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S-RNase allele identification and incompatibility group assignment in sweet cherry (*Prunus avium* L.) autochthonous genotypes

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Abstract. This work was undertaken primarily to identify the *S*-alleles and incompatibility groups in 15 autochthonous sweet cherry genotypes collected in orchards of individual growers in the Republic of Serbia, i.e. 11 genotypes in the region of Čačak ('GT-1', 'GT-2' and 'GT-4' to 'GT-12') and four genotypes in the region of Belgrade ('GT-13' to 'GT-16'). The *S*-alleles of each genotype were determined by PCR amplification of the *S-RNase* gene, with consensus primers for the second introns, as well as with allele-specific primers. The obtained results revealed eight alleles (S_1, S_2, S_3, S_4, S_5, S_6, S_9 and S_{12}), that generated the following eight *S*-genotypes: S_1S_5 ('GT-1'), S_2S_3 ('GT-7', 'GT-14', 'GT-15' and 'GT-16'), S_3S_4 ('GT-10' and 'GT-12'), S_3S_6 ('GT-4' and 'GT-13'), S_3S_9 ('GT-8'), S_3S_{12} ('GT-5' and 'GT-6'), S_6S_9 ('GT-2' and 'GT-11') and S_5S_x ('GT-9'). S_2S_3 was the most frequent allelic combination (27%), which was observed in four assessed genotypes. The *S*-allelic constitutions allowed assignment of the genotypes to their corresponding incompatibility groups as follows: III ('GT-10' and 'GT-12'), IV ('GT-7', 'GT-14', 'GT-15' and 'GT-16'), VI ('GT-4' and 'GT-13'), X ('GT-2' and 'GT-11'), XIV ('GT-1'), XVI ('GT-8') and XXII ('GT-5' and 'GT-6'). *S*-genotyping results represent important information on cross-compatibility in these local genotypes and also reveal the *S*-locus diversity of sweet cherry indigenous material.

Key words: *Prunus avium*, autochthonous genotype, *S*-allelic constitution, gametophytic self-incompatibility

Introduction

Sweet cherry (*Prunus avium* L.) is one of the economically most important members of the Rosaceae family. This fruit species may have originated within a region around the Caspian Sea and the Black Sea, and later spread across Europe resulting in local genotypes adapted to different agro-ecological conditions (Webster, 1996; Quero-García et al., 2019). Sweet cherry occurs naturally in Europe and areas of northern Afri-

ca (Faust & Surányi, 1997). Cherry germplasm in the Republic of Serbia can potentially provide wide and useful genetic variability, in particular, for fruit quality and resistance, i.e. newly released sweet cherry cultivar 'Canetova' is autochthonous genotype singled out from natural population (Fotirić-Akšić et al., 2016). Most of these traditional genotypes have been grown for familial consumption. It is also important to point out that the origin of many sweet cherry landraces is undocumented and their names confusing, due to the frequent occurrence of homonyms and synonyms.

In sweet cherry, most genotypes are self-incompatible and certain pairs of genotypes are cross-incompatible, reciprocally or unilaterally. This incompatibility is genetically determined by the multiallelic two linked genes of *S*-locus (*S-RNase* and *SFB genes*), with gametophytic action (Bošković & Tobutt, 1996; Yamane et al., 2003). Polymerase chain reaction (PCR) method has enabled the detection of 25 different S-alleles (Vaughan et al., 2008). Using this polymorphism, a total of 60 incompatibility groups (IGs) in 1,203 genotypes, then a group '0' described as universal donors with 25 genotypes and a group 'SC' with 72 self-compatible sweet cherries, have been identified so far (Schuster, 2017). In the latest review of Schuster, 18 different *S*-alleles (S_1 *to* S_7, S_9, S_{10}, S_{12} *to* S_{14}, S_{16}, S_{18}, S_{19}, S_{21}, S_{22} and S_{24}) were reported, whereas additional alleles (S_{27} to S_{32}) were found only in wild cherry (Vaughan et al., 2008). The high polymorphism of *S*-locus allows it to be used as a genetic marker for identification of sweet cherry cultivars, as well as for the study of diversity in local germplasm from Turkey (Ipek et al., 2011), Croatia (Ercisli et al., 2012), Spain (Cachi & Wünsch, 2014), Czech Republic, Poland, Ukaraine and Russia (Lisek et al., 2015), Germany (Schuster, 2017), Italy (Marchese et al., 2017). Based on the findings of Mariette et al. (2010), who reported that 19, 15 and 9 *S*-alleles were found in wild cherry, landraces and modern sweet cherry cultivars respectively, domestication and breeding had two major impacts on a decrease of diversity, as well as that the loss of diversity observed at the *S*-locus during domestication can partly be explained by the vegetative propagation.

