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Polymorphisms of the self-incompatibility locus of sweet cherry (*Prunus avium* L.) species from the VNIISPK bioresource collection

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Abstract

Self-incompatibility is a common evolutionary mechanism of flowering plants. Sweet cherry (*Prunus avium* L.) has a gametophytic type of self-incompatibility. Most of sweet cherry cultivars are self-incompatible. Although self-compatible genotypes also occur. This study presents data of S genotype investigation of 39 sweet cherry cultivars from VNIISPK bioresource collection. To identify S genotypes, PCR with consensus (PaConsI and PaConsII) and allele-specific (*S1*, *S2*, *S3*, *S4*, *S5*, *S6*, *S7*, *S9*, *S10*, *S12*, *S13*, *S14* and *S16*) primers was done. The S genotype was fully identified for 33 cultivars. 7 S alleles (*S1*, *S3*, *S4*, *S5*, *S6*, *S9*, and *S13*) were detected in the genomes of investigated sweet cherries. According to the results of allele-specific amplification, 'Venera' (*S1x*/*S4*), 'Zolotuhinskaya' (*S4x*/*S6*) and 'Viskochka' (*S3*/*S4x*) are suspected to have unique or mutant alleles of the gene. The cultivars with fully determined S-genotypes were distributed into nine incompatibility groups (III, VI, VII, IX, XV, XVII, XIX, XXI, and XLV).

Keywords: *Prunus avium* L., self-incompatibility, S-alleles, consensus primers, allele-specific primers.

Introduction

Self-incompatibility is one of the most important and widespread mechanisms used by flowering plants to prevent self-fertilization and consequently to provide the genetic diversity of populations. From the other side, sweet cherry, as other fruit crops, has too high commercial value and getting high yields is the most important task.

Sweet cherry, as the other Rosaceae, has a gametophytic type of self-incompatibility and the success of its pollination is dependent on the interaction of two genes, S-allele specific ribonuclease (*S*-RNase) and S haplotype-specific F-box protein (SFB) (Yamane*et al.*, 2003).*S-RNase* gene presented with numerous alleles,expresses into stylar tissue and non-selectively takes up into the cytoplasm of the pollen tube.Fertilization is possible only in case if S-RNases of pistil and pollen are different. In opposite way, SFB triggers off cytotoxicity of self S-RNase andthe pollen tube growth is arrested. To explain specific inhibition of the growth of self-pollen

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tubes two models of genes interaction, an inhibitor model and a receptor model, have been proposed. Presently, the inhibitor model is most favoured. According to this model, all S-RNases enter pollen tubes, but inhibitors in the pollen tube cytoplasm inhibit the activity of non-self S-RNases, as opposed to the self S-RNase.So, S locus F-box like protein and S haplotype-specific F-box like protein considered as good candidates for role of some general inhibitor molecule to take place in detoxification of non-self S-RNase (Wang *et al*., 2003; Matsumoto *et al*., 2019).

To determine the *S-RNase* gene polymorphism aset of primers for amplification of both variable and high conserved fragments were designed that made possible the PCR-based identification of *S* alleles (Tao *et al*., 1999; Wiersma*et al*., 2001; Sonneveld*et al*., 2001; Sonneveld*et al*., 2003). The development of PCR-based methods stimulated the identification of numerous*S* alleles and determination of the *S*-genotype of a wide number of sweet cherry cultivars. As a result, alleles S_1 to S_{16}(Wiersma *et al*., 2001; Sonneveld*et al*., 2001; Sonneveld*et al*., 2003), S_{17} to S_{22}(De Cuyper *et al*., 2005; Schuster *et al*., 2007; Gisbert *et al*., 2008), S_{24}(Wunsch *et al*., 2004), S_{27}, S_{30}(Vaughan *et al*., 2008) and S_{37}, S_{38} (Szikriszt*et al*., 2013) were described. Data of *S* genotype of numerous sweet cherries was received.

