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Screening of Apple Cultivars for Scab Resistance in Kazakhstan

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Abstract: Scab, caused by *Venturia inaequalis*, is the most destructive fungal disease of apple worldwide. Apple scab incidence was studied in apple orchards in the south and southeast of Kazakhstan, including the Almaty, Zhambyl, and Turkestan regions, during 2022 and 2023. Disease incidence was higher in the Zhambyl region than in the Turkestan and Almaty regions in both years. The field evaluation suggested that 19 genotypes showed resistance to apple scab. Molecular screening was carried out using eight gene-specific molecular markers (AM19, CH05e03, OPL19, Hi07f02, AL07, K08, HB09, and CH02f06). The results of the molecular screening revealed that in 38 of the 45 studied cultivars, which included 11 Kazakh cultivars and 34 foreign cultivars, the *Rvi* (*Rvi2*, *Rvi4*, *Rvi5*, *Rvi6*, *Rvi8*, *Rvi9*, *Rvi11*, *Rvi14*, and *Rvi15*) resistance genes were amplified. Resistance genes such as *Rvi2*, *Rvi4*, *Rvi6*, and *Rvi9* are still useful for breeding, but their use is recommended only in extended pyramids of multiple resistance genes. Several cultivars will be strong candidates for further breeding programs against apple scab and for the pyramiding of scab resistance genes in new cultivars.

Keywords: apple scab; *Venturia inaequalis*; resistance genes; molecular marker

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1. Introduction

Apples (*Malus domestica* L.) are among the most important fruit crops grown worldwide. The apple cultivation area in Kazakhstan is 35.7 thousand hectares, or 75% of the total field area used for the production of stone and pome fruit crops [1]. The southern and southeastern regions of Kazakhstan have the most favorable climatic conditions for growing fruit crops, in particular apple trees. To enhance the economic efficiency of apple cultivation, a critical factor is the expansion of resistant cultivars. Resistant apple cultivars fulfill requirements such as productivity, fruit quality, and resistance to diseases for sustainable agriculture production [2].

In many regions of Kazakhstan, apple scab, caused by the ascomycete fungus *Venturia inaequalis*, is the most serious disease of apple [3]. Temperate regions with humid climates are highly favorable to this disease. In cases of severe infection, production losses of up to 70% have been reported [4,5]. Most of the commercial apple cultivars are susceptible to this disease, and growers must spray fungicides several times within a season [6–9]. Applying so many treatments raises ecological problems and consumer health concerns, in addition to the direct financial costs for growers. Pathogen resistance to fungicides has become a challenging problem in the control of diseases and has threatened the performance of some

commercial fungicides [9]. Although some fungicides can still be effective against their target pathogens [10,11], the application of additional disease control strategies, such as sanitary measures in orchards, is essential. For example, leaf litter management [12–15] helps reduce disease severity and the risk of fungicide degradation. Furthermore, planting scab-resistant cultivars provides scab resistance in the long term to facilitate more sustainable apple production.

Knowledge of the pathogenicity and virulence factors needed for fungal infection is important because it represents the targets that will allow researchers to identify and deploy resistance genes against these microorganisms [16]. Phenotypically, the effects of resistance genes against *V. inaequalis* (*Rvi*) have been shown to cover a continuum from complete immunity to near-susceptibility depending on the genetic background, pathogen, and environment [17]. Currently, identifying the cultivars of apple crops that are resistant to the scab pathogen is a priority task [18].

Developing and cultivating a new apple cultivar takes a long time and involves many steps because of the biology of fruit plants. Apple trees take up to 8–10 years to fully mature, even when using early-fruiting initial forms. This limits the study of the source material using traditional genetic methods, which require not only the first seed hybrid generation but also the second and third [19,20]. A cultivated species with resistance against diseases provides an excellent opportunity to identify resistant genotypes and to use biodiversity to counter existing problems in fruit production.

Advances in technology have greatly improved the efficiency and accuracy of molecular markers, making them essential tools in plant breeding programs. Their origin, location in the genome, and the determined degree of resistance to the disease have been established [21]. Molecular markers have been developed for the most common resistance genes, which allows the identification of genotypes with target genes, their deployment in new plants, and targeted selection [22,23].

Twenty non-allelic genes that determine resistance to various scab races have been identified in apple trees, and highly informative DNA markers have been developed for most of them [24]. Molecular markers make it possible to evaluate hybrid families of seedlings for resistance in the very initial stages of plant development, significantly reducing the time needed to assess this important breeding trait.

According to a new nomenclature proposed by Bus et al. [17,25], apple scab resistance genes are named *Rvik* (*R* refers to the resistance gene, *vi* refers to *Venturia inaequalis*, and *k* refers to the differential host), and the corresponding *Avr* genes of the pathogen are named *avrRvik*.