During the last decade, the activities on collection, evaluation and utilization of autochthonous sweet cherry genotypes with good agronomic traits, as well as molecular characterization of this material have been intensified at the Fruit Research Institute, Čačak (Radičević et al., 2018; Marić et al., 2016, 2019). This work aimed to identify the *S*-alleles in fifteen autochthonous genotypes from the main sweet cherry growing regions of the Republic of Serbia (Western Serbia/Šumadija and the region of Belgrade). Therefore, determination of *S*-alleles and cross(in)compatibility groups of new sweet cherry genotypes is highly valuable tool for growers to design orchards, and for breeders to choose parents in breeding programmes.

Material and Methods

Fifteen sweet cherry genotypes (Tab. 1), corresponding to landraces or cultivars of unknown origin, were sampled in orchards of individual growers in the region of Čačak ('GT-1', 'GT-2' and 'GT-4' to 'GT-12') and the region of Belgrade ('GT-13' to 'GT-16'). Genomic DNA from the sweet cherry autochthonous material was extracted from young leaves (previously frozen in liquid nitrogen and stored at -80 °C) following the CTAB mini prep protocol described by Doyle & Doyle (1987).

S-RNase allele identification was performed by PCR using the consensus primer pairs PaConsII-F/PaConsII-R specific for the second intron and allele-specific primers for S_1 to S_7, as well as S_9, S_{10} and S_{12} (Sonneveld et al., 2001, 2003). Annealing temperatures for alleles S_1 to S_5 were reported in the study by Marić & Radičević (2014), as well as by Marić et al. (2015, 2018) for alleles S_6, S_7, S_9, S_{10} and S_{12}. Sweet cherry cultivars with known *S*-genotypes were used as standards.

PCR products obtained with the consensus primers were separated by electrophoresis in a 2% agarose gel (70 *V/cm* for 4 *h*), whereas products of allele-specific PCRs were analysed in a 1.5% agarose gel (70 *V/cm* for 2T3 *h*). PCR products were visualised by ethidium bromide staining, taking photographs under ultraviolet light in BIO-PRINT-1500/26M imaging system (Vilber Lourmat, Collégien, French Republic), and finally sized by comparison with a 1 Kb plus DNA ladder (Invitrogen, Groningen, the Netherlands).

Results and Discussion

The *S*-allele identification in the autochthonous genotypes from the main sweet cherry growing regions of the Republic of Serbia was conducted using consensus primers for the second intron of *S-RNase* in the first step, as well as the allele-specific primers for the confirmation during the second step.

The amplification of *S-RNase* with consensus primer pairs PaConsII-F/PaConsII-R resulted in two PCR products, except for genotype 'GT-9' originated from Prislonica village nearby Čačak. The size of the obtained PCR products corresponded to *S*-alleles of the assessed sweet cherry genotypes and ranged from ~580 (S_6 allele) to ~2,200 bp (S_2 or S_7 allele) (Fig. 1; Tab. 1). Based on previous studies of Sonneveld et al. (2003) and Ipek et al. (2011), small size differences were found when using primers for the second intron of some alleles, i.e. S_1 and S_3, as well as S_2 and S_7. An additional analysis with allele-specific primers was also required for discrimination of alleles S_9 and S_{10}, because according to Sonneveld et al. (2003), these alleles amplified the fragments of similar size (798 bp and 734 bp, resp.), which were difficult to distinguish on an agarose gel. The fact that amplification of the second intron disabled the discrimination of some alleles, particularly in genotypes in which *S*-allelic constitutions have not been published to date, indicates the necessity of application of allele-specific primers.

