BIOLOGIJA. 2008. Vol. 54. Nr. 1. P. 22–26

S-allele identification by PCR analysis in Lithuanian sweet cherries

Vidmantas Stanys,

Rugilė Stanytė,

Gražina Stanienė,

Jurgita Vinskienė

Department of Orchard Plant Genetics and Biotechnology, Lithuanian Institute of Horticulture, Kaunas str. 30, LT-54333 Babtai, Kaunas distr., Lithuania

The S alleles from 17 accessions of Lithuanian origin and 5 standard varieties of sweet cherry were determined using the polymerase chain reaction (PCR) method. Initially, DNA extracts were amplified with consensus primers that amplify across the first, the second, or both introns of the S-ribonuclease gene which shows a considerable length polymorphism. Because the size of PGR products in this amplification is often similar, all complicated accessions later were tested with allele-specific primers. Combinations of S alleles were identified in all the accessions studied. After the investigation, Lithuanian sweet cherries were assigned to ten different self-incompatibility groups. New combinations of S alleles were identified – S_1S_{13} ('Žemaičių juodoji') and S_6S_{16} ('Jurga','Anta'). One additional incompatibility group was proposed.

Key words: S-alleles, incompatibility genotypes, allele-specific primers, consensus primers, *Prunus avium*

INTRODUCTION

It is generally believed that sweet cherry originated in an area South of the Caucasus Mountains and possibly close to the Caspian and Black Seas from where it spread to Eastern Europe. Its distribution by man has now taken it into most countries of the world [1]. Sweet cherries were introduced to Lithuania from South and Western Europe in the 19th century from which later a population of the so-called 'Žemaičių' cherries has been formed [2]. In the beginning of the 20th century, the breeding of sweet cherry began in Lithuania and the first Lithuanian cultivars were created. Thus, the population of sweet cherry was formed at the Northern borders of their spread area. Wild sweet cherries grow only in Western and Southern Lithuania.

Gametophytic self-incompatibility in sweet cherry is controlled by a single multiallelic locus – the *S*-locus [3]. The *S*-gene product in styles is a ribonuclease enzyme [4]. Incompatibility genotypes are traditionally identified by controlled pollination, and more recently by stylar ribonuclease analysis [5, 6]. The first approaches require plants to mature enough to produce blossoms. The recent isolation of the S-RNase cDNAs of several S-alleles of sweet cherry [7–9] has allowed the identification of S-haplotypes by PCR analysis using either consensus [7, 9, 10] or allele-specific primers [8, 10]. The isolation of the genomic DNA of sweet cherry [11–13] revealed that the two introns found in sweet cherry S-RNases gene vary in size for each S-allele. This intron variability is the basis of S-allele identification by PCR analysis using consensus primers. Thus, PCR primers designed in the conserved regions of sweet cherry S-RNases that span either the first or the second intron, or both, amplify fragments of different size for each S-allele [7, 9].

In sweet cherry, up to 16 different S-alleles and 29 incompatibility groups have been reported so far using different methods [14]. However, there are very few data about the composition of S-allele in sweet cherry cultivars from Eastern Europe and no data about cultivars of Lithuanian origin.

The objective of this work was to characterize the S-allele composition in sweet cherry cultivars of Lithuanian origin using molecular approaches, to determine data on allelic frequencies and incompatibility groups.

MATERIALS AND METHODS

Plant material. Five previously genotyped cultivars were chosen to establish the fragment size according to corresponding S-RNases after amplification by PCR. They were 'Celeste' ($S_1S_{4'}$), 'Vega' (S_2S_3), 'Nadino' (S_3S_5), 'Kordia' (S_3S_6), 'Merchant' (S_4S_9). Each of these cultivars belongs to a different self-incompatibility group, and their S-allele constitution has been confirmed by various authors [5, 6, 8–10, 15]. Seventeen accessions including three land races ('Žemaičių juodoji', 'Žemaičių geltonoji','Žemaičių rožinė'), thirteen cultivars bred at the Lithuanian Institute of Horticulture, and the clone 'Hrebnickis 1' from garden of the famous Lithuanian horticulturist Adamas Hrebnickis were tested.