The reported results of the *S*-alleles identifications were summarized and analyzed, and sweet cherry genotypes were grouped on the basis of the received PCR data (Tobutt*et al*., 2001; Tobutt*et al*., 2004; Schuster 2012, 2017, 2020; Schuster *et al*., 2024).

The last overview of self-incompatibility genotypes of cultivated sweet cherries was published by Schuster in November 2024, containing information on 1700 genotypes and 26 different alleles (S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_9, S_{10}, S_{12}, S_{13}, S_{14}, S_{16}, S_{17}, S_{18}, S_{19}, S_{21}, S_{22}, S_{24}, S_{27}, S_{30}, S_{37}, $S_{38}$and three mutated alleles $S_{3'}$, $S_{4'}$, $S_{5'}$). 1583 genotypes were divided into 72 self-incompatibility groups. 26 cultivars having a unique S allele combination were included in group 0 (universal donors). 91 self-compatible genotypes were included in group SC (Schuster *et al*., 2024).

In this study, to estimate the *S-RNases* gene polymorphisms of cultivars from the VNIISPK sweet cherry collection, PCR was performed with consensus (PaConsI and PaConsII) and allele-specific (S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_9, S_{10}, S_{12}, S_{13}, S_{14}, and S_{16}) primers. A total of 39 sweet cherry genotypes were investigated. For 33 of them, *S*- genotype was determined completely, and for 4 cultivars – partially. In the case of two cultivars, ‘Bahor’ and ‘Naslazhdenie’, PCR with allele-specific primers was fruitless. So, we can conclude that none of the tested *S* alleles is present in their genomes. Also, surprisingly, in the result of amplification with primers

specific to S_1 and S_4 alleles, the size of the received PCR products was different from that was expected for the three cultivars. There are, probably, two not yet described alleles present in these genomes. In the genomes of the investigated cultivars, the presence of 7 *S* alleles (S_1, S_3, S_4, S_5, S_6, S_9, and S_{13}) was estimated. Cultivars with fully determined *S*-genotypes belong to nine incompatibility groups (III, VI, VII, IX, XV, XVII, XIX, XXI, and XLV).

Materials and Methods

Thirty-nine genotypes, cultivars and selections, from VNIISPK sweet cherry collection were studied in this work (Table 1). The majority of cultivars originate from Russia but some of them are from Ukraine or Belarus.

Genomic DNA was extracted from young leaves by CTAB method with modifications for tissue containing high polysaccharide and polyphenol components (Porebski *et al.*, 1997).

To investigate polymorphisms of the *S-RNases* gene of chosen sweet cherry genotypes, PCR with consensus (PaConsI and PaConsII) (Sonneveld*et al.*, 2003) and allele-specific (S_1, S_2, S_3, S_4, S_5, S_6, S_7, S_9, S_{10}, S_{12}, S_{13}, S_{14} and S_{16}) (Sonneveld*et al.*, 2001; Sonneveld*et al.*, 2003) primers was performed. For amplification, Set of PCR reagents with Hot Start Taq polymerase (Dia-m, Kat. KH017-002-21, Russia) was used. PCR was carried out in 25 µl total reaction mix containing 50 ng of genomic DNA, PCR reaction buffer supplemented with 2 mM $MgCl_2$, 1U of Taq polymerase, 0,5 mM dNTP and 0,5 µM of each primer. PCR conditions were: initial denaturation at 95°C for 5 min, 35 cycles at 95°C for 30 sec, primer annealing temperature 55-58°C for 30 sec, elongation at 72°C for 1 min, followed by a final elongation at 72°C for 5 min. Amplification products were analysed by 1,4 % (w/v) agarose gel electrophoresis stained with ethidium bromide and observed under ultraviolet light. Size of amplification products were estimated visually by comparison with markers of molecular weight Step50 plus (Biolabmix, Russia) and Step100 Long (Biolabmix, Russia).

Results

This study presents the results of investigation of the *S*-genotype of 39 cultivars from sweet cherry VNIISPK collection. VNIISPK (Russian Research Institute of Fruit Crop Breeding) is one of the oldest horticultural institutions in Russia, founded in 1845 (https://vniispk.ru). The bioresource collection of the Institute includes fruit, berry and ornamental crops. At present, the bioresource collection of the VNIISPK includes more than 1800 varieties, more than 2500 elite and selected forms, hybrid fund is about 25 thousand genotypes (https://vniispk.ru/pages/unu).