Therefore, discrimination and confirmation of *S*-alleles in the assessed autochthonous genotypes were conducted using the specific primers for the S_1 to S_7, as well as S_9, S_{10} and S_{12} alleles (Tab. 1). Examples of banding patterns of identified alleles are shown in Fig. 2a–h. The PCR product of č820 bp corresponded to allele S_1 and was identified in 'GT-1'. In four genotypes – 'GT-7', 'GT-14', 'GT-15' and 'GT-16', the DNA fragment of č640 bp corresponding to S_2 allele was obtained. The use of primers specific for allele S_3 enabled amplification of fragment of č960 bp in eleven genotypes 'GT-4' to 'GT-8', 'GT-10' and 'GT-12' to 'GT-16'. In two genotypes – 'GT-10' and 'GT-12', the DNA fragment of č820 bp matching to S_4 allele was detected. The PCR products of ~300 bp and ~470 bp revealed S_5 and S_6 alleles in two ('GT-1' and 'GT-9') and four ('GT-2', 'GT-4', 'GT-11' and 'GT-13') genotypes, respectively. In three genotypes – 'GT-2', 'GT-8' and 'GT-11', use of primers specific for S_9 allele enabled amplification of fragment of ~500 bp. In

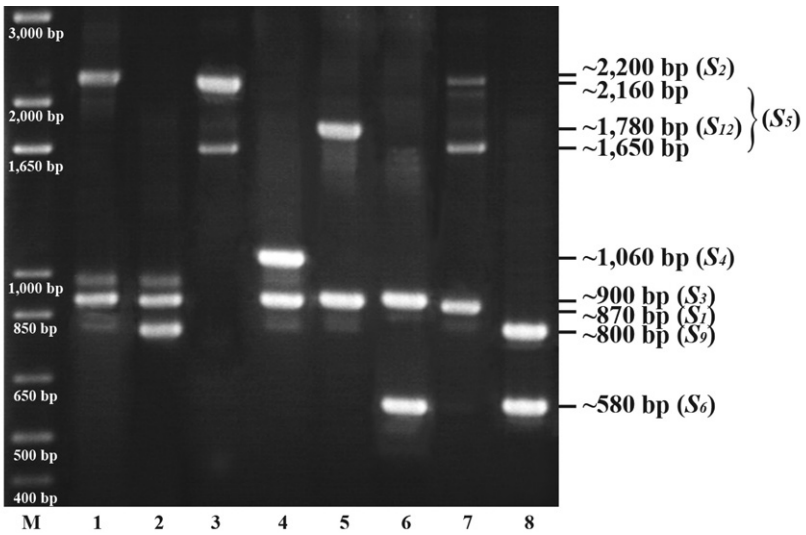

Fig. 1. PCR products of the *S-RNase* amplified fragment obtained with consensus primers for the second intron in eight autochthonous sweet cherry genotypes: 1 – 'GT-7'; 2 - 'GT-8'; 3 – 'GT-9'; 4 – 'GT-12'; 5 – 'GT-5'; 6 - 'GT-4'; 7 – 'GT-1'; 8 – 'GT-2'; M – 1Kb plus DNA ladder (Invitrogen)

Sl. 1. PCR proizvodi dobijeni umnožavanjem genomskog fragmenta S-RNaze sa konsenzus prajmerima specifičnim za drugi intron kod osam autohtonih genotipova trešnje: 1 – GT-7; 2 – GT-8; 3 – GT-9; 4 – GT-12; 5 – GT-5; 6 – GT-4; 7 – GT-1; 8 – GT-2; M – marker molekulske mase 1 Kb Plus DNA ladder (Invitrogen)

