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Assessment of Genetic Diversity in Differently Colored Raspberry Cultivars Using SSR Markers Located in Flavonoid Biosynthesis Genes

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Abstract: Raspberry is a valuable berry crop containing a large amount of antioxidants that correlates with the color of the berries. We evaluated the genetic diversity of differently colored raspberry cultivars by the microsatellite markers developed using the flavonoid biosynthesis structural and regulatory genes. Among nine tested markers, seven were polymorphic. In total, 26 alleles were found at seven loci in 19 red (*Rubus idaeus* L.) and two black (*R. occidentalis* L.) raspberry cultivars. The most polymorphic marker was *RiMY01* located in the MYB10 transcription factor intron region. Its polymorphic information content (PIC) equalled 0.82. The *RiG001* marker that previously failed to amplify in blackberry also failed in black raspberry. The raspberry cultivar clustering in the UPGMA dendrogram was unrelated to geographical and genetic origin, but significantly correlated with the color of berries. The black raspberry cultivars had a higher homozygosity and clustered separately from other cultivars, while at the same time they differed from each other. In addition, some of the raspberry cultivars with a yellow-orange color of berries formed a separate cluster. This suggests that there may be not a single genetic mechanism for the formation of yellow-orange berries. The data obtained can be used prospectively in future breeding programs to improve the nutritional qualities of raspberry fruits.

Keywords: flavonoid biosynthesis; fruit coloration; marker-assisted selection; microsatellites; *Rubus*

1. Introduction

The genus *Rubus* L. (Rosaceae, Rosoideae) is one of the most diverse in the plant kingdom and contains between 600 and 800 species grouped in 12 subgenera, which are widely distributed throughout the world from the lowland tropics to subarctic regions [1]. Among these species, red

raspberry (*Rubus idaeus* L.) and blackberry (several species in the genus *Rubus*) grown world-wide, and black raspberry (*R. occidentalis* L.) grown mainly in the United States, are of the greatest economic importance. Their berries are in great demand due to their flavor, color, and taste. In addition, they are very healthy providing a good source of antioxidants, including phenolic acids, flavonoids, anthocyanins, and carotenoids [2]. Berries contain four times more antioxidants than non-berry fruits, 10 times more than vegetables, and 40 times than cereals [3]. For this reason, berries and their products (i.e., berry juice and jam) are very often recognized as "superfoods" [4]. The popularity of this crop can be indicated by the fact that their harvest increased 1.5 times from 2010 to 2017 worldwide and exceeded 800,000 tons [5]. Russia consistently ranks first in the world for the raspberry production. The growing interest in raspberry has led not only to an increase in its production, but also to the expansion of breeding programs for the development of new cultivars. However, classical selection takes a lot of time: in red raspberry, it can take up to 15 years for development and release of a new cultivar [6]. Moreover, a specific feature in the *Rubus* spp. breeding system is that multiple species are often utilized in breeding programs [7]. Scientific achievements in molecular biology, and use of molecular markers, in particular, can accelerate the selection process, as they will allow for the assessment of the seedlings with valuable traits at a much earlier stage. Molecular genetic markers provide more reliable cultivar identification of *Rubus* species than morphological markers [8].

In order to speed up the breeding process, it is useful to have genetic linkage maps containing information about the markers associated with the most important traits, including disease and pest resistance, plant habitus, nutritional and sensory fruit quality, and plant architecture. The first genetic linkage map of *Rubus* was constructed from a cross between two *Rubus* subspecies, *R. idaeus* (cv. Glen Moy) × *R strigosus* (cv. Latham), in 2004 [9]. After that, other molecular maps for red raspberry [10–12], black raspberry [13] and tetraploid blackberry [14] appeared. Quantitative trait loci (QTL) have been identified for important traits including resistance to diseases [11,15] and pests [10], fruit anthocyanin content [16], growth characteristics [10,17], fruit color and quality traits [18]. Currently, molecular markers are routinely used in breeding raspberries for resistance to the Phytophthora root rot at the James Hutton Institute (UK), and two promising genotypes are under commercial trials, as well as markers for the quality of berries are in the process of validation [19]. If in the first reports a combination of various types of molecular markers such as AFLP and simple sequence repeat (SSR) [9,10], RAPD, and RGAP [11] were used, then the most recent molecular maps were produced using only molecular markers designed from sequenced DNA such as microsatellites or SSR markers [13,20]. SSRs are DNA tandem repeats of the 1–6 nucleotide long motifs that are very frequent in genomes. They are very polymorphic with high information content, co-dominant inheritance, locus specificity, extensive genome coverage and simple detection using labelled primers that flank the microsatellite [9,21], and their ability to distinguish even closely related individuals is particularly important for many crop species [21]. Raspberry researchers have noted the benefits of the SSR markers, but very few molecular markers still exist for *Rubus* [7,22]. It should be also acknowledged that the breeding process can be accelerated using genomic selection (e.g., [23]), an approach under rapid adoption in many species, which is based on multiple marker–trait associations and does not require linkage maps.