Currently, 20 scab resistance genes have been identified [17,26]. The new and old names of the apple resistance genes, along with their differential hosts, are listed as *Rvi1* (V_g), *Rvi2* (V_{h2}) [21], *Rvi3* ($V_{h3.1}$) [27], *Rvi4* (V_{h4}/V_{r1}) [21], *Rvi5* (V_m) [28], *Rvi6* (V_f) [21], *Rvi7* (V_{fh}) [29], *Rvi8* (V_{h8}) [21], *Rvi9* (V_{dg}) [27], *Rvi10* (V_a), *Rvi11* (V_{bj}), *Rvi12* (V_b), *Rvi13* (V_d) [21], *Rvi14* [30], *Rvi15* (V_{r2}), *Rvi16* (V_{mis}), *Rvi17* (V_{a1}) [17], and V_{d3} [31].

Major genes such as *Rvi2* [32–34], *Rvi4* [34–36], and *Rvi9* [21] have also been identified from different cultivars. The *Rvi2* and *Rvi4* genes have been mapped in the same linkage group (LG-2) at the distal end [21]. Molecular markers such as SSRs and SNPs have been identified and reported in several studies to detect these resistance genes [17,18,27,30,32–34]. The *Rvi5* gene was shown to be responsible for resistance in *Malus micromalus* and *Malus atrosanguinea* 804 by Dayton and Williams [37]. Patocchi et al. [38] used a genome scanning approach (GSA) for the identification of the molecular markers associated with this gene. They developed the SSR marker Hi07h02, which is closely linked with *Rvi5* on LG-17 at the distal end [28]. Recently, with the use of the apple genome as a reference, a 228 kb region likely containing the *Rvi5* gene was identified [39]. *Rvi6* was the first scab resistance gene identified from a wild relative (*M. floribunda*) of apples. This gene remains the most widely studied and characterized scab resistance gene in apples. It generally conditions a chlorotic reaction in resistant segregants. The molecular marker Al07 linked with this gene is positioned at 1.1 cM [40,41] and is closely linked on LG-1 [21]. The SCAR marker AL07 is

located 0.2 cM from the gene [42]. The allele *Rvi6* is determined by the presence of a 466 bp expected amplification product, while susceptible cultivars form a 724 bp product. The presence of both fragments indicates a heterozygous state for this gene [43]. Later, AM19 was found closer to a resistance gene than AL07 and was used for chromosome walking of the BAC library of 'Florina'. The resistance conferred by this gene is influenced by the gene environment [44]. A new *Rvi8* gene was discovered in *M. sieversii* accession W193B by Bus et al. [33]. It is closely linked with the *Rvi2* gene on LG-2 at the lower end [21]. It was further observed that *Rvi8* is overcome by race 8 of the *V. inaequalis* isolate NZ188B.2. In another study [27], the marker OPL19 SCAR was found to be closely related to both genes. OPL19-SCAR was initially used to identify the *Rvi2* gene in the apple genome [32]. However, a separate scab resistance factor, *Rvi8*, was subsequently identified in the vicinity of the *Rvi2* gene. It was found that the target product of the marker OPL19-SCAR—a 433 bp fragment—is amplified in carriers of the genes *Rvi2* and *Rvi8*, demonstrating different degrees of resistance against artificial infection by individual scab races. According to the data, the two resistance genes (*Rvi8* and *Rvi2*) are not dependent on one another [33]. The *Rvi11* gene was identified in *M. baccata* by Dayton and Williams [45] and was mapped to the same LG (LG-2) [21]. Gygax et al. [46] developed the first molecular marker linked to this gene. Three SSR markers, namely CH02c06, CH05e03, and CH03d01, were developed. The *Rvi11* gene has been mapped at about 0.6 cM [46]. *Rvi15* was identified from the accession GMAL 1473, a clone of R12740-7A (Russian seedling) [47,48]. It was mapped on LG-2 (at the proximal end) [21] using the progeny of a cross between 'Idared' and GMAL 2473. Two closely associated markers were identified: CH02c02a and CH02f06 [36,47]. This is the most promising resistance gene that can be incorporated relatively quickly into a new cultivar in combination with other scab resistance genes for durable resistance [49]. It should be noted that depending on the parent forms, some seedlings that do not carry known resistance genes may be resistant to the pathogen due to the presence of other genetic determinants of resistance. As a result, three putative toll interleukin1 receptor–NBS-LRR resistance genes, namely, *Rvi15-A*, *Rvi15-B*, and *Rvi15-C*, were identified in this region.

Nowadays, virulent isolates have been shown to exist for most of the scab resistance genes used in apple breeding, including some carrying multiple virulence factors [17,50,51]. These findings highlight the need to breed for durable resistance. One way to achieve durable resistance is to pyramid multiple scab resistance genes in a cultivar, and it is desirable to combine several genetic factors that control immunity in one genotype [17].