Genotyping approach and PCR reaction. Genotyping the accessions involved several stages. First the samples were amplified with the consensus primers for the first, the second or both introns. For the genotypes that were not fully resolved, allele-specific primers were used. DNA was extracted from

Corresponding author. E-mail: v.stanys@lsdi.lt

leaves (0.15 g) or buds (0.1 g) using the CTAB method according to Doyle and Doyle [16]. For identification of the different accession S-alleles, five consensus primer pairs for PCR analysis were used: EMPC2consFD-EMPC3consRD, EMPC2consFD-EMPC5consRD [17] and PruC2-PruC4R [7] flanking the second intron, PruT2-PruC4R embracing both the first and the second introns [7] and PaConsI-F-PaConsI-R corresponding for the first intron [10]. PCR reactions were performed in 20 µl of a mixture with the final concentrations: 1 × Taq polymerase reaction buffer, 0.2 mM of each dNTP, 0.2 µM of each forward and reverse primers, 1.25 units of Taq polymerase (Fermentas), 350 ng of genomic DNA. $MgCl_2$ concentration differed depending on the buffer and primer pairs: using the Taq polymerase buffer with $(NH_4)SO_4$ (for primer sets EMPC2consFD-EMPC3consRD and EMPC2consFD-EMPC5consRD) 1.625 mM $MgCl_2$ was used, and for reaction with KCl (for the rest primer sets) 1.0 mM $MgCl_2$ was employed. PCR was carried out for 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min 30 s at a primer annealing temperature, 1 min 20 s at 72 °C and a final extension step for 4 min at 72 °C. The annealing temperature for all primer sets was chosen following the author's recommendations, except for EMPC2consFD-EMPC5consRD and EMPC2consFD-EMPC3consRD where it was lowered to 50 °C and 54 °C degrees, respectively.

Allele-specific primers [8, 10] were used to find out sweet cherry S genotype still unravelled by consensus primers. Protocols for S_1 and S_2–S_6 allele identification were followed according to Sonneveld et al. [8], except the annealing temperature which was modified according to recommendations of Sonneveld et al. [10]. Protocols for S_2 and S_7 to S_{16} followed Sonneveld et al. [10]. All cultivars possessing S_4 genotype were tested for S_4 using the BFP200-BFP201 primer pair [18], following the protocol of PCR reaction suggested by authors. PCR products were separated on a 1.7% agarose gel and photographed under UV light (Herolab). The size of PCR products was estimated using the E.A.S.Y. Win 32 program.

The frequency of certain S alleles in sweet cherry cultivars of Lithuanian origin was evaluated by calculating the percentage of certain S alleles in the cherry accessions studied.

Lithuanian sweet cherry cultivars were assigned to self-incompatibility groups following the classification suggested by Schuster et al. [14].

RESULTS AND DISCUSSION

Several alleles were identified using consensus primer pairs (Table 1). S_4 and S_6 alleles were specifically distinguished from the rest using primer pairs flanking the second intron (EMPC2consFD-EMPC3consRD;EMPC2consFD-EMPC5consRD and PruC2-PruC4R). The rest alleles were not undoubtedly identified because of a similar size of the amplification products. It was difficult to separate S_1 from S_3 and S_2 from S_5 allele. Besides, amplification of S_2 and S_5 alleles was not very intensive and the bands on the agarose gel were not always visible. S_4 and $S_{4'}$ alleles could not be separated after amplification with consensus primers because they differ only by 4 bp. Authors suggesting EMPC2consFD-EMPC3consRD and EMPC2consFD-EMPC5consRD consensus primer pairs described only S_1–S_6 alleles' amplification products in sweet cherry. In our work, two additional bands, not characterised before, were found in a few accessions using the EMPC2consFD-EMPC3consRD primer pair. They were approximately 1210 bp and 520 bp in length. The smaller band (520 bp) was also amplified in the standard cultivar 'Merchant' which is known to have S_9 allele [10]. Analogously two new bands were amplified in a few accessions using another primer pair, EMPC2consFD-EMPC5consRD (approximately 1418 bp and 770 bp). Such sizes of fragments have not been described in literature before. The smaller fragment (770 bp) was clearly visible in the same accessions as a band of 520 bp after amplification with the first primer pair (EMPC2consFD-EMPC3consRD). We may conclude that S_9 allele can be successfully identified using both primer pairs (EMPC2consFD-EMPC3consRD and EMPC2consFD-EMPC5consRD).

