UDC 575.630
https://doi.org/10.2298/GENSR1701127M
Original scientific paper

DETERMINATION OF *S*-GENOTYPE IN APPLE AND SWEET CHERRY CULTIVARS RELEASED AT FRUIT RESEARCH INSTITUTE, ČAČAK

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Marić S., S. Radičević, M. Lukić, R. Cerović, S. A. Paunović (2017): *Determination of S-genotype in apple and sweet cherry cultivars released at Fruit Research Institute, Čačak.*- Genetika, Vol. 49, No.1, 127-138.
Apples and sweet cherries are generally characterized by self-incompatibility, which is controlled by the multiallelic locus *S* with gametophytic action. Thus the identification of *S*-genotype represents crucial information for fruit breeders and growers. The aim of this study was to identify the *S*-genotype of two apple ('Čačanska Pozna' and 'Čadel') and two sweet cherry ('Asenova Rana' and 'Čarna') cultivars developed at Fruit Research Institute, Čačak using the polymerase chain reaction (PCR) method with consensus and allele-specific primers. The *S*-genotype of apple cultivars 'Čačanska Pozna' (S_7S_9) and 'Čadel' (S_2S_7) were consistent with parental combinations 'Starking Delicious' (S_9S_{28}) × 'Jonathan' (S_7S_9) and 'Golden Delicious' (S_2S_3) × 'Jonathan' (S_7S_9), respectively. For sweet cherry cultivars 'Asenova Rana' ['Drogans Gelbe' (S_1S_5) × 'Majova Rana' (S_1S_4)] and 'Čarna' ['Majova Rana' (S_1S_4) × 'Bigarreau de Schrecken' (S_1S_3)], S_3S_9 and S_1S_4 were identified respectively, suggesting that the reported pedigree is erroneous. The trueness-to-type of DNA samples of both sweet cherry cultivars was confirmed by comparison of cultivar samples from more than one site. Upon this testing, the remaining inconsistency indicated that aforementioned parentages of the sweet cherry cultivars were incorrect. These discrepancies could be the result of possible pollen contamination, or mistakes either in breeders recording or in seed processing and seedling production. The results of

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S-genotyping of apple and sweet cherry cultivars provide relevant information on correct assignment of these cultivars to cross-compatibility groups, which are important for cross design in developing new cultivar, as well as for orchard management in the efficient production of fruits.

Key words: Malus × *domestica*, *Prunus avium*, planned hybridisation, self-incompatibility, PCR amplification

INTRODUCTION

Apple (*Malus* × *domestica* Borkh.) and sweet cherry (*Prunus avium* L.), like other fruit tree species of the *Rosaceae* family, exhibit gametophytic self-incompatibility (GSI), a mechanism enabling styles to reject self pollen (DE NETTANCOURT, 2001). In this system, the self-incompatibility reaction is governed by the highly polymorphic, multiallelic *S*-locus with the pistil (*S-RNase*) and the pollen-expressed (*SFB*) genes (TAKAYAMA and ISOGAI, 2005).

The rosaceous *S*-RNase protein consists of five conserved regions (C1, C2, C3, RC4 and C5) and a region of high variability (RHV), located between C2 and C3 (USHIJIMA *et al*., 1998). Contrary to apple whose *S-RNase* contains one intron in the RHV (IGIC and KOHN, 2001), the allelic forms of *S-RNase* in sweet cherry contained two introns, located between the regions coding for the signal peptide region and the mature protein (the first intron) and in the RHV region (the second intron), both of which exhibit considerable length polymorphism (SONNEVELD *et al*., 2003). The aforementioned regions are believed to be important for *S* allele recognition and for the discrimination of self from non-self pollen. Based on the diversity of nucleotide sequences of the *S-RNase*, the consensus and allele-specific PCR-based methods have been developed in order to identify *S*-genotypes of apple and sweet cherry cultivars. The use of these methods has enabled the identification of 28 *S*-alleles in apple (DREESEN *et al*., 2010), as well as 25 *S*-alleles (VAUGHAN *et al*., 2008) and 47 incompatibility groups, a group of '0' of unique *S*-genotypes and group of self-compatible cultivars in sweet cherry (SCHUSTER, 2012). The knowledge of the *S*-allelic constitution of apple and sweet cherry cultivars is therefore essential for breeders and growers to establish breeding programmes and productive orchards by defining compatible combinations.

