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S-GENOTYPING OF SOME SWEET CHERRY CULTIVARS RELEASED WITHIN BREEDING PROGRAMMES IN THE BALKAN REGION

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Abstract
Sweet cherry cultivars generally exhibit *S*-ribonuclease (*S-RNase*)-based gametophytic self-incompatibility and require pollination with pollen of compatible genotypes, which are indispensable to stable fruit production. Therefore, the determination of *S*-genotype provides relevant information for sweet cherry breeders and growers. The aim of this study was to identify the *S*-allelic constitution and incompatibility group in eight sweet cherry cultivars which were named and released at Fruit Research Institute, Čačak, Republic of Serbia ('Asenova Rana' and 'Čarna'), Research Station for Fruit Growing, Iasi, Romania ('Alexus', 'Bucium' and 'Margonia') and Fruit Growing Institute, Plovdiv, Republic of Bulgaria ('Kossara', 'Rosalina' and 'Rosita'). The use of the polymerase chain reaction (PCR) method with consensus primers for the second introns of *S-RNase*, as well as primers specific for S_1–S_7 and S_9 alleles enabled determination of the following *S*-genotypes in the assessed cultivars: S_1S_2 ('Alexus'), S_1S_4 ('Čarna'), S_2S_9 ('Kossara' and 'Rosita'), S_3S_6 ('Bucium'), S_3S_9 ('Asenova Rana' and 'Rosalina') and S_5S_6 ('Margonia'). In addition, the *S*-genotypes of two parental cultivars were reported in this manuscript for the first time – 'Boambe de Cotnari' (S_2S_7) and 'Ranna Tcherna' (S_1S_2). Based on the obtained *S*-allelic constitutions, the assessed cultivars were assigned to the following incompatibility groups: I, II, VI, IX, XI, XIV, XV, XVI and XLIII. The results generated in this study provide a valuable resource for cross design in developing new cultivars and for orchard management in the efficient high-yielding fruit production.

Keywords: *Prunus avium*, *S-RNase*, incompatibility group.

Introduction

Sweet cherry (*Prunus avium* L.) is an economically important fruit species in the Balkan countries. According to Food and Agriculture Organization of the United Nations, the average annual sweet cherry production (2010–2014) were 77,192 t, 29,196 t and 22,316 t in the Romania, Republic of Bulgaria and Republic of Serbia, respectively. Sweet cherry breeding programmes in these countries are paid particular attention to self-fertility, compact habitus, early, heavy and regular cropping, early or late ripening time, large and attractive fruits, resistance to major causal agents of economically important diseases, pests, frost and fruit cracking (Budan et al. 2013, Corneanu et al. 2016, Radičević et al. 2016). Among the most typically used conventional breeding methods are planned hybridisation, open pollination and clonal selection. The sweet cherry breeding work at Fruit Research Institute, Čačak, Republic of Serbia (FRI – Čačak) has resulted in the release of two sweet cherry cultivars (Radičević et al. 2016). Corneanu et al. (2016) reported that twenty-eight new sweet cherry cultivars were obtained and approved during 1994–2015 at Research Station for Fruit Growing, Iasi, Romania (RSFG – Iasi). The first achievements of sweet cherry breeding programme at Fruit Growing Institute, Plovdiv, Republic of Bulgaria (FGI – Plovdiv) have accomplished through

releasing of four cultivars (Malchev and Zhivondov 2016). Most sweet cherries are self-incompatible, therefore cross-compatible cultivars that flower simultaneously are planted together to allow successful cross pollination and satisfactory cropping. Gametophytic self-incompatibility mechanism in sweet cherry is governed by the multiallelic *S*-locus with *S-RNase* (Bošković and Tobutt 1996) and *SFB* genes (Yamane et al. 2003). The use of the consensus and allele-specific PCR-based methods in sweet cherry has enabled the identification of 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 (Schuster 2012). Due to high polymorphism, the *S*-locus has also been used as a genetic marker for identification of domestic and foreign sweet cherry cultivars at FRI – Čačak (Marić and Radičević 2014, Marić et al. 2015, Radičević et al. 2013, 2015). This study was aimed to determine the *S*-genotypes of sweet cherry cultivars developed within breeding programmes of three institutions (FRI – Čačak, RSFG – Iasi and FGI – Plovdiv) in the Balkan region, in order to assign the cultivars to accurate incompatibility groups for planning parental combinations in further breeding work and for orchards management by choosing the suitable pollenizer. Furthermore, these results were enabled to check the reported parental pairs of the released sweet cherry cultivars.