The larger fragment (1210 bp) was amplified in four accessions ('Jurga', 'Anta', 'Žemaičių geltonoji' and 'Hrebnickio 1') using the EMPC2consFD-EMPC3consRD primer pair. The longer band (1418 bp) with a primer pair (EMPC2consFD-EMPC5consRD) was amplified only in the cultivar 'Žemaičių geltonoji'; furthermore, it was not clearly visible. Using primers EMPC2consFD and EMPC5consRD in the cultivar 'Žemaičių juodoji', another band was amplified; its size (approximately 975 bp) did not correspond to any of S_1–S_6 alleles described before.

The primer pair PruC2–PruC4R spanning the second intron has been previously used to identify S_1–S_7, S_9–S_{10}, S_{12}–S_{14} and S_{21} alleles [19]. In our work, an unknown band of approximately 1250 bp was amplified in cultivars 'Jurga' and 'Žemaičių geltonoji'. The size of the band did not correspond to any of the previous S alleles studied using these primers. Considering that a fragment not corresponding to any of S_1–S_6 alleles was amplified also in the same cultivars using the primer pair EMPC2consFD-EMPC3consRD, it is possible to conclude that these new fragments characterise an S allele not previously investigated with these primer sets.

The length of amplification products with the primer pair PruT2-PruC4R is based on the length of both introns. These primers enable to identify clearly S_1, S_4 and S_6 alleles. The primer set PaConsI-F-PaConsI-R allowed to amplify the first intron. The length of amplification products varied from 300 bp to 520 bp because of the small size of the first intron. The difference among various alleles was insignificant. Only the band corresponding to S_3 allele was significantly smaller and therefore was clearly identified.

Using any pair of consensus primers, the number of S allele-candidates was strongly reduced, and only a few S alleles were characterised clearly. Accessions with still unclear S-allele combinations were tested with S allele-specific primers (Table 2). If the presence of S_4 allele had been confirmed with consensus primers, accessions were tested with $S_{4'}$ allele-specific primers. The $S_{4'}$ allele has not been identified in any of the Lithuanian sweet cherry cultivars tested. The presence of S_9 allele was confirmed in all accessions possessing identical fragments as in the standard cultivar 'Merchant' while amplifying with the consensus primer pairs EMPC2consFD-EMPC3consRD and EMPC2consFD-EMPC5consRD. The S_{16} allele was identified in the accessions 'Jurga', 'Anta', 'Žemaičių geltonoji' and 'Hrebnickio 1'. The S_{13} allele was found

Table 1. **Determination S alleles in sweet cherry accessions of Lithuanian origin, based on amplification with consensus primers**