Due to high polymorphism, the *S*-locus has also been used as a genetic marker for identification of domestic and foreign apple and sweet cherry cultivars, as well as for the study of genetic diversity in many countries. In apple, DREESEN *et al.* (2010) reported that all identified *S*-alleles (except S_{26}) in modern cultivars were also present in old genotypes, while 11 alleles revealed in old cultivars were not detected in modern ones. Since the modern cultivars are developed from the breeding programmes using limited number of parental cultivars, i.e. 'Golden Delicious' (S_2S_3), 'Jonathan' (S_7S_9), 'Fuji' (S_1S_9), these explain over-representation of some alleles such as S_2, S_3, S_5 and S_9 (BROOTHAERTS, 2004; DREESEN *et al*., 2010; DE ALBUQUERQUE JR. *et al*., 2011). In contrast, the parentage of old cultivars is mainly unknown, therefore autochthonous apple germplasm is rich and diverse in properties. Regarding sweet cherry, the *S*-locus was widely used to study diversity in local germplasm from Germany (SCHUSTER *et al*., 2007; SCHUSTER, 2012), Sicily (MARCHESE *et al*., 2007), Turkey (IPEK *et al*., 2011), Croatia (ERCISLI *et al*., 2012), Spain (CACHI and WÜNSCH, 2014). Based on these findings, alleles S_1, S_2, S_3 and S_{16} were highly frequent in local genetic resources from Germany, Turkey, Croatia and Sicily, respectively. In local sweet cherry cultivars from different regions of Spain, the highest *S*-allele frequencies were observed for S_3, S_6 and S_{22}.

The apple and sweet cherry breeding work at Fruit Research Institute, Čačak (FRI), Republic of Serbia has resulted in the release of two apple cultivars − 'Čačanska Pozna' ('Starking Delicious' × 'Jonathan') and 'Čadel' ('Golden Delicious' × 'Jonathan') and two sweet cherry cultivars 'Asenova Rana' ('Drogans Gelbe' × 'Majova Rana') and 'Čarna' ('Majova Rana' × 'Bigarreau Schrecken') (MILENKOVIĆ *et al*., 2006). In addition, a number of promising apple and sweet cherry selections have been singled out. The apple breeding programme at FRI has always had a goal of developing superior cultivars with enhanced quality, extended storage life and genetic resistance to diseases (LUKIĆ *et al*., 2012). In sweet cherry, the major objectives of the FRI breeding work are self-fertility, compact habitus, early, heavy and regular cropping, early or late ripening time, large and attractive fruits (BUDAN *et al*., 2013). Recently, the application of functional markers within these conventional programmes with the aim to improve efficiency by enabling early selection for adult traits and simultaneous selection for multiple traits has been started at FRI (MARIĆ *et al*., 2010; MARIĆ and LUKIĆ, 2013a, 2013b; RADIČEVIĆ *et al*., 2013; MARIĆ and RADIČEVIĆ, 2014).

This study was carried out to determine the *S*-allelic constitution of apple and sweet cherry cultivars developed at FRI, using the PCR method with consensus and allele-specific primers. In addition, these results have enabled the studying of inheritance of *S-RNase* gene, as well as the reported parentage of assessed cultivars to be checked.

MATERIALS AND METHODS

Plant material and extraction of genomic DNA

Ten apple and sweet cherry cultivars, including parents and derivatives, were sampled from the FRI collections at Zdravljak and Ljubić facilities, near Čačak. Fresh leaves of the assessed cultivars were collected, frozen in liquid nitrogen and stored at −80°C until required. Frozen samples were ground with four ball-bearings in Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) and DNA was extracted according to the method described in DOYLE and DOYLE (1987), with the addition of β-mercaptoethanol (0.5% for apple and 1% for sweet cherry) and polyvinylpyrrolidone (2% PVP 40) in the extraction buffer.