Material and methods

Plant material and DNA extraction

Fifteen sweet cherry cultivars, including parents ('Bigareau Burlat', 'Bigarreau de Schrecken', 'Boambe de Cotnari' 'Drogans Gelbe', 'Majova Rana', 'Ranna Tcherna' and 'Van') and derivatives ('Alexus', 'Asenova Rana', 'Bucium', 'Čarna', 'Kossara', 'Margonia', 'Rosalina' and 'Rosita'), were sampled from the collections FRI – Čačak, RSFG – Iasi and FGI – Plovdiv (Table 1 and 2). Young leaves of the assessed cultivars were collected, frozen in liquid nitrogen and stored at –80°C prior to DNA extraction. Frozen samples were ground in Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) and genomic DNA was then extracted according to the method reported by Doyle and Doyle (1987), with the modification of the extraction buffer which included addition of β-mercaptoethanol (1%) and polyvinylpyrrolidone (2% PVP 40). DNA samples in TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA) were kept at –20°C until use.

Consensus and allele-specific PCR analysis of the S-RNase

S-genotyping of assessed sweet cherry cultivars was based on the methods of Sonneveld et al. (2001, 2003). In order to identify the *S-RNase* alleles, the PCRs were performed with the consensus primer pairs specific for the second intron (PaConsII-F + -R) and primers specific for alleles S_1 to S_7, as well as for S_9 allele (Sonneveld et al., 2001, 2003). For the aforementioned alleles, the following annealing temperatures were used: 64°C for S_1, 60°C for S_2, 66°C for S_3, 63°C for S_4, 52°C for S_5, 65°C for S_6, 59°C for S_7 and 61°C for S_9. Sweet cherry cultivars with known *S*-genotypes were used as standards.

Detection and visualization of the PCR products

PCR products of *S-RNase* gene obtained with the consensus and allele-specific primers were separated by electrophoresis in 2% (70 V for 4 h) and 1.5% (70 V for 2–3 h) agarose gels, respectively. The gels were stained with ethidium bromide, and obtained DNA fragments were visualized using 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-allele detection with consensus and allele-specific PCR assays

The use of consensus primers for the second introns and allele-specific primers allowed the identification of *S-RNase* alleles in the released and parental sweet cherry cultivars.

The amplification of the second intron of *S-RNase* with PaConsII-F + -R primers resulted in two PCR products, which corresponded to *S*-alleles of the assessed sweet cherry cultivars, except for parental cultivars 'Bigarreau Schrecken', 'Boambe de Cotnari' and 'Van'. The size of PCR product for the the second intron ranged from ~570 (S_6 allele) to ~2,380 bp (S_7 allele) (Figure 1). Amplification of the

second intron disabled the identification of the S_1/S_3 alleles and S_2/S_7, therefore discrimination of these alleles required additional analysis. As reported earlier (Sonneveld et al. 2003, Schuster et al. 2007, Ipek et al. 2011), small size differences were found when using consensus primers for the amplification of the second intron of the S_1 and S_3, as well as S_2 and S_7 alleles. Confirmation of *S-RNase* alleles of the assessed sweet cherry cultivars was conducted using the specific primers for the S_1 to S_7, as well as S_9 allele. The PCR product of ~820 bp corresponded to allele S_1 and was identified in two released cultivars – 'Alexus' and 'Čarna' (Figure 2a), and five parental cultivars – 'Bigarreau de Schrecken', 'Drogans Gelbe', 'Majova Rana', 'Ranna Tcherna' and 'Van'. In three released cultivars – 'Alexus', 'Kossara' and 'Rosita' (Figure 2b), and parental cultivars – 'Boambe de Cotnari' and 'Ranna Tcherna', the PCR product of ~640 bp corresponding to S_2 allele was obtained. Use of S_3 allele-specific primers enabled amplification of fragment of ~960 bp in three released cultivars – 'Asenova Rana', 'Bucium' and 'Rosalina' (Figure 2c), and three parental cultivars – 'Bigareau Burlat', 'Bigarreau de Schrecken' and 'Van'. In 'Čarna' (Figure 2d) and 'Majova Rana', the PCR product of ~820 bp corresponding to S_4 allele was detected. The DNA fragment of ~300 bp corresponding to S_5 allele was identified in 'Margonia' (Figure 2e) and 'Drogans Gelbe', whereas a fragment of ~470 bp corresponding to S_6 allele was identified in two released cultivars – 'Bucium' and 'Margonia' (Figure 2f). In parental cultivar 'Boambe de Cotnari', the PCR product of ~580 bp corresponding to S_7 allele was identified. Use of S_9 allele-specific primers enabled amplification of fragment of ~500 bp in four released cultivars – 'Asenova Rana', 'Kossara', 'Rosalina' and 'Rosita' (Figure 2g), and 'Bigareau Burlat' as parental cultivar. The size of PCR products for aforementioned *S*-alleles were in agreement with results stated by Sonneveld et al. (2001, 2003), who reported that DNA fragment of 820, 640, 960, 820, 300, 470, 584 and 495 bp corresponded to S_1, S_2, S_3, S_4, S_5, S_6, S_7 and S_9 alleles, respectively.

