosses in which one parental cultivar is 'Heimanns Konserven Weichsel', were insufficient for clarification. Therefore, further crosses with diverse cultivars are needed.

*S*-haplotype data for 'Čačanski Rubin' ($S_1S_4S_{13}'S_{36a}$) and 'Iskra' ($S_1S_{13}S_{36b}S_{36b2}$) suggest that 'Köröser Weichsel' ($S_1S_4S_{35}S_{36b}$) is their male and female parent, respectively.

## Conclusion

This study represents an important foundational stage in the characterisation of sour cherry cultivars developed and utilized through the FRI breeding programme. It provides valuable insights for growers and breeders, facilitating the effective management of released and parental cultivars to achieve high-yielding sour cherry production, as well as supporting the planning of future crosses. Furthermore, *S*-locus polymorphism was employed in this study to verify the parentage of the released sour cherry cultivars.

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# GENETIČKI ASPEKTI DETERMINACIJE *S*-HAPLOTIPA SORTI VIŠNJE STVORENIH U INSTITUTU ZA VOĆARSTVO, ČAČAK

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## Rezime

Višnja (*Prunus cerasus* L.) je koštičava vrsta voćaka od značajnog potencijala da zadrži istaknuto mesto u voćarskoj proizvodnji Republike Srbije, zahvaljujući kako ekonomskom značaju, tako i dugoj tradiciji gajenja. Višegodišnji oplemenjivački rad na višnji u Institutu za voćarstvo, Čačak je rezultirao priznavanjem pet sorti – Čačanski rubin, Iskra, Nevena, Sofija i Šumadinka. Identifikacija *S*-haplotipa kod priznatih i roditeljskih sorti predstavlja ključni korak u njihovoj molekularnoj karakterizaciji i od velikog je značaja kako za oplemenjivače, tako i za proizvođače, budući da višnja, kao tetraploidna vrsta, ispoljava gametofitnu samo-inkompatibilnost regulisanu ekspresijom dva multialelna gena *S*-lokusa. Imajući u vidu njenu poliploidnu prirodu, genetička osnova samooplodnosti/samobesplodnosti, kao i identifikacija *S*-haplotipa, složenija je u poređenju sa diploidnim vrstama roda *Prunus*. Cilj ovog istraživanja bio je da objedini postojeća saznanja i prikaže nove rezultate identifikacije *S*-haplotipa (aleli *S-RNaze* i *SFB* gena) kod priznatih i roditeljskih sorti u okviru oplemenjivačkog programa višnje Instituta za voćarstvo, Čačak. Identifikacija *S*-haplotipa zasnovana je na metodi lančane reakcije polimeraze (PCR metoda), uz primenu tri para konsenzus prajmera specifičnih za prvi i drugi intron *S-RNaze*, alel-specifičnih prajmera za *S-RNazu* i *SFB*, kao i genetičkih testova za razlikovanje funkcionalnih i nefunkcionalnih alelnih formi oba gena *S*-lokusa. U okviru ovog istraživanja određen je kompletan *S*-haplotip sorte Čačanski rubin ($S_1S_4S_{13}'S_{36a}$), dok su po prvi put identifikovana tri *S*-haplotipa sorte Heimanns Konserven Weichsel ($S_9S_{13}S_{36b2}$). Pored toga, analizirano je poreklo priznatih sorti na osnovu identifikovanih *S*-haplotipova, jer se *S*-lokus, zbog visoke polimorfnosti i potvrđenog nasleđivanja u skladu sa Mendelovim zakonima, primenjuje kao funkcionalni DNK marker u genotipizaciji i proučavanju pedigrea sorti. *S*-haplotip sorti Nevena ($S_4S_9S_{36b}S_{36b2}$) i Šumadinka ($S_1S_{13}S_{36b}S_{36b2}$) u potpunosti odgovara roditeljskoj kombinaciji Köröser Weichsel ($S_1S_4S_{35}S_{36b}$) × Heimanns Konserven Weichsel ($S_9S_{13}S_{36b2}S_x$). Takođe, *S*-haplotip sorte Sofija ($S_1S_{13}'S_{36b2}S_x$), nastale iz ukrštanja Čačanski rubin ($S_1S_4S_{13}'S_{36a}$) × Heimanns Konserven Weichsel ($S_9S_{13}S_{36b2}S_x$), u skladu je sa roditeljskim *S*-haplotipovima. Na osnovu *S*-haplotipova, sorta Köröser Weichsel ($S_1S_4S_{35}S_{36b}$) je potencijalno muški, odnosno ženski roditelj sorti Čačanski rubin ($S_1S_4S_{13}'S_{36a}$) i Iskra ($S_1S_{13}S_{36b}S_{36b2}$), po redosledu. Rezultati ovog istraživanja su od posebnog značaja za planiranje ukrštanja u budućim oplemenjivačkim programima, kao i za pravilno određivanje adekvatnih sortnih kompozicija oprašivača u komercijalnim zasadima, u cilju povećanja ukupnog nivoa proizvodnje ove vrste voćaka.

**Ključne reči**: *Prunus cerasus* L., *S-RNaze, SFB*, *S*-genotipizacija, gametofitna samo-inkompatibilnost, oplemenjivanje višnje