Accession	Primers										Conclusion
	EMPC2consFD EMPC3consRD		EMPC2consFD EMPC5consRD		PruT2 PruC4R		PruC2 PruC4R		PaConsI-F PaConsI-R		
	No of bp	Candidates	No of bp	Candidates	No of bp	Candidates	No of bp	Candidates	No of bp	Candidates	
Celeste	838	$S_{4'}$	1048	$S_{4'}$	1434	$S_{4'}$	911	$S_{4'}$	492	$S_{4; 616}$	**$S_1S_{4'}$**
	635	$S_{1; 3}$	853	$S_{1; 3}$	1200	S_1	730	$S_{1; 3}$	434	$S_{1; 9; 10}$	
Vega	2245	$S_{2; 5}$	856	$S_{1; 3}$	1104	$S_{3; 9; 10}$	731	$S_{1; 3}$	415	$S_{2; 7; 9; 12}$	**S_3**
	648	$S_{1; 3}$							303	S_3	
Nadino	2289	$S_{2; 5}$	1919	$S_{2; 5}$	1062	$S_{3; 9; 10}$	729	$S_{1; 3}$	452	$S_{1; 5; 10; 14}$	**S_3**
	645	$S_{1; 3}$	882	$S_{1; 3}$					303	S_3	
Merchant	841	S_4	1066	S_4	1466	S_4	911	S_4	500	$S_{4; 6}$	**S_4S_9**
	535	S_9*	775	S_9*	1064	$S_{3; 9; 10}$	623	$S_{9; 10}$	409	$S_{2; 7; 9; 12}$	
Kordia	627	$S_{1; 3}$	931	$S_{1; 3}$	1104	$S_{3; 9; 10}$			506	$S_{4; 6}$	**S_3S_6**
	330	S_6	573	S_6	989	S_6			300	S_3	
Jurga	1208	S?	554	S_6	979	S_6	1299	Sx	508	$S_{4; 6}$	**S_6**
	322	S_6					433	S_6	477	$S_{5; 14; 16}$	
Vytėnų rožinė	613	$S_{1; 3}$	865	$S_{1; 3}$	1086	$S_{3; 9; 10}$	730	$S_{1; 3}$	501	$S_{4; 6}$	**S_3S_6**
	316	S_6	554	S_6	955	S_6	425	S_6	303	S_3	
Vytėnų juodoji	625	$S_{1; 3}$	858	$S_{1; 3}$	1195	S_1	660	$S_{9; 10}$	438	$S_{1; 10}$	**S_1S_9**
	520	S_9*	765	S_9*	1087	$S_{3; 9; 10}$			418	$S_{2; 7; 9; 12}$	
Agila	817	S_4	1048	S_4					510	$S_{4; 6}$	**S_4S_9**
	532	S_9*	795	S_9*					422	$S_{2; 7; 9; 12}$	
Jurgita	520	S_9*	793	S_9*	1070	$S_{3; 9; 10}$			508	$S_{4; 6}$	**S_6S_9**
	310	S_6	573	S_6	962	S_6			421	$S_{2; 7; 9; 12}$	
Vasarė	840	S_4	748	S_9*					425	$S_{2; 7; 9; 12}$	**S_4S_9**
	518	S_9*									
Mindaugė	613	$S_{1; 3}$	880	$S_{1; 3}$	1047	$S_{3; 9; 10}$	728	$S_{1; 3}$	506	$S_{4; 6}$	**S_3S_6**
	309	S_6	578	S_6	968	S_6	430	S_6	303	S_3	
Norta	2310	$S_{2; 5}$	755	S_9*	1050	$S_{3; 9; 10}$	642	$S_{9; 10}$	426	$S_{2; 7; 9; 12}$	**S_9**
	523	S_9*									
Lukė	330	S_6	560	S_6	940	S_6			500	$S_{4; 6}$	**S_6**
									406	$S_{2; 7; 9; 12}$	
Germa	800	S_4	1048	S_4					506	$S_{4; 6}$	**S_4S_9**
	536	S_9*	795	S_9*					420	$S_{2; 7; 9; 12}$	
Seda	810	S_4	875	$S_{1; 3}$	1064	$S_{3; 9; 10}$			520	$S_{4; 6}$	**S_3S_4**
	617	$S_{1; 3}$							303	S_3	
Meda	631	$S_{1; 3}$	875	$S_{1; 3}$	1042	$S_{3; 9; 10}$	735	$S_{1; 3}$	448	$S_{1; 5; 10}$	**S_3**
									303	S_3	
Anta	1216	Sx	560	S_6					500	$S_{4; 6}$	**S_6**
	317	S_6							471	$S_{5; 14; 16}$	
Žemaičių juodoji			957	Sy	1200	S_1	728	$S_{1;3}$	444	$S_{1; 5; 10}$	S_1
			865	$S_{1; 3}$					398	$S_{2; 7; 12}$	
Žemaičių geltonoji	1212	Sx	1418	Sx	1723	Sx	1238	Sx	467	$S_{1; 5; 14; 16}$	**S_3**
	631	$S_{1; 3}$	882	$S_{1; 3}$	1039	$S_{3; 9; 10}$	732	$S_{1; 3}$	303	S_3	
Žemaičių rožinė	618	$S_{1;3}$	882	$S_{1; 3}$	1175	S_1	729	$S_{1; 3}$	440	$S_{1; 5; 10}$	**S_1S_3**
									303	S_3	
Hrebnickio1	1254	Sx							492	$S_{14; 16}$	
									432	$S_{2; 7; 9; 12}$	

* Fragment size of S_9 allele amplifying with these primers (not described in literature).