This study presents the results of a molecular genetic analysis of apple cultivars to identify *Rvi2*, *Rvi4*, *Rvi5*, *Rvi6*, *Rvi8*, *Rvi9*, *Rvi11*, and *Rvi15* genes that are promising for further breeding, determining their resistance to scab and the incidence of apple scab in the most important apple growing areas of Kazakhstan.

2. Materials and Methods

2.1. Disease Monitoring

Eight apple orchards in the Almaty, Turkestan, and Zhambyl regions (Table 1) were monitored for the incidence of apple scab through phytopathological studies in 2022 and 2023. A total of 45 apple cultivars, which included 11 Kazakh cultivars and 34 foreign cultivars, were investigated in the current study.

2.2. Field Evaluation

The phytopathological assessment was conducted from 10 June to 30 August in 2022 and 2023 to study the incidence of apple scab. The number of trees per cultivar for disease evaluation is presented in Table 1. The susceptible cultivars 'Golden Delicious' and 'Idared' served as positive controls. Scab incidence was defined as the percentage of infected leaves (infected leaves/all leaves). A leaf was considered infected if there were matte, olive green-to-black-colored lesions on it, indicating active sporulation [52].

Table 1. Experimental sites included in this study.

Breeding Program	Geographical Site *	Coordinates	Precipitation May–August (mm)	Number of Trees for Sampling **
Kazygurt district, Kyzylkiyan rural district, "Akniyet Agro Orchard" LLP	Turkestan region	N 41°36′8.594″ E 69°21′58.013″	926.8	8
Saryagash district, Zhemisti rural areas, Regional Branch, "Saryagash" LLC, "Kazakh Fruit and Vegetable Research Institute" (KazF&VRI)	Turkestan region	N 41°32′2.545″ E 69°21′36.069″	902.8	8
Tulkubas district, Shakpak Baba rural areas, "Koktal" Peasant Farm	Turkestan region	N 41°29′2.69″ E 70°31′11.46″	945.3	8
Merke district, Merke rural areas, "Merke experimental farm" LLP	Zhambyl region	N 42°48′.584″ E 73°10′.387″	925.2	3
Yenbekshikazakh district, Baidibek bi rural areas, "Akkazy" Peasant Farm	Almaty region	N 43°39′.930″ E 77°86′.171″	902.8	8
Yenbekshikazakh district, Baidibek bi rural areas, "Ermek" Peasant Farm	Almaty region	N 43°32′49.344″ E 77° 52′ 3.468″	906.9	8
Yenbekshikazakh district, Koram rural areas, "Zhetysu Trade"	Almaty region	N 43°31′48.31″ E 78°11′48.09″	908.9	9
Talgar District, Regional Branch, "Talgar" of KazF&VRI (Pomological Garden)	Almaty region	N 43°17′27″ E 77°12′15″	90.36	45

* Site names are used in the following tables and text to identify the respective breeding programs. ** Number of trees used for disease assessment.

2.3. Collection of Plant Materials, DNA Extraction, and Detection of Rvi Genes with Molecular Markers

Three leaf samples from each of three trees per cultivar were collected from apple orchards located in the Almaty, Zhambyl, and Turkestan regions and from the pomological garden of the Kazakh Fruit and Vegetable Research Institute. DNA was isolated from fresh leaves of apple cultivars. For the identification of the scab resistance genes *Rvi2/Rvi8*, *Rvi2/Rvi4/Rvi9/Rvi11*, *Rvi5*, *Rvi11*, *Rvi14*, and *Rvi15*, the following markers were applied: OPL19, CH05e03, Hi07h02, K08, HB09, and CH02f06, respectively. AL07 and AM19 SCAR markers were used to identify *Rvi6* [17,18,27,30,43,46,53,54].

Extraction was conducted using the method of Doyle et al. [55] with a modification that included an additional purification of the samples. A modified CTAB method was used by introducing an additional component, polyvinylpyrrolidone (1%), into the composition of the lysis buffer, which provided a DNA yield of sufficient purity for PCR amplification.