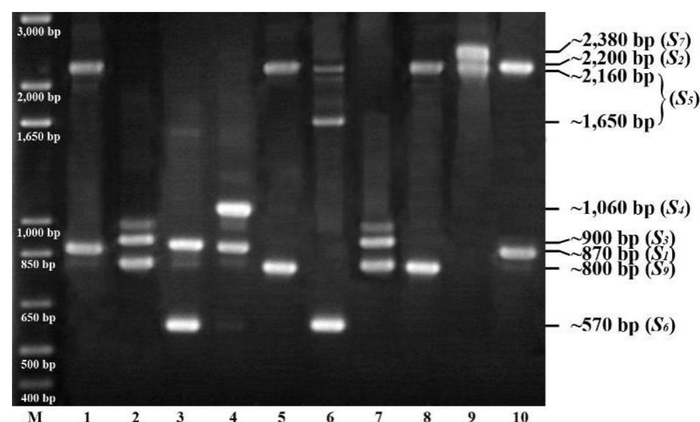

Figure 1. PCR products of the *S-RNase* amplified fragment obtained with consensus primers for the second intron of the assessed sweet cherry cultivars: 1 – 'Alexus', 2 – 'Asenova Rana', 3 – 'Bucium', 4 – 'Čarna', 5 – 'Kossara', 6 – Margonia', 7 – 'Rosalina', 8 – 'Rosita', 9 – 'Boambe de Cotnari', 10 – 'Ranna Tcherna'; 1Kb plus DNA ladder (M).

S-genotypes, incompatibility groups and assessment of parentage

The *S*-genotype of each released and parental cultivar was determined after combining the results obtained upon amplification with the consensus and the allele-specific primers (Table 1 and 2). Out of fifteen sweet cherry cultivars, the *S*-genotypes for six released cultivars – 'Alexus' (S_1S_2), 'Bucium' (S_3S_6), 'Kossara' (S_2S_9), 'Margonia' (S_5S_6), 'Rosalina' (S_3S_9) and 'Rosita' (S_2S_9), and two parental cultivars – 'Boambe de Cotnari' (S_2S_7) and 'Ranna Tcherna' (S_1S_2) are published in this paper for the first time. The following *S*-allelic constitutions were confirmed: S_1S_3 ('Bigarreau de Schrecken' and 'Van'), S_1S_4 ('Čarna' and 'Majova Rana'), S_1S_5 ('Drogans Gelbe') and S_3S_9 ('Asenova Rana' and 'Bigareau Burlat'), and these results were in agreement with the findings reported by Schuster (2012), Marić and Radičević (2014) and Marić et al. (2017). According to the genotypes detected, all

assessed cultivars were assigned to their coresponding incompatibility groups (IGs), which were previously described by Schuster (2012). Therefore, the nine following IGs were determined: I (‘Alexus’ and ‘Ranna Tcherna’), II (‘Bigarreau de Schrecken’ and ‘Van’), VI (‘Bucium’), IX (‘Čarna’ and ‘Majova Rana’), XI (‘Boambe de Cotnari’), XIV (‘Drogans Gelbe’), XV (‘Margonia’), XVI (‘Asenova Rana’, ‘Rosalina’ and ‘Bigareau Burlat’) and XLIII (‘Kossara’ and ‘Rosita’). This study revealed that ‘Kossara’ and ‘Rosita’ sharing the same *S*-genotype and belong to the same IG, which was consistent with results reported by Zhivondov et al. (2011), who stated that ‘Rosita’ was not suitable pollenizer for ‘Kossara’.

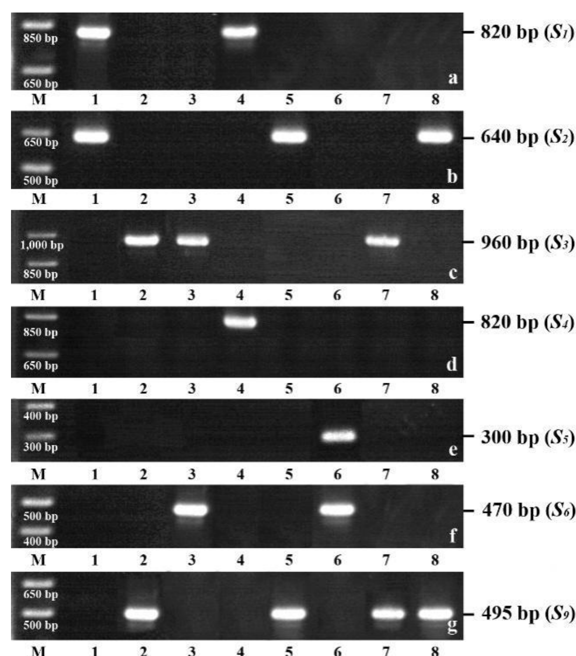

Figure 2. PCR products of the *S-RNase* amplified fragment obtained with primers specific for alleles S_1 (a), S_2 (b), S_3 (c), S_4 (d), S_5 (e), S_6 (f) and S_9 (g) in released sweet cherry cultivars: 1 – ‘Alexus’, 2 – ‘Asenova Rana’, 3 – ‘Bucium’, 4 – ‘Čarna’, 5 – ‘Kossara’, 6 – Margonia’, 7 – ‘Rosalina’, 8 – ‘Rosita’; 1Kb plus DNA ladder (M).