Allele-specific PCR analysis of S-RNase in apple

The allele specific-primers used to amplify genomic fragment of *S-RNase* were reported in the study by BROOTHAERTS (2003). PCRs were carried out in a 25 µl volume with 50–100 ng genomic DNA, 0.5 µM of each primer, 0.2 mM of each dNTP, 1× PCR reaction buffer, 2.5 mM $MgCl_2$ and 0.625 U *Taq* DNA polymerase (Qiagen GmbH, Hilden, Germany). Amplification was performed in *TPersonal* Biometra thermal cyclers programmed for 30 cycles of denaturation of 1 min at 94°C, annealing of 1 min at an optimal temperature for each primer combination (56°C – S_2 allele; 55°C – S_3 allele; 62°C – S_7 and S_9 alleles; 60°C – S_{28} allele), and extension of 1 min at 68°C, with a final 15-min extension step at 72°C (BOŠKOVIĆ R., personal communication). Apple cultivars with known *S*-genotypes were used as standards.

Consensus and allele-specific PCR analysis of S-RNase in sweet cherry

Identification of the *S*-alleles was based on the methods described by SONNEVELD *et al.* (2001, 2003). The PCRs were carried out by using the consensus primer pairs specific for the first (PaConsI-F + -R, SONNEVELD *et al*., 2003) and the second introns (PaConsII-F + -R, SONNEVELD *et al*., 2003), as well as the allele-specific primers for S_1, S_3, S_4, S_5, S_9 and S_{10} (SONNEVELD *et al*.,

2001, 2003). The following annealing temperatures for the allele-specific primers were used: 64°C for S_1, 66°C for S_3, 63°C for S_4, 52°C for S_5, 61°C for S_9 and S_{10}. Sweet cherry cultivars with known *S*-allelic constitutions were used as standards.

Detection and visualization of PCR products

PCR products of *S-RNase* gene obtained with allele-specific primers in apple and sweet cherry cultivars were separated by electrophoresis in a 1.5% agarose gel (70 V for 2–3 h), whereas DNA fragments obtained with consensus primers specific for the first and second introns of *S-RNase* in sweet cherry were separated by electrophoresis in 2% agarose gel (70 V for 4 h). Fragments were visualized by ethidium bromide staining, photographed under ultraviolet light in BIO-PRINT-1500/26M (Vilber Lourmat) imaging system and sized by comparison with a 1 Kb plus DNA ladder (Invitrogen, Groningen, the Netherlands).

RESULTS AND DISCUSSION

S-genotypes of parental and derived apple cultivars

The use of primers specific to allele S_2 enabled the amplification of *S-RNase* genomic fragment which was ~450 bp in 'Golden Delicious' and 'Čadel' (Figure 1a). The fragment of ~500 bp was obtained upon amplification of *S-RNase* with S_3 allele-specific primers only in 'Golden Delicious' and was absent from all other cultivars (Figure 1b). The PCR product of ~300 bp corresponded to allele S_7 and was identified in 'Jonathan', 'Čačanska Pozna' and 'Čadel' (Figure 1c). Using primers designed to amplify S_9 allele, the PCR product of ~340 bp was amplified from 'Starking Delicious', 'Jonathan' and 'Čačanska Pozna' (Figure 1d). In 'Starking Delicious', the amplified product of ~300 bp which corresponds to S_{28} allele was identified (Figure 1e). The allelic patterns are scored in Table 1. The size of the obtained fragments for the assessed *S* alleles was in agreement with the findings of BROOTHAERTS (2003), who reported size of PCR product of 449, 500, 302, 343 and 304 bp for alleles S_2, S_3, S_7, S_9 and S_{28}, respectively.

Table 1. S-genotypes of the parental and derived apple cultivars determined by the S-allele-specific PCR analysis

Cultivar	Cross	*S*-allele					*S*-genotype
		S_2	S_3	S_7	S_9	S_{28}	
'Golden Delicious'		+	+				S_2S_3
'Jonathan'				+	+		S_7S_9
'Starking Delicious'					+	+	S_9S_{28}
'Čačanska Pozna'	'Starking Delicious' × 'Jonathan'			+	+		S_7S_9
'Čadel'	'Golden Delicious' × 'Jonathan'	+		+			S_2S_7