The negative control for the studied loci was the cultivar 'Golden Delicious'. Primer sequences and their sizes are shown in Table 2. The primers used in this study were synthesized by Sigma–Aldrich (Darmstadt, Germany). A 15 µL PCR reaction mixture contained 20 ng of genomic DNA, 1.5 µL of dNTPs, 2.5 µL of $MgCL_2$, 10 µL of each primer, 1 µL of Taq polymerase, and 2.5 µL of 10x Taq buffer (+$(NH_4)_2SO_4$, -KCL). All PCR components were manufactured by Thermo Fisher Scientific, Waltham, MA, USA. Amplification was carried out in a thermal cycler according to the following programs: marker CH05e03: 1 cycle, 2 min 30 s 94 °C, 33 cycles (30 s 94 °C, 30 s 55 °C, 1 min 72 °C), 5 min 72 °C; marker AL07-SCAR: 1 cycle 10 min 95 °C, 35 cycles (30 s 95 °C, 1 min 59 °C, 2 min 72), 10 min 72 °C; marker K08-SCAR: 1 cycle 5 min 94 °C, 35 cycles (40 s 94 °C, 1 min 64 °C, 2 min 72 °C), 10 min 72 °C; marker OPL19-SCAR: 1 cycle 2 min 45 s, 40 cycles (55 s 94 °C, 55 s 55 °C, 1 min 39 s 72 °C), 10 min 72 °C; marker Hi07f02-SSR: 1 cycle 2 min 30 s 94 °C, 30 cycles (30 s 94 °C, 45 s 56 °C, 1 min 72 °C),10 min 72 °C; marker AM19 SCAR: 1 cycle 5 min 94 °C, 35 cycles (1 min 94 °C, 1 min 58 °C, 2 min 72 °C), 7 min 72 °C; marker HB09-SSR: 1 cycle 5 min 94 °C, 35 cycles (40 s 94 °C, 1 min 64 °C, 1 min 72 °C), 10 min 72 °C; marker CH02f06-SSR: 1 cycle 3 min 94 °C, 39 cycles (30 s 94 °C, 1 min 55 °C, 1 min 72 °C), 10 min 72 °C. Agarose gel (2%) electrophoresis was used to separate DNA fragments. The Gene Ruler family of 100 bp DNA ladders (Thermo Fisher Scientific) was used to estimate the sizes of DNA samples.

Table 2. Characteristics of molecular markers for apple scab resistance genes.

Resistance Genes	Marker Name	Marker Type	Size of Allele (bp)	Primer Sequence (5′→3′)	References
Rvi6 (*Vf*)	AM19	SCAR	526	F: CGTAGAACGGAATTTGACAGTG R: GACAAGGGCTTAAGTGCTCC	Bus et al. [17,18]
Rvi2, *Rvi4*, *Rvi9*, *Rvi11*	CH05e03	SSR	172	F: CGAATATTTTCACTCTGACTGGG R: CAAGTTGTTGTACTGCTCCGAC	Gygax et al. [46] Patocci et al. [27]
Rvi2, *Rvi8*	OPL19	SCAR	433	F: ACCTGCACTACAATCTTCACTAATC R: GACTCGTTTCCACTGAGGATATTTG	Bus et al. [17] Patocci et al. [27]
Rvi5	Hi07f02	SSR	226	F: ATTTGGGGTTTCAACAATGG R: GTTTCGGACATCAAACAAATGTGC	Silfverberg-Dilworth et al. [53]
Rvi6	AL07	SCAR	466	F: TGGAAGAGAGATCCAGAAAGTG R: CATCCCTCCACAAATGCC	Tartarini et al. [54]
Rvi11	K08	SCAR	743	F: GAACACTGGGCAAAGGAAAC R: TAAAAGCCACGTTCTCTCGC	Gygax et al. [46]
Rvi14	HB09	SSR	210	F: GCTCAAAATACTGAAGCCTTGC R: GGGGAAGCAGGATGGTTACT	Soufflet et al. [30] Patocchi et al. [27]
Rvi15	CH02f06	SSR	147	F: CCCTCTTCAGACCTGCATATG R: ACTGTTTCCAAGCGCTCAGG	Patocci et al. [48] Patocci et al. [27]

2.4. Data analysis

Data analysis was performed based on the results of molecular analysis. The visualization of PCR products was achieved through electrophoresis. The banding pattern of each amplified PCR product was scored as "+", indicating the presence of resistance, or "−", indicating the absence of the resistance gene. Genetic distance was evaluated through Popgen software (version 1.32, Yeh et al., 2000 [56]) by calculating the Dice coefficient [57]. This calculated index was used to develop the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The dendrogram was drawn in Molecular Evolutionary Genetics Analysis (MEGA software, version 11, Tamura et al., 2021 [58]).

3. Results

3.1. Field Evaluation of Apple Cultivars for Scab Resistance

Apple scab did not develop in many of the orchard–year combinations included in this study (Tables 3–5). Specifically, the susceptible control 'Idared' developed disease only in 3 of 14 orchard–year combinations that included this cultivar. Similarly, the other susceptible control 'Golden Delicious' developed disease only in 10 of 16 orchard–year combinations. Based on the limited disease development, conclusions about phenotypic host resistance in the test cultivars must be interpreted with caution. Among the test cultivars, 'Maksat', 'Kamila', 'Diana', 'Saltanat', 'Korey', 'Mutsu', 'Talgarskoye', 'Tulpan', 'Williams Pride', 'Piros', 'Honeycrisp', 'SuperChief', SQ159 (Natyra), 'Modi', 'Golden Resistant', and 'Prima' did not develop scab in our trials. However, among these cultivars, only 'Korey' was tested in more than two orchard–year combinations. Several other cultivars developed low levels of disease incidence (5% or less), with the caveat of low disease pressure across most trials as explained above.