The color of the berries not only affects their attractiveness but also serves as an indicator of the content of biologically active compounds. For example, the content of anthocyanins in raspberry berries varies widely from 2 to 325 mg/100 g depending on the color of the berries [24]. Flavonols and anthocyanins are synthesized in the flavonoid pathway, and its enzymes are well characterized. Kassim et al. [16] mapped QTLs for individual anthocyanin pigments in raspberry. The genes of various enzymes of flavonoid biosynthesis were also identified in red [18] and black [25] raspberry and blackberry [26]. Besides the structural genes, regulatory genes are important in the biosynthesis of flavonoids. The late flavonoid biosynthetic genes are activated by the ternary transcriptional MYB-bHLH-WD40 (MBW) complex comprising three classes of regulatory proteins including R2R3-MYBs, bHLHs, and TTG1 (WD40) [27]. Transcription factor genes, such as *MYB10*, *bHLH* and *bZIP*, have also been identified in the *Rubus* species [18,26].

There are several studies that used random genomic SSR markers to assess genetic diversity in cultivars within [8,28] and between [29] different species. However, we are unaware of studies in which genetic diversity would be assessed using markers located in genes of any metabolic pathway and the biosynthesis of flavonoids, in particular. In this study, we developed SSR markers using nucleotide sequences of structural and regulatory genes of flavonoid biosynthesis in *Rubus* and *Fragaria* (strawberry) available at the National Center for Biotechnology Information (NCBI) GenBank database to test whether genetic variation associated with these genes correlate with a variation of berry colors. These markers were genotyped in 19 raspberry cultivars from different geographic regions (Russia, Poland, Italy, Switzerland, UK, and USA) and two cultivars of black raspberry. If alleles at these loci correlate with the content of biologically active substances, they could subsequently be used to optimize selection for valuable traits associated with color and, indirectly, with the content of flavonoids, by accelerating selection via screening genotypes at early stages.

2. Materials and Methods

2.1. Plant Materials

Nineteen cultivars of red raspberry (Amira, Anne, Babye Leto II, Beglyanka, Brilliantovaya, Bryanskoe Divo, Gerakl, Glen Ample, Marosejka, Meteor, Oranzhevoe Chudo, Pingvin, Polka, Poranna Rosa, Solnyshko, Sugana, Tarusa, Zheltyj Gigant, and Zolotaya Osen) and two cultivars of black raspberry (Cumberland and Jewel) were chosen to genotype SSR loci located in the flavonoid biosynthesis genes. These cultivars have a wide range of fruit color from yellow to black with various geographic and genetic origins, but cultivars of Russian origin from two raspberry breeding centers (Bryansk and Moscow) dominated in the list (Table 1). Raspberry plants used in this study were kindly provided by Dr. I. A. Pozdniakov (OOO Microklon, Pushchino, Russia). Each cultivar represented a microclonally vegetatively propagated line containing practically genetically identical plants. Therefore, a single specimen per culture was used for further DNA isolation and genotyping.

Table 1. Parentage and fruit color of the *Rubus* cultivars used in the study.