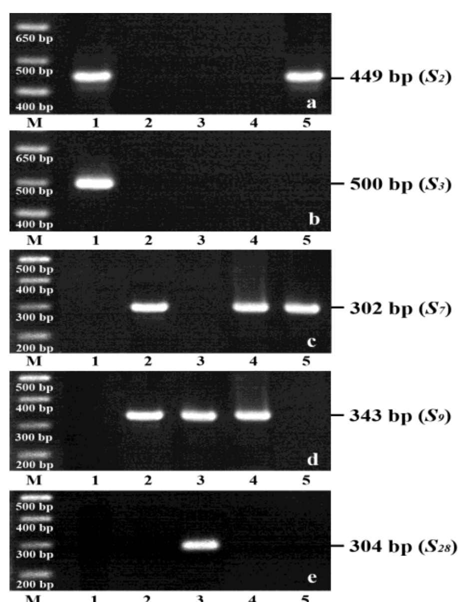

Figure 1. PCR products of the *S-RNase* amplified fragment obtained with primers specific for alleles S_2 (a), S_3 (b), S_7 (c), S_9 (d) and S_{28} (e) in the parental and derived apple cultivars: 1 – 'Golden Delicious'; 2 – 'Jonathan'; 3 – 'Starking Delicious'; 4 – 'Čačanska Pozna'; 5 – 'Čadel'; 1Kb plus DNA ladder (M).

As a result of the analysis in this work, *S*-genotypes for apple cultivars developed at the FRI were identified for the first time. Namely, S_7S_9 and S_2S_7 genotypes were determined for 'Čačanska Pozna' and 'Čadel', respectively. The *S*-genotypes for the three parental cultivars ('Golden Delicious' – S_2S_3, 'Jonathan' – S_7S_9 and 'Starking Delicious' – S_9S_{28}) were in agreement with the results reported by BROOTHAERTS and VAN NERUM (2003), BROOTHAERTS (2004) and DRESSEN *et al*. (2010). According to the pedigree of 'Čačanska Pozna' (originated from a seedling of the cross 'Starking Delicious' × 'Jonathan'), the S_9 allele was inherited from 'Starking Delicious' (S_9S_{28}) and S_7 allele from Jonathan' (S_7S_9). For 'Čadel', which originated from a seedling of the cross 'Golden Delicious' × 'Jonathan', the S_2 allele was inherited from 'Golden Delicious' (S_2S_3) and S_7 allele from 'Jonathan' (S_7S_9). Based on aforementioned results, *S*-genotypes of FRI apple cultivars were consistent with the parentage. Simultaneously, these results are a confirmation that *S-RNase* is inherited in a Mendelian fashion.

As showed, the cultivars 'Čačanska Pozna' and 'Jonathan' possess the same *S*-alleles (S_7 and S_9), which means that there is a complete gametophytic incompatibility between them. This is very important information for breeders when choosing cultivars for cross-fertilization, especially as 'Jonathan' is one of the four most frequently used cultivars as the progenitor in pedigrees of 50 apple cultivars (NOITON and ALSPACH, 1996). In addition, the results obtained in this study revealed that the FRI apple breeding programme was following the same pattern which was conducted in different countries. Despite the huge genetic variability of apple germplasm in the world, the genetic base of cultivated apples has greatly eroded over time, because the breeders have used a very limited gene pool. In earlier programmes, 'Cox's Orange Pippin' (S_5S_9), 'Golden Delicious' (S_2S_3), 'Jonathan' (S_7S_9) and 'McIntosh' ($S_{10}S_{25}$) dominated as parental cultivars

(NOITON and ALSPACH, 1996), whilst 'Gala' (S_2S_5) and 'Fuji' (S_1S_9) are now the most commonly used cultivars (DE ALBUQUERQUE JR. *et al*., 2011). Consequently, this strategy has led to breeding of mutually incompatible cultivars, because of the few *S*-alleles which occur at a high frequency. Therefore, apple breeding programmes need new germplasm to improve current cultivars which are in demand by both market and industry (MARIĆ and LUKIĆ, 2015).

***S*-genotypes of parental and derived sweet cherry cultivars**

The identification of *S*-alleles of the assessed sweet cherry cultivars was conducted in two stages. In the first stage, the first and the second introns of *S-RNase* were amplified using consensus primers, followed by the next stage in which the allele-specific primers were used.