Table 3. Results of monitoring apple genotypes for scab disease incidence (%) in the Turkestan region.

	Turkestan Region					
	Akniyet Agro Orchards, Kazygurt District		**Saryagash, Saryagash District**		**Koktal, Tulkubas District**	
	2022	**2023**	**2022**	**2023**	**2022**	**2023**
Star Crimson	8.75	0	18.75	9.37	0	0
Idared	20.88	0	0	0	0	0
Gala	5.62	0	0	0	0	0
Fuji	0	0	0	0	0	0
Golden Delicious	0	0	21.25	9.22	0	0
Pink Lady	0	0	0	0	0	0
Landsberger Renette	0	0	20.65	19.36	0	0

Table 4. Results of monitoring apple genotypes for scab disease incidence (%) in the Almaty region.

	Almaty Region							
	Akkazy, Yenbekshikazakh District		**Ermek, Yenbekshikazakh District**		**Zhetysu Trade, Yenbekshikazakh District**		**Talgar, Talgar District**	
	2022	**2023**	**2022**	**2023**	**2022**	**2023**	**2022**	**2023**
Ainur	-*	-	-	-	-	-	4.98	0
Aigul	-	-	-	-	-	-	4.69	0
Maksat	-	-	-	-	-	-	0	0
Zaman	-	-	-	-	-	-	5.06	0
Kamila	-	-	-	-	-	-	0	0
Diana	-	-	-	-	-	-	0	0
Malus sieversii	-	-	-	-	-	-	10.63	4.29
Danalyk	-	-	-	-	-	-	5.03	0
Saltanat	-	-	-	-	-	-	0	0
Kandil Sinap	-	-	-	-	-	-	4.38	5.63
Golden Delicious	21.88	10.63	0	0	9.36	4.38	48.75	28.75
Granny Smith	-	-	-	-	-	-	10.08	9.63
Korey	0	0	0	0	0	0	0	0
Mutsu	-	-	-	-	-	-	0	0
Landsberger Renette	-	-	-	-	-	-	4.68	5.06
Star Crimson	19.48	4.38	0	0	0	0	0	9.68
Stark's Earliest	-	-	-	-	9.79	9.88	5.06	9.38
Talgarskoye	-	-	-	-	-	-	0	0
Tulpan	-	-	-	-	-	-	0	0
Williams Pride	-	-	-	-	-	-	0	0
Gala	0	0	0	0	9.36	5.05	4.98	27.90
Pestrushka	-	-	-	-	-	-	4.38	0
Idared	0	0	0	0	0	0	5.03	9.65
Pink Lady	-	-	-	-	-	-	4.89	0
Piros	-	-	-	-	-	-	0	0

Table 4. *Cont.*

	Almaty Region							
	Akkazy, Yenbekshikazakh District		Ermek, Yenbekshikazakh District		Zhetysu Trade, Yenbekshikazakh District		Talgar, Talgar District	
	2022	2023	2022	2023	2022	2023	2022	2023
Braeburn	-	-	-	-	-	-	5.03	0
Honeycrisp	-	-	-	-	-	-	0	0
Jeromine	-	-	-	-	-	-	4.83	0
SuperChief	-	-	-	-	-	-	0	0
Wilton's Star	-	-	-	-	-	-	4.78	0
Red Topaz	-	-	-	-	-	-	54.97	0
SQ159 (Natyra)	-	-	-	-	-	-	0	0
Santana	-	-	-	-	-	-	4.78	0
Deljonca	-	-	-	-	-	-	4.38	0
Fuji	0	0	0	0	0	0	4.78	9.48
Pinova	-	-	-	-	-	-	5.03	
Nicola	-	-	-	-	-	-	18.70	19.86
Modi (*Rvi6* control)	-	-	-	-	-	-	0	0
Quinte	0	0	0	0	9.89	4.89	4.78	0
Jonagold	-	-	-	-	-	-	20.05	4.38
Voskhod	-	-	-	-	-	-	4.98	0
Babuskino	-	-	-	-	-	-	21.38	4.38
Red Delicious	0	0	0	0	4.68	4.48	49.50	0
Golden Resistant	-	-	-	-	-	-	0	0
Prima (*Rvi6* control)	-	-	-	-	-	-	0	0

* "-" means that these genotypes were not grown in the Almaty region.

Table 5. Results of monitoring apple genotypes for scab disease incidence (%) in the Zhambyl region.