Cultivar	Abbr.	Genetic Origin and Background	Fruit Color	Origin
		R. idaeus (red raspberry)		
Amira	Ami	Polka × Tulameen	red	Italy
Anne	Ann	Amity × Glenn Garry	yellow	USA
Babye Leto II	BL2	Autumn Bliss × Babye Leto	red	Russia (Bryansk)
Beglyanka	Beg	Kostinbrodskaya × Novost Kuzmina	orange	Russia (Bryansk)
Brilliantovaya	Bri	open pollination of interspecific hybrids	red	Russia (Bryansk)
Bryanskoe Divo	BrD	47-18-4 (open pollination)	light-red	Russia (Bryansk)
Gerakl	Ger	Autumn Bliss × 14-205-4	red	Russia (Bryansk)
Glen Ample	GAm	SCRI7326EI × SCRI7412H16	dark red	UK
Marosejka	Mar	7324/50 × 7331/3	light-red	Russia (Moscow)
Meteor	Met	Kostinbrodskaya × Novost Kuzmina	red	Russia (Bryansk)
Oranzhevoe Chudo	OrC	Shapka Monomaha (open pollination)	orange	Russia (Bryansk)
Pingvin	Pin	interspecific hybrid	dark red	Russia (Bryansk)
Polka	Pol	P89141(open pollination)	red	Poland
Poranna Rosa	PoR	83291 × ORUS 1098-1	yellow	Poland
Solnyshko	Sol	Kostinbrodskaya × Novost Kuzmina	red	Russia (Bryansk)
Sugana	Sug	Autumn Bliss × Tulameen	light-red	Switzerland
Tarusa	Tar	Stolichnaya × Shtambovyj-1	red	Russia (Moscow)
Zheltyj Gigant	ZhG	Marosejka × Ivanovskaya	yellow	Russia (Moscow)
Zolotaya Osen	ZOs	13-39-11 (open pollination)	yellow	Russia (Bryansk)
		R. occidentalis (black raspberry)		
Cumberland	Cum	Gregg selfed	blue-black	USA
Jewel	Jew	(Bristol × Dundee) × Dundee	black	USA

2.2. Simple Sequence Repeat (SSR) Marker and Polymerase Chain Reaction (PCR) Primer Development

The WebSat software [30] was used to detect SSR loci in the nucleotide sequences of *Rubus* and *Fragaria* × *ananassa* (the garden strawberry or simply strawberry, a widely grown hybrid species of the genus *Fragaria*) flavonoid biosynthesis genes available at the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) (Table 2). The Primer 3 software (http://primer3.org) was used to design appropriate polymerase chain reaction (PCR) primers based on the sequences flanking the SSR loci. The minimum number of motifs used to select the SSR locus was nine for mono-nucleotide repeats, five for di-nucleotide motifs, three for tri-, and tetra-, and two for penta-, and hexa-nucleotide repeats. Primers were designed using the following criteria: primer length of 18–27 bp (optimally 22 bp), GC content of 40%–80%, annealing temperature of 57–68 °C (optimally 60 °C), and expected amplified product size of 100–400 bp. Primers for the *RiG001* locus were as in [8]. Primers were synthesized by Syntol Company (Moscow, Russia) and are summarized in Table 2.

2.3. DNA Isolation, PCR Amplification and Fragment Analysis

A single DNA sample per each cultivar was produced from young expanding leaves representing a single plant per each cultivar. Total genomic DNA was extracted using the STAB method [31]. The quality and quantity of extracted DNA were determined by the NanoDrop 2000 spectrophotometer (ThermoFisher). The final concentration of each DNA sample was adjusted to 50 ng/μL in TE buffer before the PCR amplification.

For genotyping, PCR was performed separately for each primer pair using a forward primer labeled with the fluorescent dye 6-FAM and an unlabeled reverse primer (Syntol, Russia). The PCR amplification was performed in a total volume of 20 μL consisted of 50 ng of genomic DNA, 10 pmol of the labeled forward primer, 10 pmol of an unlabeled reverse primer, and PCR Mixture Screenmix (Eurogen, Russia). After an initial denaturation at 95 °C for 3 min, DNA was amplified during 33 cycles in a gradient thermal cycler (Bio-Rad, Hercules, CA, USA) programmed for a 30 s denaturation step at 95 °C, a 20 s annealing step at the optimal annealing temperature of the primer pair and a 35 s extension step at 72 °C. A final extension step was done at 72 °C for 5 min.

The PCR generating clear, stable, and specific DNA fragments within an expected length (200–400 bp) were considered as successful PCR amplifications. If a primer pair failed three times to amplify template DNA that was amplified with other primers, then it was scored as a null genotype.

Separation of amplified DNA fragments was performed in an ABI 3130xl Genetic Analyzer using S450 LIZ size standard (Syntol Company, Moscow, Russia). Peak identification and fragment sizing were done using the Gene Mapper v4.0 software (Applied Biosystems, Foster, CA, USA).

2.4. Genetic Data Analysis

Genetic parameters were calculated for 21 raspberry cultivars based on seven SSR polymorphic loci. The allele frequencies, number of alleles, observed (H_o) and expected (H_e) heterozygosities, and polymorphic information content (PIC) were calculated using the PowerMarker v.3.25 software [32]. This software was also used to estimate pairwise Nei's standard genetic distances between each pair of cultivars and to generate a UPGMA dendrogram, which was visualized using the Statistica software (TIBCO Software Inc., Palo Alto, CA, USA).