Most of the tested sweet cherry cultivars originate from Russia. 4 cultivars, 'Gostinets', 'Sopernitsa', 'Medunitsa', and 'Minchanka', originate from Belarus. Another 4 cultivars, 'DonetskiiVelikan', 'Vasilisa', 'Alenushka', and 'Naslazhdenie', originate from Ukraine. Also, one cultivar from a private collection from Ukraine ('Sweet Cherry from Donetsk') was tested. At the first step, PCR was performed with consensus primers PaConsI and PaConsIIcreated Sonneveld(Sonnevelde*t al.*, 2003). These two pairs of primers were designed to amplify regions of the two introns of the *S-RNase* gene. In the result of amplification with consensus primers a set of PCR products ranged from 303 to 523bp for PaConsI primers, and from 577 to 2 383 bp for PaConsII primers (Sonnevelde*t al.*, 2003). Applying of consensus primers allows identifying some allelesin genomes, including S_3 and S_6 alleles, and predicting otheralleles.Exploitation of the consensus primers gives an opportunity to reduce the number of checked alleles.But, anyway, allele-specific amplification is necessary to avoid mistakes.

In this study, the S_3 allele was detected using PaConsI primers in the genomes of 21 tested sweet cherries. The S_6allele was identified using PaConsII primers in the genomes of 19 sweet cherries (Table 1). Received data were confirmed by S_3 and S_6 allele-specific amplification.

After that, based on data of PCR with consensus primers, amplification with S_1, S_2, S_4, S_5, S_7, S_9, S_{10}, S_{12}, S_{14} and S_{16} allele specific primers was performed.

The alleles S_1, S_2, S_5, S_9, and S_{10} more frequently occur in genomes of sweet cherries and that is why they were tested at first. As a result of the analysis, only alleles S_1, S_4, S_5 and S_9 were detected in the genomes of investigated cultivars. The S_4 allele was detected in the genomes of 16 genotypes. The S_1, S_5, and S_9 alleles were present in the genomes of few sweet cherry cultivars (the S_1 allele was detected in 2 cultivars, the S_5 allele wasdetected in 5cultivars, and the S_9 allele wasdetected in 1 cultivar) (Table 1). Surprisingly, in the result of amplification with primers specific to S_1 and S_4 alleles, the size of the three PCR products, one in amplification with S_1 primers and two with S_4 primers, was different from that was expected (Figure 1). The expected size of PCR products after allele-specific amplification with S_1 and S_4 primers is 820 b.p. for both primer pairs.The amplification with S_1 primers with the DNA of 'Venera' cultivar results in the PCR product size approximately 100 bp less than predicted. Also, the amplification with S_4 allele specific primers with DNA of 'Zolotuhinskaya' and 'Viskochka' results in synthesis of PCR product size approximately 200 bp less than was expected. In these two cases, specific amplification took place. To avoid mistakes, PCRs were repeated. In the case of S_4 allele specific PCR, the size of amplified products with DNA 'Zolotuhinskaya' and 'Viskochka' was identical. That also excludes mistakes of amplification.