Table 2. **Determination S alleles in sweet cherry of Lithuanian origin, based on amplification with allele-specific primers**

Accession	Amplification with allele-specific primers														Conclusion
	S_1	S_2	S_3	S_4	$S_{4'}$	S_5	S_6	S_7	S_9	S_{10}	S_{12}	S_{13}	S_{14}	S_{16}	
Celeste	+		–		+		–		–	–				–	$S_1S_{4'}$
Vega	–	+	+			–		–	–	–	–				S_2S_3
Nadino	–	–	+			+			–	–			–		S_3S_5
Merchant		–	–	+	–		–	–	+	–	–				S_4S_9
Kordia	–		+	–			+		–	–					S_3S_6
Jurga				–		–	+						–	+	S_6S_{16}
Norta		+	–			–		–	+	–	–				S_2S_9
Lukė		+		–			+	–	–		–				S_2S_6
Meda	–		+			+			–	–					S_3S_5
Žemaičių juodoji	+	–	–			–		–		–	–	+	–	–	S_1S_{13}
Žemaičių geltonoji	–		+			–			–	–			–	+	S_3S_{16}
Hrebnickio1		–				–		–	–	–	+	–	–	+	$S_{12}S_{16}$
Anta				–		–	+						–	+	S_6S_{16}
Agila				+	–				+						S_4S_9
Vasarė				+	–				+						S_4S_9
Germa				+	–				+						S_4S_9
Seda				+	–										S_3S_4
Vytėnų juodoji	+								+						S_1S_9
Jurgita							+		+						S_6S_9

in the cultivar 'Žemaičių juodoji', and S_{12} was identified in the accession 'Hrebnickio 1'.

Ten different S alleles were identified in Lithuanian sweet cherries (Figure). The frequency of various S alleles partially characterises the level of breeding progress in Lithuania. The S_3, S_9 alleles are more frequently found, and the S_4, S_5 alleles are exclusively found only in cultural sweet cherry cultivars [20]. The frequency of S_3 and S_9 alleles was 17.6% and of S_4 11.9% in the Lithuanian cultivars studied. The S_7, S_{10}, S_{12}, S_{13}, S_{16} alleles are more common in wild sweet cherry populations [20]. The S_7 and S_{10} alleles were not found in any of the Lithuanian cultivars. The S_{13} and S_{16} alleles were identified in land races 'Žemaičių juodoji' and 'Žemaičių geltonoji'. 'Hrebnickio1', which is characterised as very resistant to cold and is intended to be grown as a rootstock, was the only accession possessing both S alleles typical of wild sweet cherries.

Lithuanian sweet cherries were assigned to eleven different self-incompatibility groups from the 38 currently known (Table 3). Two new combinations of S alleles were found – S_1S_{13} ('Žemaičių juodoji') and S_6S_{16} ('Jurga', 'Anta'). The cultivars

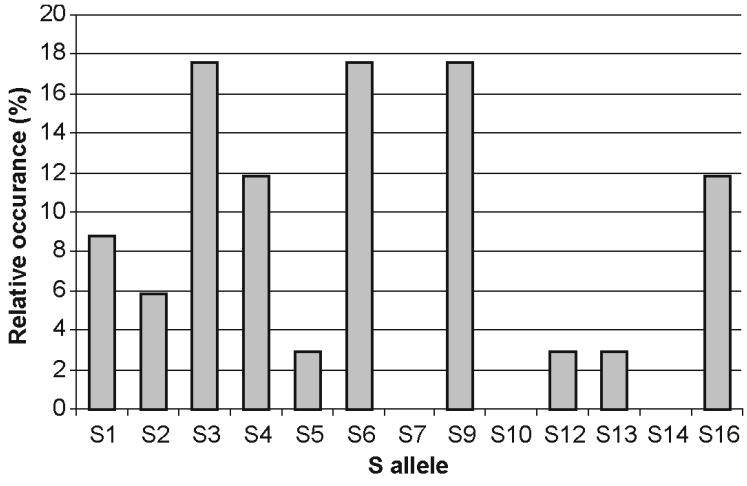

Figure. Comparison of the relative occurrence of S alleles in Lithuanian sweet cherry accessions

Table 3. **Incompatibility groups identified by PCR analysis in the sweet cherry accessions analysed**

Incompatibility group	Allele pair	Variety
II	S_1S_3	Žemaičių rožinė
III	S_3S_4	Seda
VI	S_3S_6	Vytėnų rožinė, Mindaugė
VII	S_3S_5	Meda
X	S_6S_9	Jurgita
XVIII	S_1S_9	Vytėnų juodoji
XXI	S_4S_9	Agila, Vasarė, Germa
XXIII	S_3S_{16}	Žemaičių geltonoji
XXV	S_2S_6	Lukė
XXXVII*	S_6S_{16}	Jurga, Anta
0 (Universal donors in group I–XXXVII)	S_2S_9	Norta
	S_1S_{13}	Žemaičių juodoji
	$S_{12}S_{16}$	Hrebnickio1

* new incompatibility group

'Jurga' and 'Anta' could form a new XXXVII self-incompatibility group. The cultivars 'Žemaičių juodoji' and 'Norta' were assigned to group 0 (Universal donors in group I–XXXVII). Combinations of S alleles in this group are represented by one accession only and thus can currently be regarded as unique genotypes and therefore as universal pollinators.