The pedigree of three cultivars from the Republic of Bulgaria (‘Kossara’, ‘Rosalina’ and ‘Rosita’) and one cultivar from the Romania (‘Alexus’) was confirmed by their *S*-genotyping. ‘Kossara’ was inherited one *S*-allele from each parent (S_2 from ‘Ranna Tcherna’ and S_9 from ‘Bigareau Burlat’). For the open-pollinated cultivars ‘Alexus’, ‘Rosalina’ and ‘Rosita’, the *S*-alleles inherited from female parents were confirmed (S_1 from ‘Lijana’, S_3 from ‘Van’ and S_9 from ‘Bigareau Burlat’, respectively). *S*-genotypes of two cultivars from the Republic of Serbia (‘Asenova Rana’ and ‘Čarna’) and two cultivars from the Romania (‘Bucium’ and ‘Margonia’) suggested incorrectly reported parents for these cultivars. ‘Asenova Rana’ (S_3S_9) and ‘Margonia’ (S_5S_6) revealed *S*-genotypes that could not be explained by proposed parentage [‘Drogans Gelbe’ (S_1S_5) × ‘Majova Rana’ (S_1S_4) and ‘Van’ (S_1S_3) O.P., respectively]. Marić et al. (2017) reported that these discrepancies could be resulted from pollen contamination, information presented by the breeders or technical errors in the processing of seedlings. Based on *S*-allelic constitutions of ‘Bucium’ (S_3S_6) and ‘Čarna’ (S_1S_4), the possible female parents may be ‘Van’ (S_1S_3) and ‘Majova Rana’ (S_1S_4), respectively. Since ‘Bucium’ and ‘Čarna’ had not inherited any *S*-allele of their respective male parent, ‘Boambe de Cotnari’ (S_2S_7) and ‘Bigarreau de Schrecken’ (S_1S_3) could not be in their pedigrees.

This study reports the first part of comprehensive *S*-genotype screening of sweet cherry genetic resources developed in the Balkan countries, which will be useful for the cross design in the further breeding programmes and for orchard management of these cultivars. For some of the assessed cultivars, no information on their *S*-genotypes was available in the literature. Hence, the crucial

genetic compatibility information for released cultivars 'Alexus', 'Bucium', 'Kossara', 'Margonia', 'Rosalina' and 'Rosita', as well as parental cultivars 'Boambe de Cotnari' and 'Ranna Tcherna', was provided in this manuscript.

Table 1. *S*-genotypes and incompatibility groups of sweet cherry cultivars released in the Balkan countries

Origin*	Cultivar	Parentage	*S*-genotype	IG**
RO	'Alexus'	'Lijana' O.P.***	S_1S_2	I
RS	'Asenova Rana'	'Drogans Gelbe' × 'Majova Rana'	S_3S_9	XVI
RO	'Bucium'	'Van' × 'Boambe de Cotnari'	S_3S_6	VI
RS	'Čarna'	'Majova Rana' × 'Bigarreau de Schrecken'	S_1S_4	IX
BG	'Kossara'	'Ranna Tcherna' × 'Bigareau Burlat'	S_2S_9	XLIII
RO	'Margonia'	'Van' O.P.	S_5S_6	XV
BG	'Rosalina'	'Van' O.P.	S_3S_9	XVI
BG	'Rosita'	'Bigareau Burlat' O.P.	S_2S_9	XLIII

*Country according ISO 3166 code list.
**Incompatibility group, according to Schuster (2012).
***By open-pollination.

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

Cultivar	*S*-genotype	IG	Reference for *S*-genotype
'Bigareau Burlat'	S_3S_9	XVI	Schuster (2012); confirmed in this study
'Bigarreau de Schrecken'	S_1S_3	II	Schuster (2012); confirmed in this study
'Boambe de Cotnari'	S_2S_7	XI	This study
'Drogans Gelbe'	S_1S_5	XIV	Schuster (2012); confirmed in this study
'Lijana'	S_1S_6	XX	Schuster (2012)
'Majova Rana'	S_1S_4	IX	Marić and Radičević (2014);confirmed in this study
'Ranna Tcherna'	S_1S_2	I	This study
'Van'	S_1S_3	II	Schuster (2012); confirmed in this study

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