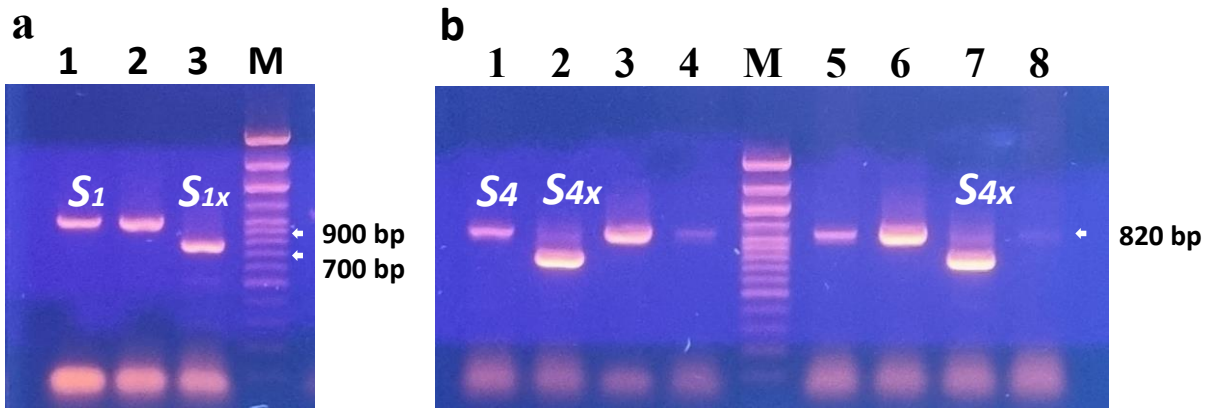

Figure 1. PCR amplification of cultivars used *S1* and *S4* allele specific primers. (a)PCR with *S1* allele specific primers. 1 –'BrianskayaRosovoya', 2 – 'Gostinits', 3 – 'Venera', M - markers of molecular weight Step100 Long (Biolabmix, Russia);(b) PCR with *S4* allele specific primers.1 – 'Irinka', 2 – 'Zolotuhinskaya', 3 – 'Medunitsa', 4 – 'PamiatiZhukova', 5 – 'LubimitsaAstahova', 6 – 'Alenushka', 7 – 'Viskochka', 8 – 'PamiatiChernishevskogo', M - markers of molecular weight Step100 Long (Biolabmix, Russia).

The results of the amplification using consensus primers PaConsI and PaConsIIof these genotypes were also not entirely typical.No PCR product was detected after amplification with PaConsI primers using DNA of 'Zolotukhinskaya' cultivar (Figure2).The size of the PCR product after amplification with Venera DNA was around 500 b.p., which could suggest the presence of multiple alleles, including *S1*, *S4* and *S6*.The amplification with DNA from 'Viskochka' resulted in two PCR products, one approximately 300 b.p. and the other around 500 b.p.(Figure 2).The detection of a 300 b.p. PCR product indicates the presence of the *S3* allele in the genome. This was also confirmed by allele-specific amplification. Thus, the sizes of the PCR products amplified using PaConsI primers corresponded to alleles *S1*, *S3*, *S4* and *S6*. The only unexpected result was the absence of PCR products when 'Zolotukhinskaya' DNA was used as the template.

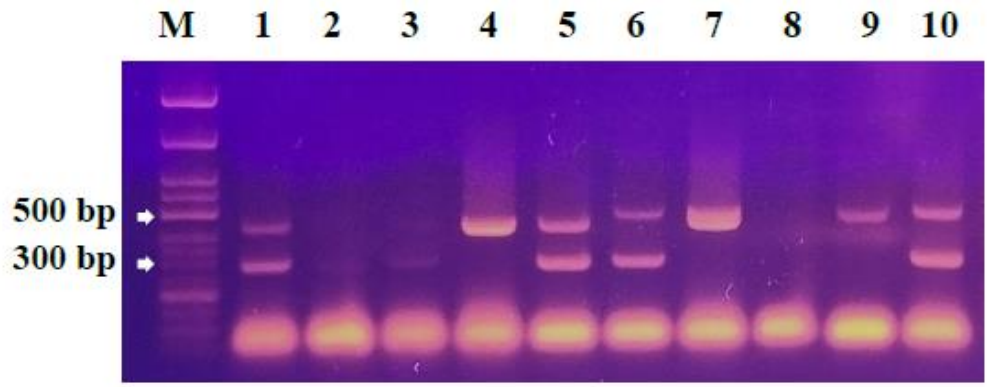

Figure 2. PCR amplification of cultivars used consensus PaConsI primers. 1 – 'Cheremashnaya', 2 – 'Raditsa', 3 – 'Sopernitsa', 4 – 'Venera', 5 – 'Viskochka', 6 – 'Kompaktnaya', 7 – 'Irinka', 8 – 'Zolotuhinskaya', 9 – 'Medunitsa', 10 – 'Lena', M - markers of molecular weight Step50 Plus (Biolabmix, Russia)