The amplification of the first and the second introns of *S-RNase* with consensus primers (PaConsI-F + -R and PaConsII-F + -R) resulted in two PCR products, which corresponded to *S*-alleles of the assessed sweet cherry cultivars, except for 'Drogans Gelbe' for the first intron and 'Bigarreau Schrecken' for the second intron. The size of PCR product for the first intron ranged from ~300 to ~520 bp (Figure 2) and the second intron ranged from ~800 to ~2,200 bp (Figure 3). Amplification of the first intron was not enough to discriminate the S_1 allele from the S_5 allele, which is in agreement with the results reported by SONNEVELD *et al*. (2003) and IPEK *et al*. (2011). On the other hand, amplification of the second intron enabled identification of the cultivars carrying S_4 and S_5 alleles, but discrimination of S_1 from S_3 required additional analysis (Table 2). Difficulty in identification of S_1 and S_3 alleles by PCR amplification with consensus primers for the second intron were also reported by SONNEVELD *et al*. (2003), SCHUSTER *et al*. (2007) and IPEK *et al*. (2011). The sizes of PCR products for S_9 and S_{10} alleles, obtained with both consensus primers, were too close to discriminate them on an agarose gel. In order to confirm *S*-allelic constitutions of the assessed sweet cherry cultivars determined by consensus primers, the genomic fragment of *S-RNase* was amplified using the specific primers for the S_1, S_3, S_4, S_5, S_9 and S_{10} alleles. The *S*-genotype of each cultivar was determined after combining the results of the consensus and the allele-specific primers (Table 2).

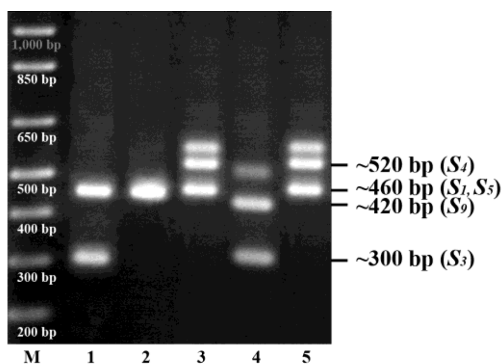

Figure 2. PCR products of the *S-RNase* amplified fragment obtained with consensus primers for the first intron of the parental and derived sweet cherry cultivars: 1 – 'Bigarreau Schrecken'; 2 – 'Drogans Gelbe'; 3 – 'Majova Rana'; 4 – 'Asenova Rana'; 5 – 'Čarna'; 1Kb plus DNA ladder (M).

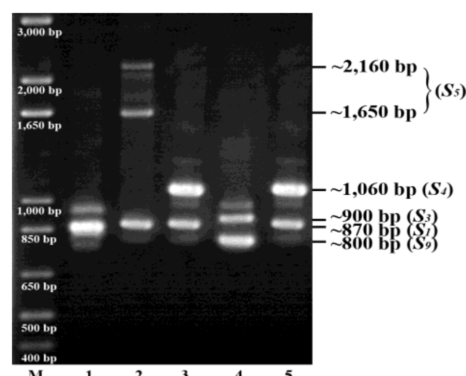

Figure 3. PCR products of the *S-RNase* amplified fragment obtained with consensus primers for the second intron of the parental and derived sweet cherry cultivars: 1 – 'Bigarreau Schrecken'; 2 – 'Drogans Gelbe'; 3 – 'Majova Rana'; 4 – 'Asenova Rana'; 5 – 'Čarna'; 1Kb plus DNA ladder (M).

Table 2. S-genotypes and incompatibility groups of the parental and derived sweet cherry cultivars

Cultivar	Cross	Results with consensus primer for the first intron		Results with consensus primer for the second intron		*S*-allele results based on specific primers						*S*-genotype	Incompatibility group
		Allele 1	Allele 2	Allele 1	Allele 2	S_1	S_3	S_4	S_5	S_9	S_{10}		
'Bigarreau Schrecke'		S_1	S_3	*Difficult to distinguish*	*Difficult to distinguish*	+	+					S_1S_3	II
'Drogans Gelbe'		*Difficult to distinguish*	*Difficult to distinguish*	S_1	S_5	+			+			S_1S_5	XIV
'Majova Rana'		S_1	S_4	S_1	S_4	+		+				S_1S_4	IX
'Asenova Rana'	'Drogans Gelbe' × 'Majova Rana'	S_3	S_9/S_{10}	S_3	S_9/S_{10}		+			+	–	S_3S_9	XVI
'Čarna'	'Majova Rana' × 'Bigarreau Schrecken'	S_1	S_4	S_1	S_4	+		+				S_1S_4	IX