Table 2. Data on nine simple sequence repeat (SSR) loci located in the flavonoid biosynthesis genes and their polymerase chain reaction (PCR) primer pairs used to study genetic diversity in *Rubus* cultivars.

Locus	Gene	Species	NCBI GenBank Accession Number	Motif and Number of Repeats	Location in the Gene	PCR Primer Nucleotide Sequence		T *, °C	Allele Size, bp		
									Expected	Observed	
						Forward	Reverse			Red Raspberry	Black Raspberry
RiG001	aromatic polyketide synthase (PKS3)	*R. idaeus*	AF292369	$(AT)_6$	intron	TGTCCGATCCTTTTC TTTGG	CGCTTCTTGATCCTT GACTTGT	55	345	349, 350, 351	0
RcFH01	flavanone-3-hydroxylase (F3H)	*R. coreanus*	EU255776	$(TATG)_3$	intron	GGTCCAAGTGCATTCCA TATTAC	GTTCTTGAATCTCCC GTTGCT	60	262	255, 265, 271	255, 271
FaFS01	flavonol synthase (FLS)	*Fragaria* × *ananassa*	DQ834905	$(CT)_{12}$	intron	CATCCCTAATGCCCTA GTCATC	TGTACTTCGGTGGA TTCTCCTT	60	304	323, 328	323
FaFS02	flavonol synthase (FLS)	*F. ananassa*	DQ834905	$(GGAAG)_2$	exon	AAGCTCCTCAAACAA ATCTTCG	GTAGTTAATGGCAGAA GGTGGC	60	273	255, 271	271
RiAS01	anthocyanidin synthase (ANS)	*R. idaeus*	KX950789	$(ATCTC)_2$	exon	TCAACAAGGAGAAGGT GAGGAT	CCGTTAGGAGAGATGAA AGCAG	60	334	309, 333, 358	309
FaAR01 **	anthocyanidin reductase (ANR)	*F. ananassa*	DQ664193	$(TGCTG)_2$ $(CATTT)_2$	exon intron	AATCTGCTTCTGGTCG GTACAT	AGAGAGTATGGTCTTC GCCTTG	60	244	250	250
RhUF01 **	UDP-glucose flavonoid 3-O-glycosyltransferase-like protein (UFGT)	*R. hybrid*	JF764808	$(GAG)_7$ $(ACAAGC)_2$	exon	AGGAGCTGAAGAAAAG ACTCCA	AAAGTCCTCTAGGTT TCCCCTG	60	275	267, 270	269, 270
RiMY01 **	transcription factor MYB10	*R. idaeus*	EU155165	$(TAATA)_2$ $(CT)_7$ $(AT)_{15}$	introns	GTTCCTCTCCAAGCA GGTTATT	TGCAAAGTCTCCTCTC TTGATG	59	330	323, 325, 327, 329, 331, 333, 341, 342	358
RiTT01	transparent testa glabra 1 (TTG1) protein	*R. idaeus*	HM579852	$(CAC)_5$	exon	ACTCCACACAAGAATC CCATCT	CTGTTGTTCAAGACCG AAATTG	60	379	379	379

* Optimal annealing temperature. ** Two or three SSRs in these loci were amplified simultaneously by a single pair of primers.

3. Results

3.1. Polymorphism and Genetic Diversity Analysis

Nine SSR markers (six based on *Rubus* and three on *Fragaria* nucleotide sequences of the flavonoid biosynthesis genes) were used to estimated genetic diversity in 19 raspberry (*R. idaeus*) and two black raspberry (*R. occidentalis*) cultivars. All PCR primer pairs amplified one or two alleles. In raspberries, two loci (*RiTT01* and *FaAR01*) were monomorphic, and other seven were polymorphic. In black raspberry cultivars, the *RiG001* was not amplified at all, six loci were monomorphic and only two polymorphic (Table 2). In total, 26 alleles were found in seven polymorphic microsatellite loci. The number of alleles per locus varied from two per locus (*FaFS02* and *FaFL01*) to nine per locus (*RiMY01*) with an average number of 3.7 alleles per locus (Table 3). The *RiMY01* locus was the most polymorphic. In general, the SSR loci located in introns were more polymorphic than loci in exons.

Table 3. Parameters of genetic variation for seven polymorphic SSR loci in 21 *Rubus* cultivars.