As expected, the sizes of the PCR products obtained using PaConsII primers corresponded to alleles S_1, S_3, S_4 and S_6 (Figure 3). The detection of a 570 bp product indicated the presence of allele S_6 in the 'Zolotukhinskaya' genome. Products with sizes of 800–900 bp indicated the presence of alleles S_1 and S_3, and products with sizes of 900–1000 bp indicated the presence of alleles S_3 and S_4 (Sonneveld*et al.*, 2003). All results were rechecked and refined using allele-specific primers.

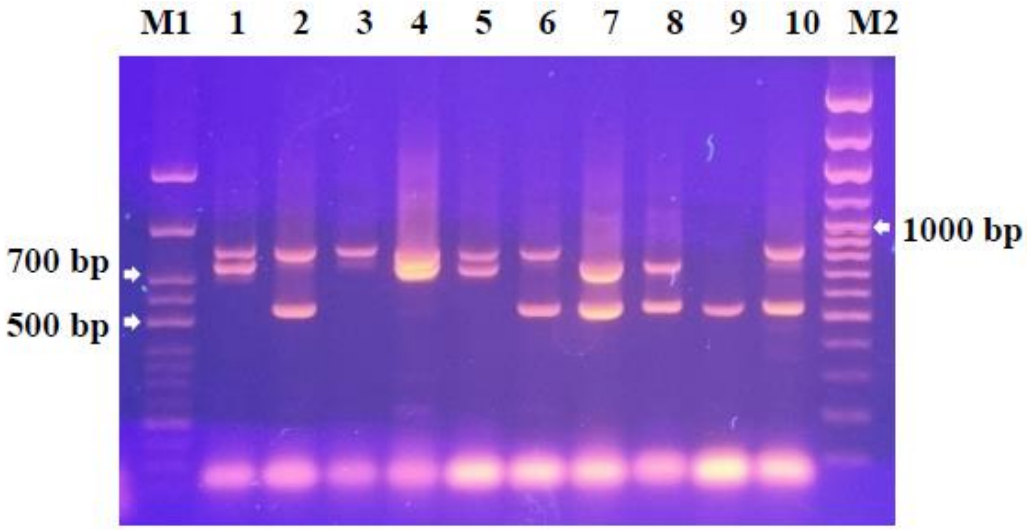

Figure 3. PCR amplification of cultivars used consensus PaConsII primers.1 – 'Cheremashnaya', 2 – 'Raditsa', 3 – 'Sopernitsa', 4 – 'Venera', 5 – 'Viskochka', 6 – 'Kompaktnaya', 7 – 'Irinka', 8 – 'Zolotuhinskaya', 9 – 'Medunitsa', 10 – 'Lena',M1 - markers of molecular weight Step50Plus (Biolabmix, Russia); M2 - markers of molecular weight Step100 Long (Biolabmix,Russia).

Alleles S_7, S_{12}, S_{13}, S_{14}, and S_{16} are rarely detected in sweet cherry genomes. Cultivars with not yet established S genotype were tested by PCR with these allele specific primerslast. S_{13} allele was detected in the genomes of three cultivars, 'Kormilitsa', 'Iput' and 'Mak'. Alleles S_7, S_{12}, S_{14}, and S_{16} were not identified in the tested genomes.The S genotype of the two cultivars, 'Bahor' and 'Naslazhdenie', is unclear because no one of the tested *S* alleles was detected in their genomes. The S genotype of the four cultivars, 'Odrinka','Preludia', 'Minchanka', and 'Valentina', was partially identified. Further investigation of their S allele polymorphisms is necessary.