Received 20 January 2008
Accepted 28 February 2008

References

1. Webster AD. Fundamentals of Temperate Zone Tree Fruit Production. Leiden, Backhuys Publishers, 2005: 1–11.
2. Lukoševičius A. Lietuvos pomologija.Vilnius. Mokslo ir enciklopedijų leidykla, 1996: 59–119.
3. Nettancourt de D. Monographs on Theoretical and Applied Genetics 3. Springer-Verlag, Berlin–Heidelberg–New York, 1977.
4. McClure BA, Haring V, Ebert PR et al. Nature 1989; 342: 955–7.
5. Bošković R, Tobutt KR. Euphytica, 1996; 90: 245–50.
6. Bošković R, Tobutt KR. Theor Appl Genet 2001; 103: 475–85.
7. Tao R, Yamane H, Sugiura A et al. J. Am Soc Hort Sci 1999a; 124: 224–33.
8. Sonneveld T, Robbins TP, Bošković R et al. Theor Appl Genet 2001; 102: 1046–55.
9. Wiersma PA, Wu Z, Zhou L et al. Theor Appl Genet 2001; 102: 700–708.
10. Sonneveld T, Tobutt KR, Robbins TP. Theor Appl Genet 2003; 107: 1057–70.
11. Tao R, Yamane H, Akira H. Plant Physiol 1999b; 121: 1057.
12. Yamane H, Tao R, Murayama H. J Hort Sci Biotechnol 2000; 75: 562–7.
13. Wünsch A, Hormaza JI. Theor Appl Genet 2004a; 108: 299–305.
14. Schuster M, Flachowsky H, Köhler D. Plant Breeding 2007; 126: 533–40.
15. Schmidt H, Wolfram B, Bo ković R. Erwerbsobstbau 1999; 41: 42–5.
16. Doyle JJ, Doyle JL. Focus 1990; 12(1): 13–5.
17. Sutherland BG, Robbins TP, Tobutt KR. Plant Breeding 2004; 123: 582– 4.
18. Zhu Mo, Xiaoming Zhang, Kaichun Zhang et al. Plant Mol Biol Rep 2004; 22: 387–98.
19. Wünsch A, Hormaza JI. Plant Breeding 2004b; 123: 327–31.
20. De Cuyper B, Sonneveld T, Tobutt KR. Molecular Ecology 2005; 14: 945–55.

Vidmantas Stanys, Rugilė Stanytė,
Gražina Stanienė, Jurgita Vinskienė

S ALELIŲ IDENTIFIKAVIMAS PGR METODU LIETUVIŠKOSE TREŠNĖSE

S a n t r a u k a

Panaudojant polimerazinę grandininę reakciją (PGR) nustatyti S aleliai 17-oje lietuviškos kilmės ir 5 standartinėse trešnių veislėse. Iš pradžių DNR buvo pagausinta S aleliams identifikuoti naudojant bendruosius pradmenis. Šie pradmenys pagausina DNR ties pirmuoju, antruoju arba ties abiem S-ribonukleazės geno intronais, kur stebimas fragmentų ilgio polimorfizmas. Kadangi DNR pagausinimo produktų dydis dažnai buvo panašus, visi sunkiai identifikuojami pavyzdžiai vėliau buvo ištirti su S aleliams specifiniais pradmenimis. Nustatyti visų tirtų pavyzdžių S aleliai. Tyrimo rezultatai leido priskirti lietuviškas trešnes dešimčiai savinesuderinamumo grupių. Buvo pasiūlyta papildoma savinesuderinamumo grupė. Identifikuotos iki šiol neaptiktos S alelių kombinacijos: S_1S_{13} ('Žemaičių juodoji') ir S_6S_{16} ('Jurga', 'Anta').