Locus	Location in the Gene	Major Allele Frequency	Number of Alleles	Heterozygosity		Polymorphism Information Content (PIC)
				Expected (H_e)	Observed (H_o)	
RiG001	intron	0.81	4	0.33	0.19	0.31
RcFH01	intron	0.74	3	0.41	0.52	0.35
FaFS01	intron	0.76	2	0.36	0.48	0.30
FaFS02	exon	0.98	2	0.05	0.05	0.05
RiAS01	exon	0.79	3	0.36	0.19	0.33
RhUF01	exon	0.90	3	0.18	0.00	0.17
RiMY01	introns	0.29	9	0.84	0.57	0.82
Mean		0.75	3.71	0.36	0.29	0.33

There were cultivar-specific alleles, such as a unique allele 358 at the *RiMY01* locus found only in black raspberry, and alleles 267 and 269 at the *RhUF01* locus found only in the red raspberry Meteor and Jewel cultivars, respectively. Meteor contained also a unique allele 333 at the *RiMY01* locus.

Parameters of genetic variation for seven polymorphic SSR loci in 21 *Rubus* cultivars are presented in Table 3. Expected heterozygosity (H_e) ranged from 0.05 in the *RiMY01* locus up to 0.84 in the *RiMY01* locus with an average value of 0.36. Observed heterozygosity was zero in the *RhUF01* locus and ranged from 0.05 in the *FaFS02* locus to 0.57 in the *RiMY01* locus with an average value of 0.29. The observed heterozygosity was lower than expected in four microsatellite loci and on average (Table 3). On average, the expected and observed heterozygosities were higher for the SSRs in introns (0.49 and 0.44, respectively) compared to the SSRs in exons (0.20 and 0.08, respectively). The average PIC was 0.332 and varied from 0.05 in the *FaFS02* locus to 0.82 in the *RiMY01* locus (Table 3).

3.2. Cluster Analysis

A UPGMA dendrogram was constructed for 21 raspberry cultivars based on seven SSR markers located in the genes of the flavonoid biosynthesis (Figure 1). The dendrogram clearly separates red and black raspberries. Among the red raspberry cultivars, there is a group of cultivars with yellow-orange colored berries (Anne, Poranna Rosa, Orangevoe Chudo, and Zolotaya Osen), which forms a separate cluster. The same group includes also the Bryanskoe Divo cultivar with light red berries. At the same time, the Zheltyj Gigant (yellow berries) and Beglyanka (orange berries) were not included in this group. Separation of cultivars did not follow their genetic origin. The cultivars Beglyanka, Solnyshko, and Meteor having the same genetic origin from the Kostinbrodskaya × Novost Kuzmina cross were completely separated from each other. In addition, the Babye Leto 2 also having an ancestral hybrid (Autumn Bliss × (September × (Kostinbrodskaya × Novost Kuzmina))) turned out to differ mostly from other raspberry cultivars. Gerakl and Sugana both also having Autumn Bliss as their parent species

were significantly separated. At the same time, close similarities have been observed for cultivars from different geographic regions. No genetic differences were found between the Orangevoe Chudo (Russia) and Poranna Rosa (Poland) cultivars, and between the Amira (Italy) and Tarusa (Russia) cultivars, although they have different genetic origins. The Brilliantovaya and Pingvin cultivars were also identical and were obtained with the use of interspecific hybrids.