The PCR product of ~820 bp corresponded to allele S_1 and was identified in 'Bigarreau Schrecken', 'Drogans Gelbe', 'Majova Rana' and 'Čarna' (Figure 4a). Use of S_3 allele-specific primers enabled amplification of fragment of ~960 bp from 'Bigarreau Schrecken' and 'Asenova Rana' (Figure 4b). In 'Majova Rana' and 'Čarna', the PCR product of ~820 bp corresponding to S_4 allele was obtained (Figure 4c). The DNA fragment of ~300 bp corresponding to S_5 allele and a fragment of ~500 bp corresponding to S_9 allele were identified in 'Drogans Gelbe' and 'Asenova Rana', respectively (Figure 4d–e). Similarity in size of products for S_9 and S_{10} alleles required

additional PCR with primers specific for S_{10}. The absence of amplification with S_{10} allele-specific primers was another confirmation that S_9 is the second allele in 'Asenova Rana'. The aforementioned size of PCR product for the assessed S alleles were consistent with those of SONNEVELD *et al*. (2001, 2003), who stated that fragment of 820, 960, 820, 300 and 495 bp corresponded to S_1, S_3, S_4, S_5, S_9 alleles, respectively.

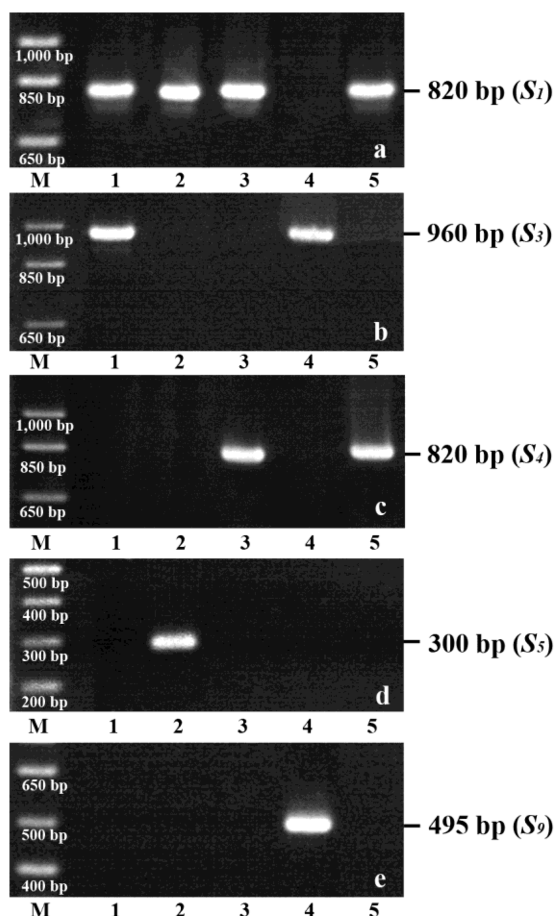

Figure 4. PCR products of the *S-RNase* amplified fragment obtained with primers specific for alleles S_1 (a), S_3 (b), S_4 (c), S_5 (d) and S_9 (e) in the parental and derived sweet cherry cultivars: 1 – 'Bigarreau Schrecken'; 2 – 'Drogans Gelbe'; 3 – 'Majova Rana'; 4 – 'Asenova Rana'; 5 – 'Čarna'; 1Kb plus DNA ladder (M).

Previous studies showed that some S-alleles are more frequent in some regions and that certain alleles are specific to a particular geographic region. Among the identified S-alleles, a relatively higher frequency of occurrence (> 20%) of alleles S_1 and S_3, as well as for S_1, S_3 and S_4 were reported respectively by TOBUTT *et al*. (2004) and LISEK *et al*. (2015), while the S_9 allele

occurred with similar frequency (about 6%). CACHI and WÜNSCH (2014) showed that alleles S_3 and S_6 are highly frequent all over Europe, while in northern and central Europe the most frequent are S_1, S_3, S_4 and S_6, compared to southern Europe where S_3, S_6 and S_{22} alleles are the most common. The authors also stated that the geographical distribution of *S*-alleles may indicate a common origin of genotypes spread in closer areas and on the other hand a correlation between certain *S*-alleles and different climatic conditions.