So, as a result of the performed analysis, seven*S* alleles (S_1, S_3, S_4, S_5, S_6, S_9, and S_{13}) were detected in the genomes of the investigated sweet cherry cultivars. Alleles S_2, S_7, S_{10}, S_{12}, S_{14}, and S_{16} were not present in any tested genotypes. For two cultivars, 'Bahor' and 'Naslazhdenie', PCR with allele-specific primers did not produce any results, although PCR with consensus primers was successful. For four cultivars, only one *S* allele was identified. The S-genotype of the three genotypes needs further study due to atypical amplification with S_1 and S_4 allele specific primers. The S-genotype of the 'Venera' cultivar is shown in Table 1 as S_{1x}/S_4. S-genotypes of cultivars 'Zolotuhinskaya' and 'Viskochka' are shown as S_{4x}/S_6 and S_3/S_{4x}, respectively. For 30 sweet cherry cultivars, self-incompatible groups were determined. Nine incompatibility groups (III, VI, VII, IX, XV, XVII, XIX, XXI, and XLV) were detected.Received data was summarized in Table 1.

Table 1. Results of S-genotyping of sweet cherry cultivars.

Cultivar	S allele													S-genotype	Incomp. group
	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_9	S_{10}	S_{12}	S_{13}	S_{14}	S_{16}		
Odrinka			+											S_3	-
Kormilitsa				+							+			S_4/S_{13}	XLV
PamiatyAstahova			+			+								S_3/S_6	VI
Fatezh			+			+								S_3/S_6	VI
Mak			+								+			S_3/S_{13}	XIX
Brianskaya Rosovaya	+			+										S_1/S_4	IX
Donetckii Velikan				+				+						S_4/S_9	XXI
Gostinets	+			+										S_1/S_4	IX
Zaria Vostoka			+			+								S_3/S_6	VI
Iput			+								+			S_3/S_{13}	XIX
Pamiaty Nikitina				+		+								S_4/S_6	XVII
Geroinya			+		+									S_3/S_5	VII
Krasnaya Gorka				+		+								S_4/S_6	XVII
Tutchevka				+		+								S_4/S_6	XVII
SeianetsChernishevskogo					+	+								S_5/S_6	XV
Sweet cherry from Donetsk					+	+								S_5/S_6	XV
Cheremashnaya			+	+										S_3/S_4	III
Raditsa			+			+								S_3/S_6	VI
Sopernitsa			+		+									S_3/S_5	VII
Venera	x			+										S_{1x}/S_4	-
Dar Cheliabinsku			+	+										S_3/S_4	III
Kompactnaya			+			+								S_3/S_6	VI
Irinka				+		+								S_4/S_6	XVII
Zolotuhinskaya				x		+								S_{4x}/S_6	-
Medunitsa				+		+								S_4/S_6	XVII
Lena			+			+								S_3/S_6	VI
Aelita					+	+								S_5/S_6	XV
Preludia			+											S_3	-
Pamiati Zhukova				+		+								S_4/S_6	XVII
Lubimitsa Astahova			+	+										S_3/S_4	III
Alenushka			+	+										S_3/S_4	III
Viskochka			+	x										S_3/S_{4x}	-
Bahor														-	-
Pamiati Chernishevskogo			+	+										S_3/S_4	III
Naslazhdenie														-	-
Brianochka			+			+								S_3/S_6	VI
Minchanka						+								S_6	-
Valentina			+											S_3	-
Vasilisa			+			+								S_3/S_6	VI

x- the amplification with allele specific primers was successful, but the size of the PCR product was different from expected.

Discussion

Sweet cherry (*Prunus avium* L.) is an important fruit crop. Most sweet cherries are self-incompatible. Sweet cherry has a gametophytic type of self-incompatibility controlled by S allele specific ribonuclease (S-RNase) and S haplotype-specific F-box protein (SFB). As we wrote above, self-incompatibility is a very significant mechanism in the evolution of flowering plants. Self-incompatibility matters in the prevention or reduction of self-fertilization. Knowledge of the S-genotype of cultivars gives us the opportunity to predict the effectiveness

of fertilization. At the same time, the S genotype of sweet cherry cultivars is almost never taken into account in the breeding process. So, some rare *S* alleles can be lost. Maintenance of sweet cherry cultivars containing rare and unique *S* alleles in their genomes is important.