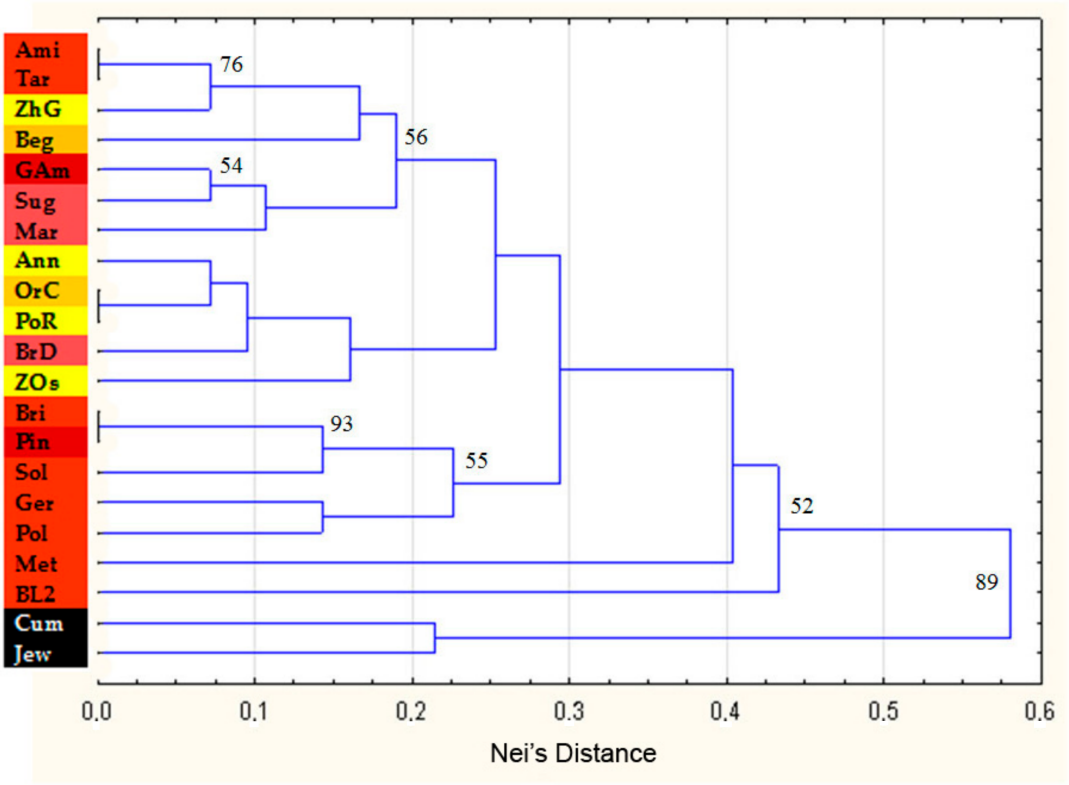

Figure 1. The UPGMA dendrogram of the 21 *Rubus* cultivars based on pairwise Nei's standard genetic distances calculated using seven SSR markers located in the flavonoid biosynthesis genes. Left column shows the colors of the cultivar berries. Only bootstrap values larger than 50% are presented. See Table 1 for the full cultivar names.

4. Discussion

SSR markers (microsatellites) are widely used in genetic diversity studies, QTL and genetic mapping, molecular-assisted selection (MAS), and cultivar identification, because they are multi-allelic, co-dominant, highly informative, relatively accurate and easily detected [33]. SSR markers have been often used to map different types of *Rubus* [9,13], fingerprinting germplasm [34], and in studies of the genetic diversity and population structure within [28] and among [29] *Rubus* species. However, genetic diversity has not previously been studied in terms of any specific metabolic pathway genes that determine valuable breeding traits.

In this study, we report on the evaluation of a number of red and black raspberry cultivars using SSR loci representing known sequences of the flavonoid biosynthesis pathway genes, which synthesize biologically active substances with high antioxidant activity—flavonols and anthocyanins. Among these microsatellite loci, six (*RcFH01*, *FaFS01*, *FaFS02*, *RiAS01*, *FaAR01*, and *RhUF01*) were located in the structural genes of the flavonoid biosynthesis (*F3H*, *FLS*, *ANS*, *ANR*, and *UFGT*) and two (*RiMY01* and *RiTT01*) in the regulatory genes (*MYB10* and *TTG1*). Flavanone-3-hydroxylase (F3H) is a key enzyme in the flavonoid biosynthesis in plants, as it catalyzes formation of 3-hydroxy flavonol, a common precursor of anthocyanins, flavanols, and proanthocyanidins [35]. Particular attention was paid to the flavonol synthase gene, for which two loci were used. Flavonol synthase (FLS) is an important enzyme

of flavonoid pathway that catalyzes the formation of flavonols from dihydroflavonols, and thus may influence anthocyanin levels, as dihydroflavonols are intermediates in the production of both colored anthocyanins and colorless flavonols [36]. The anthocyanidin synthase (ANS) leads to the synthesis of the anthocyanidin, the first colored compound in the anthocyanin biosynthetic pathway, from which anthocyanidin reductase catalyzes the formation of proanthocyanidins (condensed tannins) [37]. The last common step for the production of stable anthocyanins is the glycosylation by the enzyme UDP-glucose/flavonoid 3-O-glucosyl transferase (UFGT) [38].

In addition, loci were used on the sequence of two transcription factors (MYB 10 and TTG1) that belong to the MBW complex, which regulates the production of the late biosynthetic genes [27]. For comparison, we also used a pair of primers designed for the *RiG001* locus using the sequence of the *R. idaeus* aromatic polyketide synthase (*PiPKS3*) gene, which was not amplified in blackberry cultivars [8]. The *RiPKS3* gene differed from the *RiPKS1* gene, encoding a typical chalcone synthase (CHS) catalyzing the first step of flavonoid biosynthesis, in four amino acid positions and produced in vitro predominantly p-coumaryltriacetic acid lactone and low levels of chalcone [39]. Within the PCR fragment amplified by the primers for the *RiG001* locus the sequence of the *RiPKS3* gene (NCBI GenBank AF292369) differed from the *RiPKS1* gene sequence (AF292367) by a two nucleotide long deletion (2 bp) and a single nucleotide insertion. Three alleles (349, 350, and 351 bp) were obtained for this locus (Table 2).