	Zhambyl Region	
	Merke Experimental Farm, Merke District	
	2022	2023
Red Delicious	29.75	4.63
Star Crimson	19.65	9.63
Golden Delicious	19.86	4.38

3.2. Molecular Screening of Apple Cultivars for Scab Resistance

As a result of the molecular screening of the 45 Kazakh and foreign cultivars, it was found that 38 apple cultivars contained *Rvi* resistance genes. To identify carriers of the *Rvi2* + *Rvi8* gene, PCR analysis of apple genotypes was carried out using the SCAR marker OPL19 (Table 6), which had an expected PCR product size of 433 bp. The presence of the OPL19-SCAR marker in the genomes of the cultivars 'Prima' and 'Modi' was previously confirmed by other researchers [21,22,49,50]. We showed that 24 apple cultivars are carriers of this gene: 'Ainur', 'Aigul', 'Kamila', 'Diana', 'Saltanat', 'Korey', 'Mutsu', 'Landsberger Renette', 'Star Crimson', 'Stark's Earliest', 'Talgarskoye', 'Williams Pride', 'Gala', 'Idared', 'Pink Lady', 'Braeburn', 'Jeromine', 'SuperChief', SQ159 (Natyra), 'Fuji', 'Pinova', 'Modi', 'Voskhod', and 'Prima'.

Table 6. Results of the identification of scab resistance genes of apple genotypes.

Cultivar	Origin *	*Rvi2*/*Rvi8*	*Rvi2*	*Rvi4*	*Rvi9*	*Rvi11*	*Rvi5*	*Rvi6*		*Rvi11*	*Rvi14*	*Rvi15*
		OPL19	CH05e03				Hi07f02	AL07	AM19	K08	HB09	CH02f06
		433 bp	163 bp	172 bp	169 bp	160 bp	220 bp	466 bp	526 bp	743 bp	210 bp	135–158 bp
Ainur	KZ	+ **	+	– **	–	–	–	–	–	–	–	+
Aigul	KZ	+	+	–	–	–	–	–	–	–	+	–
Maksat	KZ	–	–	–	–	–	–	–	–	–	–	+
Zaman	KZ	–	–	+	+	–	–	–	–	–	+	–
Kamila	KZ	+	–	–	–	–	–	–	–	+	+	+
Diana	RU	+	–	–	–	–	+	–	–	+	–	–
Malus sieversii	KZ	–	–	–	–	–	–	–	–	–	–	–
Danalyk	KZ	–	–	–	–	–	+	+	+	–	–	–
Saltanat	KZ	+	–	+	+	–	+	–	–	+	–	–
Kandil Sinap	UA	–	+	+	–	–	–	–	–	+	–	+
Golden Delicious	US	–	–	–	–	–	–	–	–	–	–	–
Granny Smith	AU	–	–	+	+	–	–	–	–	+	–	–
Korey	JP	+	–	–	–	–	–	–	–	+	+	–
Mutsu	JP	+	–	–	–	–	–	–	–	+	–	–
Landsberger Renette	DE	+	–	–	–	–	–	–	–	–	–	–
Star Crimson	US	+	–	–	+	–	–	–	–	+	–	–
Stark's Earliest	US	+	+	+	+	–	+	–	–	+	+	+
Talgarskoye	KZ	+	–	–	–	–	–	–	–	+	+	+
Tulpan	KZ	–	–	–	–	–	–	–	–	–	–	–
Williams Pride	US	+	–	–	–	–	+	+	+	+	+	–
Gala	NZ	+	–	+	–	–	–	–	–	–	–	+
Pestrushka	RU	–	–	–	+	–	–	–	–	+	+	–
Idared	US	+	–	–	–	–	+	–	–	+	+	–
Pink Lady	AU	+	–	+	+	–	–	–	–	–	+	–
Piros	DE	–	–	–	+	–	–	–	–	–	+	+
Braeburn	NZ	+	–	+	+	–	–	–	–	–	–	+
Honeycrisp	US	–	–	+	+	–	–	–	–	–	–	–
Jeromine	US	+	–	+	–	–	+	–	–	+	+	–
SuperChief	US	+	–	+	–	–	+	–	–	+	+	–
Wilton's Star	NL	–	–	–	–	–	–	–	–	–	–	+
Red Topaz	CZ	–	–	–	–	–	–	–	–	+	+	+
SQ159 (Natyra)	DE	+	–	–	–	–	–	+	+	+	–	+
Santana	NL	–	–	–	–	–	–	+	+	–	–	–
Deljonca	DE	–	–	–	–	–	+	–	–	–	–	+
Fuji	JP	+	+	+	+	–	–	–	–	–	–	–
Pinova	DE	+	+	+	–	–	–	–	–	+	–	+
Nicola	CA	–	–	–	–	–	–	–	–	–	–	–
Modi (*Rvi6* control)	IT	+	–	+	+	+	–	+	+	–	–	–
Quinte	CA	–	–	–	+	–	–	–	–	–	–	–
Jonagold	US	–	–	–	–	–	–	–	–	–	–	–
Voskhod	KZ	+	–	–	–	–	–	–	–	+	–	–
Babuskino	RU	–	–	–	–	–	–	–	–	–	–	–
Red Delicious	US	–	–	–	–	–	–	–	–	+	–	–
Golden Resistant	US	–	–	–	–	–	–	–	–	–	–	–
Prima (*Rvi6* control)	US	+	+	–	–	–	–	+	+	–	–	–