Tab. 1. *S-RNase* alleles identification in the autochthonous sweet cherry genotypes with consensus and allele-specific primers
Tab. 1. Identifikovanje alela S-RNaze primenom konsenzus i alel-specifičnih prajmera kod autohtonih genotipova trešnje

		Amplification with PaConsII-F and PaConsII-R primers *Amplifikacija sa PaConsII-F i PaConsII-R prajmerima*		Amplification with allele-specific primers *Amplifikacija sa alel-specifičnim prajmerima*									
Genotype *Genotip*	Origin *Poreklo*	Allele 1 *Alel 1*	Allele 2 *Alel 2*	S_1	S_2	S_3	S_4	$S5$	$S6$	$S7$	$S9$	$S10$	$S12$
'GT11'	Čačak (town)/*Čačak (grad)*	S_3/S_3	S_5	+		–		+					
'GT-2'	Čačak (Jezdina)	S_6	S_9/S_{10}						+		+	–	
'GT-4'	Čačak (Trbušani)	S_1/S_3	S_6	–		+			+				
'GT-5'	Čačak (Trbušani)	S_1/S_3	S_{12}	–		+							+
'GT-6'	Čačak (Trbušani)	S_1/S_3	S_{12}	–		+							+
'GT-7'	Čačak (Jezdina)	S_2/S_7	S_1/S_3	–	+	+				–			
'GT-8'	Čačak (Prislonica)	S_1/S_3	S_9/S_{10}	–		+					+	–	
'GT-9'	Čačak (Prislonica)	S_5	Sx	–	–	–	–	+	–	–	–	–	–
'GT-10'	Čačak (Prislonica)	S_1/S_3	S4	–		+	+						
'GT-11'	Čačak (Trbušani)	S_6	S_9/S_{10}						+		+	–	
'GT-12'	Čačak (Trbušani)	S_1/S_3	S_4	–		+	+						
'GT-13'	Belgrade (Grocka)	S_1/S_3	S_6	–		+			+				
'GT-14'	Belgrade (Grocka)	S_2/S_7	S_1/S_3	–	+	+				–			
'GT-15'	Belgrade (Grocka)	S_2/S_7	S_1/S_3	–	+	+				–			
'GT-16'	Belgrade (Grocka)	S_2/S_7	S_1/S_3	–	+	+				–			

'GT-5' and 'GT-6', the PCR product of č560 bp corresponding to S_{12} allele was identified. The absence of amplification with S_1, S_3, S_7 and S_{10} allele-specific primers was another confirmation that S_3, S_1, S_2 and S_9 were present in abovementioned genotypes. The fragment sizes of identified S-alleles in autochthonous genotypes were consistent with results from Sonneveld et al. (2001, 2003).

The *S*-allelic constitution of each autochthonous genotype was determined after combining the results obtained upon amplification with the consensus and the allele-specific primers (Tab. 1–2) and published in this paper for the first time, as follows: S1S5 ('GT-1'), S_2S_3 ('GT-7', 'GT-14', 'GT-15' and 'GT-16'), S_3S_4 ('GT-10' and 'GT-12'), S_3S_6 ('GT-4' and 'GT-13'), S_3S_9 ('GT-8'), S_3S_{12} ('GT-5' and 'GT-6'), S_6S_9 ('GT-2' and 'GT-11') and S_5S_x ('GT-9'). Therefore, a total of eight alleles were found in this germplasm survey, that generated eight *S*-allelic constitutions. Based on identified *S*-alleles, the genotypes were assigned to their corresponding IGs, previously reported by Schuster (2017). Therefore, the following seven IGs (Tab. 2) were determined: III ('GT-10' and 'GT-12'), IV ('GT-7', 'GT-14', 'GT-15' and 'GT-16'), VI ('GT-4' and 'GT-13'), X ('GT-2' and 'GT-11'), XIV ('GT-1'), XVI ('GT-8') and XXII ('GT-5' and 'GT-6').