In addition to the sequences of the genes of the *Rubus* plants (*R. idaeus*, *R. coreanus*, and *R. hybrid*), we used the sequences of the genes from *Fragaria* × *ananassa*, which is a close relative of *Rubus* from the same sub-family, Rosoideae. The *Rubus* and *Fragaria* both have the same base chromosome number $1n = 7$, similar morphology and chloroplast and nuclear DNA phylogenies [13].

Among three most economically important types of raspberry, 19 cultivars of red raspberry with a wide range of berry color from various world breeding centers and two cultivars of black raspberry are mostly used. Both species, red (*R. idaeus*) and black (*R. occidentalis*) raspberry belong to the same subgenus *Idaeobatus* (raspberries) and are diploids ($2n = 2x = 14$), while blackberry species vary greatly in ploidy [34].

In our study, the average number of alleles for seven polymorphic SSR loci in the flavonoid biosynthesis genes was 3.71, the mean H_o and H_e were 0.286 and 0.360, respectively, and the mean PIC was 0.332. These values were generally lower than previously reported for *R. idaeus* [8] and *R. coreanus* [29], but quite comparable with the data for black raspberry cultivars [28]. Perhaps, this is due to the fact that red raspberry cultivars are, for the most part, complex hybrids with a limited genetic pool [34], and the selection for berries quality has further reduced their diversity. The level of expected heterozygosity (H_e) was higher than observed (H_o) both on average and in most individual loci. These data are different from other studies of the *Rubus* species, where these parameters were approximately equal [8,29], or even higher [28]. However, unlike those studies, where population samples were used, a collection of different cultures was used in this study, which is not a population sample, but a mixture of genotypes with different genetic background and origin. Therefore, it is expected to observe excess of expected heterozygosity in comparison to observed heterozygosity due to Wahlund effect.

Only the *RiMY01* locus was highly polymorphic (PIC = 0.82). This locus had three SSR regions, two of which represent dinucleotide repeats. These data coincide with the results of Castillo et al. [8], in which all three highly informative markers (PIC = 0.78–0.82) represented dinucleotide repeats. In *R. coreanus*, among five highly polymorphic markers (PIC > 0.7), four represented dinucleotide repeats, and one trinucleotide repeats [29]. The high variation of the *RiMY01* locus can be explained by its location in the first intron of the transcription factor MYB10. SSR markers located in introns were more variable in comparison to those located in exons (expected and observed heterozygosities averaged 0.49 and 0.44 vs. 0.20 and 0.08, respectively). Our results are in agreement with those of Garcia-Gomez et al. [40], which showed that SSRs in introns had a higher level of heterozygosity compared to SSRs in exons in Prunus species—0.65 vs. 0.17, respectively. Similar results were also

obtained in maize [41]. Significantly higher variation was observed also for SNPs in noncoding regions compared to coding ones [42]. In general, introns are more variable than exons, as they are under less selection pressure during the evolutionary process [43].

The length of most alleles at the *RiMY01* locus differ from each other by two nucleotide-long steps, which is consistent with dinucleotide repeats of the SSR motifs in this locus. However, imperfect repeats also often occur in the raspberry SSR loci. For instance, Fernandez et al. [34] has previously reported the alleles with length different by consecutive one nucleotide-long steps in the *Rubus57a* and *Rub5a* markers. This single nucleotide stepwise variation is expected for *Rub5a*, which is a SSR marker with a mononucleotide motif, but *Rubus57a* is a SSR marker with a dinucleotide motif. We also observed a few alleles with imperfect repeats, such as the unique allele 267 of the *RhUF01* locus in the Meteor cultivar, for which the perfect allele size is 270 following the trinucleotide motif GAG stepwise allelic variation.