The main aim of this study was to investigate S-genotype polymorphisms of sweet cherry cultivars and selections from the VNIISPK fruit crop bio resource collection. The sweet cherry collection includes not only cultivars that originate from Russia, but also those from Belarus and Ukraine. Previously, the analysis of *S-RNase* gene polymorphisms of cultivars of VNIISPK breeding was performed (Bezlepkina*et al.*, 2020), so these genotypes were not included in the investigation. However, this data should be considered when analyzing the VNIISPK collection of sweet cherry genotypes generally.

This paper represents data of S genotype analysis of 39 sweet cherry cultivars. The presence of thirteen S alleles in genotypes was checked. Seven of them (S_1,S_3, S_4, S_5, S_6, S_9, and S_{13}) were detected. Alleles S_3, S_4, and S_6 were the most common. The S_3, S_4, and S_6 alleles were detected in the genomes of 21, 16, and 19 cultivars, respectively. The S_1, S_5, S_9, and S_{13}alleles were identified in the genomes of sweet cherry collection much less frequently. They were detected in 2, 5, 1, and 3 genotypes, respectively.

In a previous study (Bezlepkina*et al.*, 2020), we tested nine sweet cherry cultivars of VNIISPK breeding. They were 'Adelina' (S_3/S_5), 'Poezia' (S_3/S_5), 'Malish' (S_6), 'PodarokOrlu' (S_9), 'OrlovskayaRozovaya' (S_6/S_x), 'OrlovskayaYantarnaya' (S_6), 'OrlovskayaFeia' (S_3/S_5), 'Trosnianskaya' (S_5/S_6), and 'Siana' (S_3/S_6).

Considering the data of the previous S genotype investigation, allele S_3 is present in 25 out of 48 tested genotypes of the VNIISPK sweet cherry collection. This is 52% of the investigated genomes. The allele S_4 was detected in 16 out of 48 genomes. This is 33% of the tested genotypes. In 50 % of the investigated sweet cherry genomes, the allele S_6 was detected. The S_5allele is the next one by the presence in the genomes of sweet cherry cultivars from the VNIISPK collection. The S_5 allele was detected in 19% of genotypes. The S_3, S_4, S_5 and S_6 alleles were more prevalent in the genomes of sweet cherry cultivars. Each of the S_1, S_9 and S_{13} alleles were detected in 4% of investigated genomes (Figure 4).