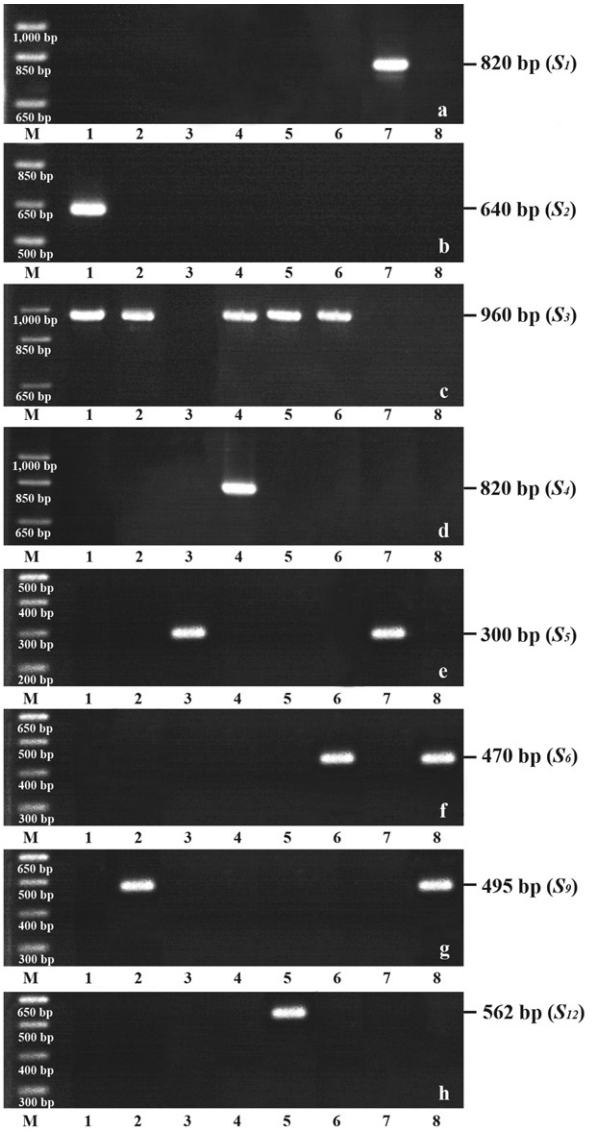

Fig. 2. PCR products of the *S-RNase* amplified fragment obtained with primers specific for alleles S_1 (a), S_2 (b), S_3 (c), S_4 (d), S_5 (e), S_6 (f), S_9 (g) and S_{12} (h) in eight autochthonous sweet cherry genotypes: 1 – 'GT-7'; 2 – 'GT-8'; 3 – 'GT-9'; 4 – 'GT-12'; 5 – 'GT-5'; 6 – 'GT-4'; 7 – 'GT-1'; 8 – 'GT-2'; M – 1Kb plus DNA ladder (Invitrogen)

Sl. 2. PCR proizvodi dobijeni umnožavanjem genomskog fragmenta S-RNaze sa prajmerima specifičnim za alele S_1 (a), S_2 (b), S_3 (c), S_4 (d), S_5 (e), S_6 (f), S_9 (g) i S_{12} (h) kod osam autohtonih genotipova trešnje: 1 – GT-7; 2 – GT-8; 3 – GT-9; 4 – GT-12; 5 – GT-5; 6 – GT-4; 7 – GT-1; 8 – GT-2; M - marker molekulske mase 1 Kb Plus DNA ladder (Invitrogen)

Tab. 2. *S*-allelic constitution and incompatibility group of the assessed sweet cherry genotypes

Tab. 2. S-alelna konstitucija i grupa inkompatibilnosti autohtonih genotipova trešnje