The black raspberry cultivars were highly homozygous: six out of eight loci were monomorphic (Table 2). High homozygous in black raspberry has been also found earlier by Lewers and Weber [44]. They noticed that the level of homozygosity for the black raspberry was 80%, but only 40% for the red raspberry. The 21 SSR loci were unable to distinguish between six of the black raspberry cultivars [28]. However, the black raspberry cultivars Cumberland and Jewel were well discriminated in this study. Despite the small number of loci used in our study, these two cultivars were also separated by two loci: *RcFH01* and *RhUF01*. In our study the red raspberry cultivars were easily discriminated from the black raspberry cultivars by a unique black raspberry specific allele 358 at the *RiMY01* locus and the allele 309 at the *RiAS01* locus, which occurred almost exclusively in the black raspberry cultivars, except the red raspberry cultivar Babye Leto 2. In addition, the *RiG001* locus was not amplified in black raspberry. The same was observed also in 48 earlier tested blackberry cultivars [8]. Thus, in respect to this locus, the black raspberry is closer to the wild blackberry than to the red raspberry, although it belongs to different subgenera. No amplification of RiG001 and the unique allele 358 at the *RiMY01* locus can be used to separate the red raspberry cultivars from the black ones.

Cluster analysis of the SSR markers located in the genes of the biosynthesis of flavonoids showed a clear separation of the black raspberry (*R. occidentalis*) cultivars with black colored berries from the red raspberry (*R. idaeus*) cultivars with berries colored from yellow to dark red (Figure 1). It is important to note also that five cultivars with berries of similar shades of light red color (three with yellow berries, one with orange, and another with light red color) having completely different origin still clustered together into one sub-group. Perhaps, gene-targeted markers [45] such as SSR loci in the genes of the biosynthesis of flavonoids reflect better their genetic similarity for traits, such as color of their berries, likely controlled or affected by these genes, than random genomic SSR markers.

Castillo et al. [8] found that the primocane fruiting (fall fruiting) raspberry cultivars were grouped into a separate cluster. In Fernandez et al. [34] studies, it was shown that the majority of primocane-fruiting material from various breeding programs, as well as some very early ripening floricane-fruiting genotypes are grouped into one cluster. This shows that cultivars can be grouped according to a particular trait regardless of their origin. At the same time, two cultivars with yellow and orange-colored fruits (Zheltyj Gigant and Beglyanka) fell into another group of red-colored fruits. Perhaps, for a clearer separation, it is necessary to use additionally more polymorphic markers, including other genes of the biosynthesis of flavonoids not represented in this study.

Moreover, it is possible that the yellow color of the raspberry fruits can be obtained by two or more mechanisms. For example, primocane fruiting cultivars were also distributed in two different groups [34]. The genetic mechanisms for the formation of yellow color in raspberry fruit have not yet been fully studied. Although assumptions on this topic were made back in the 1930s, it was not until 2016 when an inactive anthocyanidin synthase (ANS) allele was identified in yellow raspberry [46]. A 5 bp insertion in the coding region of gene creates a premature stop codon resulting in a truncated amino acid sequence of the defective ANS protein. However, other mechanisms are also possible, such

as the combinations of recessive and dominant alleles, or the transcription factors that may lead to a huge variety of berry colors in raspberry.

The clustering along the flavonoid pathway also showed that there is a lack of connections between cultivars of the related origin. This is exactly the opposite data compared to the analyses carried out on randomly selected SSR markers evenly distributed across the genome. For example, Fernandez et al. [34] demonstrated that one cluster is almost entirely composed of cultivars from the Scottish raspberry breeding program or cultivars based on their germplasm. From the point of view of MAS the use of gene-targeted markers to assess genotypes for particular breeding traits is preferable to the use of random SSR markers. Graham et al. [9] suggested in 2004 that *Rubus idaeus* due to the diploid set of chromosomes ($2n = 2x = 14$) and a very small genome (275 Mb) may be used as a model species for the Rosaceae. For many years, this was impeded by the lack of the full-genome *Rubus* sequence, although the genomes of other Rosaceae species have been already sequenced, such as apple in 2010, strawberry in 2011, pear and peach in 2013 [47]. However, the situation is changing with genomes of *R. occidentalis* [48] and *R. idaeus* [49] having been recently published. This will facilitate developing gene-targeted markers that can advance breeding *Rubus* for important traits including those related to the nutritional value of their berries.

5. Conclusions

In this study, we demonstrated that a set of gene-targeted SSR markers representing structural and regulatory genes of flavonoid biosynthesis could potentially allow more informative and meaningful evaluation of the genetic relationship between different cultivars of red and black raspberries that reflect the color of their berries and possibly also their nutritional value. However, the study did not compare this set of gene-targeted markers with an analysis of the same germplasm set using neutral markers. A comparative analysis using a set of neutral SSR markers would seem to be important to support this particular conclusion. The developed primer set can be potentially used for MAS in the *Rubus* breeding programs for improving the nutritional quality of fruits. This first requires confirmation that the SSR alleles identified correlate with differences in the content of flavonoids. Additional studies and further development of these gene-targeted markers are needed to validate this approach.

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