As a result of this study, the *S*-allelic constitutions of three parental cultivars and two cultivars released at FRI were determined. The *S*-allelic constitutions of all parental cultivars (‘Bigarreau Schrecken’ – S_1S_3, ‘Drogans Gelbe’ – S_1S_5 and ‘Majova Rana’ – S_1S_4) were consistent with the results from SCHUSTER (2012) and MARIĆ and RADIČEVIĆ (2014). Based on parental genotypes, ‘Asenova Rana’ was expected to be S_1S_4 or S_4S_5, but S_3S_9 was observed. In addition, cultivar ‘Čarna’ was expected to be S_1S_3 or S_3S_4, but S_1S_4 was identified. Since *S*-allele constitutions for sweet cherry cultivars developed at FRI had not been previously described, the result obtained in this work suggests incorrectly reported parents for these cultivars. In order to be confident that samples were true-to-type, DNA samples from two sources were compared. Since inconsistency remained after the testing, the reported pedigree was presumed to be incorrect. In effect, errors in pollen contamination, errors in the information presented by the breeders or technical errors in the processing of seedlings may explain the discrepancies. Based on *S*-allelic constitution, ‘Majova Rana’ may be the possible female parent of ‘Čarna’. The knowledge of *S*-genotype allows ‘Asenova Rana’ and ‘Čarna’ to be assigned to incompatibility groups XVI and IX, respectively.

ACKNOWLEDGEMENTS

This work was conducted under Research Project *TR–31064: Development and preservation of genetic potential of temperate zone fruits*, supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

Received August 04th, 2016
Accepted December 25th, 2016

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DETERMINACIJA *S*-GENOTIPA SORTI JABUKE I TREŠNJE STVORENIH U INSTITUTU ZA VOĆARSTVO, ČAČAK

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Izvod

Jabuka (*Malus* × *domestica* Borkh.) i trešnja (*Prunus avium* L.) su samobesplodne vrste voćaka, čija je auto-inkompatibilnost gametofitnog tipa, regulisana polimorfnim *S*-lokusom. Stoga je identifikacija *S*-genotipa od krucijalnog značaja za oplemenjivače i proizvođače. Cilj ovog rada je *S*-genotipizacija sorti jabuke ('Čačanska pozna' i 'Čadel') i trešnje ('Asenova rana' i 'Čarna') stvorenih u Institutu za voćarstvo, Čačak, primenom lančane reakcije polimeraze (PCR metoda) sa konsenzus i alel-specifičnim prajmerima. Kod sorti jabuke utvrđeno je da njihovi *S*-genotipovi odgovaraju roditeljskim, odnosno da su alelne konstitucije S_7S_9 sorte 'Čačanska pozna' i S_2S_7 sorte 'Čadel' u skladu sa alelnim konstitucijama sorti koje su u njihovom pedigreu ['Starking Delicious' (S_9S_{28}) × 'Jonathan' (S_7S_9) i 'Golden Delicious' (S_2S_3) × 'Jonathan' (S_7S_9), resp.]. *S*-genotipizacijom sorti trešnje 'Asenova rana' i 'Čarna' utvrđene su alelne konstitucije S_3S_9 i S_1S_4, kao i neslaganja sa *S*-alelnim konstitucijama deklarisanih roditeljskih sorti ['Drogans Gelbe' (S_1S_5) × 'Majova rana' (S_1S_4) za sortu 'Asenova rana'; 'Majova rana' (S_1S_4) × 'Bigarreau de Schrecken' (S_1S_3) za sortu 'Čarna']. Rezultati *S*-genotipizacije sorti jabuke i trešnje su od velikog značaja za planiranje ukrštanja u okviru oplemenjivačkih programa, kao i za kreiranje adekvatne sortne kompozicije u komercijalnim zasadima.

Primljeno 04. VIII 2016.
Odobreno 25. XII 2016.