Genotype *Genotip*	S-allelic constitution *S-alelna konstitucija*	Incompatibility group *Grupa inkompatibilnosti*
'GT-1'	S_1S_5	XIV
'GT-2'	S_6S_9	X
'GT-4'	S_3S_6	VI
'GT-5'	S_3S_{12}	XXII
'GT-6'	S_3S_{12}	XXII
'GT-7'	S_2S_3	IV
'GT-8'	S_3S_9	XVI
'GT-9'	S_5S_x	/
'GT-10'	S_3S_4	III
'GT-11'	S_6S_9	X
'GT-12'	S_3S_4	III
'GT-13'	S_3S_6	VI
'GT-14'	S_2S_3	IV
'GT-15'	S_2S_3	IV
'GT-16'	S_2S_3	IV

The genotype 'GT-9' (S_5S_x) gave two typical bands in the S_5 position with consensus primers for the second intron of *S-RNase* (~2,160 bp and a second, lower band of ~1,650 bp; Fig. 1) and PCR product (300 bp; Fig. 2e) upon amplification with S_5 allele-specific primers, while the second allele is still unknown. Sonneveld et al. (2003) reported that the lower band (~1,650 bp) may be an artefact resulting from secondary structure of the DNA or perhaps some of the top band products are able to run faster on an agarose gel as a result of having a different conformation. Further, the band/bands corresponding to one allele on the agarose gel could mean either that two *S*-alleles are the same in sizes of the second introns, or the primers do not match the sequence of the second allele. Marić et al. (2019) reported similar results for Macedonian autochthonous genotypes 'OCK-2' and 'Ohridska Crna', which gave a single band in the S_4 position (S_4S_x). Therefore, cloning and sequencing will provide their further characterization and possible identification of the second allele in these genotypes.

In the group of 14 Serbian autochthonous genotypes ('GT-9' was excluded because the second allele was not determined), the alleles S_1, S_2, S_3, S_4, S_5, S_6, S_9 and S_{12} occurred with frequency of 3.6%, 14.3%, 39.3%, 7.1%, 3.6%, 14.3%, 10.7% and 7.1%, respectively. Therefore, the most frequent *S*-allele in the Serbian autochthonous material assessed in this

work is S_3 and S_2S_3 allelic constitution was observed in four genotypes. Marchese et al. (2017) speculated about the higher frequency of some *S*-alleles in specific areas and assumed that some alleles may be linked with traits of adaptation to the environmental conditions or could be the result of selection events. Also, the same authors reported that the most frequent S-alleles in Italian sweet cherry germplasm were S_3, S_6, S_{13} and S_{16} in descending order. Cachi & Wünsch (2014) found that alleles S_3 and S_6 were highly incident all over Europe, but others were more specific to its northern or southern part. For Croatian sweet cherry germplasm, Ercisli et al. (2012) reported that S_3 and S_{12} were the most frequent alleles (39% and 19%, resp.). Tobutt et al. (2004) stated a relatively higher frequency of occurrence (> 20%) of alleles S_1 and S_3 in 247 cultivars of different origins, while Lisek et al. (2015) pointed out the presence of alleles S_3 (34.4%), S_1 (25.0%) and S_4 (21.9%) in cultivars originated from Central Europe.

This study represents the first report on *S*-allele diversity of Serbian autochthonous sweet cherry genotypes, as well as the first fundamental stage of molecular characterization of the genetic resources linked with the history and traditions of our territory.

Conclusion

Findings of the present work provides valuable insight into the S-allelic constitutions of fifteen Serbian autochthonous sweet cherry genotypes, useful for choosing pollenisers, designing crosses within breeding programmes, population studies and conservation of *S*-alleles. The genetic richness of cherry germplasm in the Republic of Serbia is still rather unexplored, despite the large variability in terms of useful agronomic traits. Therefore, accurate germplasm identification and future studies should aim to continue research in the field of conserving through long-term conservation, molecular fingerprints and increase in the number of genotypes.

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IDENTIFIKOVANJE ALELA *S-RNAZE* I GRUPE INKOMPATIBILNOSTI AUTOHTONIH GENOTIPOVA TREŠNJE (*Prunus avium* L.)