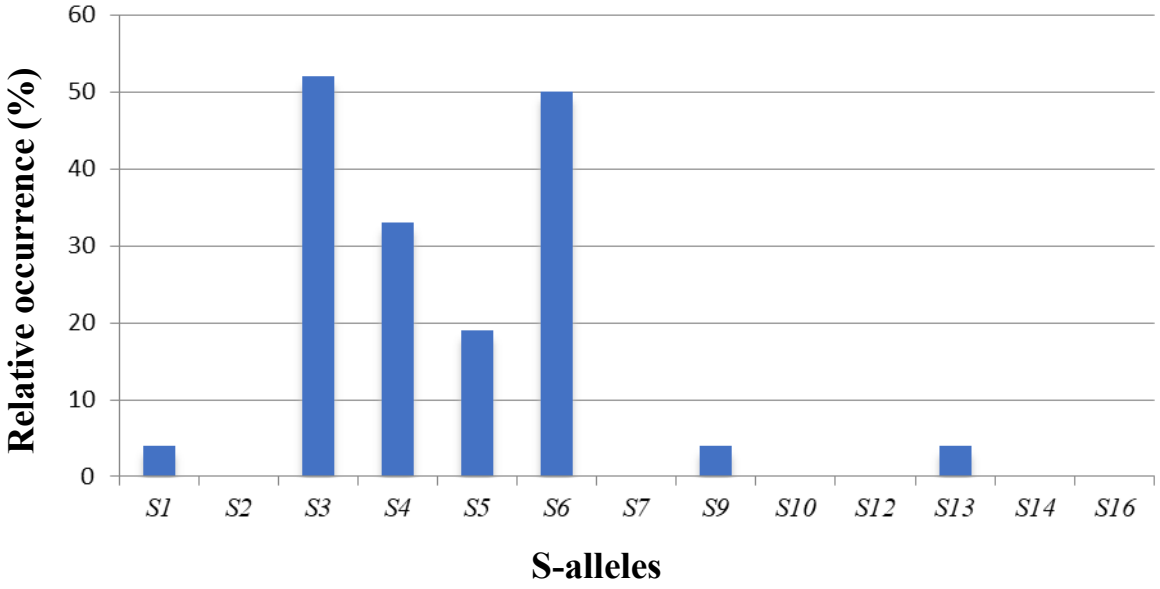

Figure 4. The frequency of S-alleles in the VNIISPK sweet cherry collection.

S_1, S_3, S_4, S_5, S_6, and S_9 alleles are the most common in sweet cherry genotypes worldwide (Schuster *et al.*, 2024). In this regard, the presence of S_3, S_4, S_5, and S_6 alleles in a large number of sweet cherry genotypes from the VNIISPK bioresource collection was expected.The S_1, S_9, and S_{13} alleles are relatively poorly represented in the genomes of sweet cherry cultivars from the VNIISPK bioresource collection. However, the most interesting cultivars are sweet cherries, in the genome of which unique alleles of the *S-RNase* gene have been identified. These are'Venera' (S_{1x}/S_4), 'Zolotuhinskaya' (S_{4x}/S_6), 'Viskochka' (S_3/S_{4x}) and'OrlovskayaRozovaya' (S_6/S_x). As well as cultivars in which allelic set of the *S-RNase* gene has not been fully determined that may indicate the presence of rarer alleles of the gene in the genome.In the process of selective breeding for a complex of economically valuable traits, the allelic polymorphism of the *S-RNase* gene is most likely to decrease. In this regard, the preservation of sweet cherry genotypes with rare and unique *S* alleles is of great importance.

Also, in the result of S genotype analysis presented in this article, incompatibility groups were established for 30 sweet cherry cultivars. A total of nine incompatibility groups were identified. There are III (S_3/S_4), VI (S_3/S_6), VII (S_3/S_5), IX (S_1/S_4), XV (S_5/S_6), XVII (S_4/S_6), XIX (S_3/S_{13}), XXI (S_4/S_9), and XLV (S_4/S_{13}) incompatibility groups. The sweet cherry cultivars of VNIISPK breeding tested previously belong to the VI, VII, and XV in-compatibility groups (Bezlepkina*et al.*, 2020). The incompatibility groups III (S_3/S_4), VI (S_3/S_6), and XVII (S_4/S_6) are the most numerous. This was expected due to the frequent occurrence of the S_3, S_4, and S_6alleles in the genomes of the investigated sweet cherry cultivars.

Conclusions

To summarize, a total of 39 cultivars were tested. The presence of the thirteen S alleles was checked, and seven of them (*S1*, *S3*, *S4*, *S5*, *S6*, *S9*, and *S13*) in the sweet cherry's genomes were detected. The *S3*, *S4*, *S5*, and *S6* alleles were present in 52%, 33%, 19%, and 50% of the investigated genomes, respectively. The *S1*, *S9*, and *S13* alleles were identified in the genomes of sweet cherry collection much less frequently. 'Venera' (*S1x*/*S4*), 'Zolotukhinskaya' (*S4x*/*S6*) and 'Viskochka' (*S3*/*S4x*) are suspected to have unique or mutant alleles of the gene. In 6 genotypes, the allele set of the gene was not fully determinedthat may indirectly indicate the presence of more rarely occurring alleles of the gene in the genome.CultivarswithfullyestablishedS-genotypesbelongtonineincompatibilitygroups (III, VI, VII, IX, XV, XVII, XIX, XXI, andXLV). The most numerous incompatibility groups are III (*S3*/*S4*), VI (*S3*/*S6*), and XVII (*S4*/*S6*).

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