* KZ—Kazakhstan; RU—Russia; UA—Ukraine; US—United States; AU—Australia; JP—Japan; DE—Germany; NZ—New Zealand; NL—the Netherlands; CZ—Czech Republic; IT—Italy; CA—Canada. ** "+"—amplification; "–"—no amplification.

The SSR molecular marker CH05e03 was developed for *Rvi11* by Gygax et al. [46]. This marker has been mapped to LG-2 of the apple genome [39]. To identify the *Rvi2* + *Rvi4* + *Rvi9* + *Rvi11* gene, the expected amplification fragment sizes of CH05e03 were 163, 172, 169, and 160 bp, respectively. A 163 bp PCR product is characteristic of *Rvi2* gene carriers [27,46]. Seven apple cultivars, i.e., 'Ainur', 'Aigul', 'Kandil Sinap', 'Stark's Earliest', 'Fuji', 'Pinova', and 'Prima', were found to be carriers of *Rvi2*. *Rvi4* was detected in 14 apple cultivars, i.e., 'Zaman', 'Saltanat', 'Kandil Sinap', 'Granny Smith', 'Stark's Earliest', 'Gala', 'Pink Lady', 'Braeburn', 'Honeycrisp', 'Jeromine', 'SuperChief', 'Fuji', 'Pinova', and 'Modi', forming a band measuring 172 bp. For *Rvi9* gene carriers, the amplification fragment measured 169 bp, and this fragment was detected in 13 apple cultivars: 'Zaman', 'Saltanat', 'Granny Smith', 'Star Crimson', 'Stark's Earliest', 'Pestrushka', 'Pink Lady', 'Piros', 'Braeburn', 'Honeycrisp', 'Fuji', 'Modi', and 'Quinte'. The *Rvi11* gene was only detected in the 'Modi' cultivar, and the expected amplification fragment was 160 bp.

The molecular marker Hi07h02 was designed for *Rvi5* by Patocchi et al. in 2009 [27]. The expected size of the amplification fragments was 220 bp. Nine apple cultivars were

classified as carriers of this gene: 'Diana', 'Danalyk', 'Saltanat', 'Stark's Earliest', 'Williams Pride', 'Idared', 'Jeromine', 'SuperChief', and 'Deljonca'.

The primer AL07 is codominant, AM19 is dominant, and both are specific to the *Rvi6* gene. The AL07 primer amplifies 466 bp (resistant) and 724 bp products linked to pathogen susceptibility, while the AM19 primer amplifies a 526 bp fragment associated with resistance. The resistance gene *Rvi6* was detected in six cultivars ('Danalyk', 'Williams Pride', SQ159 (Natyra), 'Santana', 'Modi', and 'Prima') using the AL07 and AM19 markers.

PCR amplification using the SCAR primer K08 was performed to identify carriers of the *Rvi11* gene. The expected size of the amplification fragment was 743 bp. The *Rvi11* resistance gene was detected in 20 cultivars: 'Kamila', 'Diana', 'Saltanat', 'Kandil Sinap', 'Granny Smith', 'Korey', 'Mutsu', 'Star Crimson', 'Stark's Earliest', 'Talgarskoye', 'Williams Pride', 'Pestrushka', 'Idared', 'Jeromine', 'SuperChief', 'Red Topaz', SQ159 (Natyra), 'Pinova', 'Voskhod', and 'Red Delicious'.

PCR amplification using the SSR primer HB09 was performed to identify carriers of the *Rvi14* gene. The expected size of the amplification fragment was 210 bp. The *Rvi14* resistance gene was detected in 14 cultivars, i.e., 'Aigul', 'Zaman', 'Kamila', 'Korey', 'Stark's Earliest', 'Talgarskoye', 'Williams Pride', 'Pestrushka', 'Idared', 'Pink Lady', 'Piros', 'Jeromine', 'SuperChief', and 'Red Topaz'.

To identify carriers of the *Rvi15* gene, PCR amplification was performed using the SSR primer CH02f06. The expected size of the amplification fragment was 158 bp. The *Rvi15* resistance gene was detected in 14 cultivars, i.e., 'Ainur', 'Maksat', 'Kamila', 'Kandil Sinap', 'Stark's Earliest', 'Talgarskoye', 'Gala', 'Piros', 'Braeburn', 'Wilton's Star', 'Red Topaz', SQ159 (Natyra), 'Deljonca', and 'Pinova'.