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Rezime

Trešnja (*Prunus avium* L.) je među najranijim stonim voćem na tržištu, veoma cenjenog i atraktivnog ploda. Smatra se da je poreklom iz područja između Kaspijskog jezera i Crnog mora, kao i da je njeno kasnije gajenje širom Evrope dovelo do stvaranja velikog broja lokalnih genotipova prilagođenih različitim agroekološkim uslovima. Samobesplodna je vrsta voćaka, čija je samo-inkompatibilnost gametofitnog tipa, regulisana ekspresijom dva vezana gena S-lokusa koji kontrolišu komponente stubića (*S-RNaza*) i polena (*SFB*). Prisustvo istog *S*-haplotipa u polenu i stubiću dovodi do zaustavljanja rasta polenove cevčice, što ima za posledicu izostajanje oplođewnja, nezavisno od toga da li se radi o polenu iste sorte ili druge sorte iste grupe inkompatibilnosti. Na osnovu *S*-genotipa, sorte trešnje se klasifikuju u različite grupe inkompatibilnosti kojih je do sada poznato 60 (označene od I do LX), pored grupe samooplodnih sorti, kao i grupe „0“, kojoj pripadaju sorte retkog *S*-genotipa koji im omogućava da budu potencijalno dobri oprašivači sortama drugih grupa.

U radu su predstavljeni rezultati identifikacije *S*-alela i grupe inkompatibilnosti 15 autohtonih, odnosno genotipova trešnje nepoznatog porekla (vode poreklo sa stabala o kojima poznati podaci datiraju iz prvih decenija XX veka) sakupljenih u zasadima individualnih proizvođača glavnih proizvodnih rejona ove vrste voćaka, koji se nalaze na područjima Zapadne Srbije/Šumadije i beogradskog Podunavlja – 11 genotipova u okolini Čačka (GT-1, GT-2 i GT-4 do GT-12) i četiri genotipa u Grockoj (GT-13 do GT-16). Genomska DNK ispitivanih genotipova je izolovana iz uzoraka mladog lista, primenom modifikovane CTAB mini prep metode. Identifikacija .S-alela sprovedena je PCR umnožavawem fragmenata *S- RNaze* uz korišćewe konsenzus prajmera (PaConsI-F i PaConsI-R) specifičnih za drugi intron (određeni potencijalni iS'-aleli), kao i alel-specifičnih prajmera (potvrđeni S'-aleli). U ispitivanom autohtonom materijalu detektovano je osam *S*-alela (S_1, S_2, S_3, S_4, S_5, S_6, S_9 i S_{12}) i ustanovqeno da je S_3 najzastupljeniji alel, sa frekvencijom od 39,3%. *S*-genotipizacijom su utvrđene sledeće alelne konstitucije: S_1S_5 (GT-1), S_2S_3 (GT-7, GT-14, GT-15 i GT-16), S_3S_4 (GT-10 i GT-12), S_3S_6 (GT-4 i GT-13), S_3S_9 (GT-8), S_3S_{12} (GT-5 i GT-6), S_6S_9 (GT-2 i GT-11) i S_5S_x (GT-9), kao i da je S_2S_3 najzastupljenija alelna konstitucija, potvrđena kod četiri genotipa (27%). Na osnovu određenih *S*-alelnih konstitucija, autohtoni genotipovi su svrstani u sedam grupa inkompatibilnosti – III, IV, VI, X, XIV, XVI i XXII. Rezultati ovog rada su od posebnog značaja za planiranje ukrštanja u okviru budućih oplemenjivačkih programa koji podrazumevaju uključenje autohtonih genotipova, kao i za određivanje sortnih kompozicija oprašivača u proizvodnim zasadima ovih genotipova. Istovremeno su omogućili utvrđivanje učestalosti pojedinih *S*-alela, odnosno genotipova u autohtonom materijalu trešnje.

Ključne reči: *Prunus avium*, autohtoni genotip, *S*-alelna konstitucija, gametofltna samoinkompatibilnost