UPGMA cluster analysis based on the presence or absence of resistance genes revealed that SQ159 (Natyra) and 'Williams Pride' formed a distinct cluster. The remaining 43 cultivars formed two large subclusters, one with 20 cultivars and the other with 23 cultivars. Several genotypes clustered together closely, including *Malus sieversiii* and 'Golden Delicious' (Figure 1).

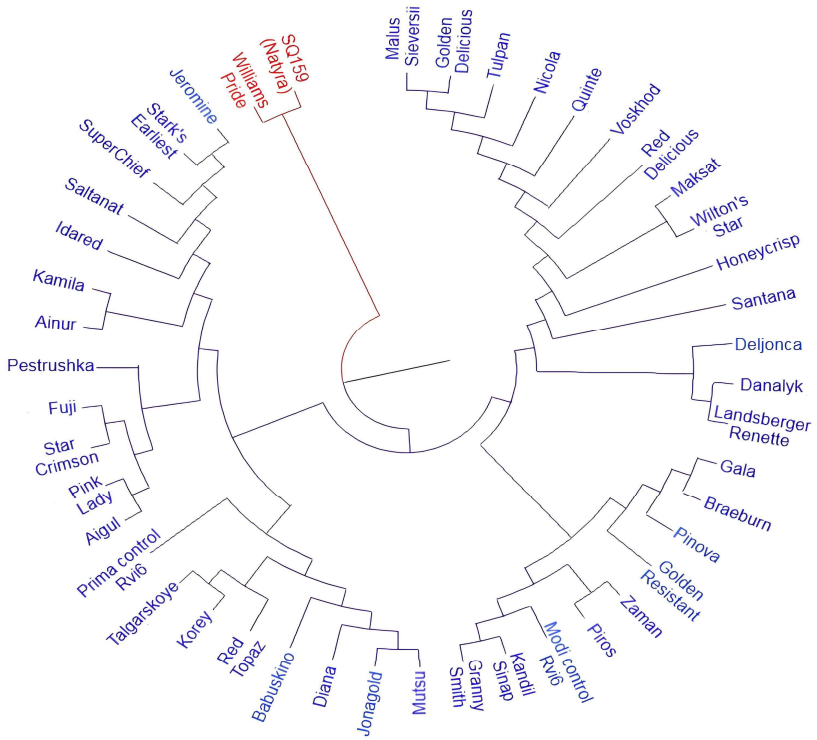

Figure 1. UPGMA (Unweighted Pair–Cluster Method using Arithmetic Averages) dendrogram based on the presence or absence of apple scab resistance genes in 45 apple cultivars or accessions assessed in southern and southeastern Kazakhstan.

4. Discussion

Progress in fruit breeding strongly depends on the availability of a rich diversity of genetic resources [3].

Many resistance genes have been identified from apple germplasm and are effective against only a few isolates *V. inaequalis* [59]. Hence, such resistance genes may not be suitable for apple breeding against scab. Because the *Rvi2* gene and the *Rvi4* gene give resistance that has been overcome by race 2 [32,34] and isolate 1797-9 [34–36], respectively, highly informative SNPs for *Rvi2* (*FBsnRvi2-7* and *FBsnRvi2-8)* and *Rvi4* (*FBsnRvi4-1*, ARGH37, and TNL1) have been developed.

Up to now, *V. inaequalis* isolates have commonly been used in genetic experiments for their known specific ability to overcome one of the apple scab resistance genes represented in the differential host set [17]. The identification of differential hosts with monogenic resistances will assist in the monitoring of pathogen populations to determine the potential of specific *Rvi* genes, currently the main sources of resistance in apple breeding. The *Rvi9* gene generally conditions a chlorotic reaction in resistant segregants. Caffier et al. reported that *Rvi9* gene resistance has been overcome by *V. inaequalis* isolate 1639 [50]. However, isolate 1639 has not spread and therefore has a limited presence in apple growing areas [27]. Bus et al. showed that isolate 1639 has also overcome the *Rvi2* gene [17]. *Rvi9* as well as *Rvi2* and *Rvi8* map to the same genomic region of apple and condition very similar stellate necrotic resistance reactions [17,33,58]. The study by Luby et al. in the Silk Road apple collection of *Malus sieversii* from Central Asia demonstrated that *Rvi8* is prevalent in the Kazakh accessions sampled [60]. In our study, this gene was found in six Kazakh local cultivars ('Ainur', 'Aigul', 'Kamila', 'Saltanat', 'Talgarskoe', and Voskhod'). The *Rvi15* gene provides full resistance to apple scab [48], and there are no reports yet on the breaking of this resistance. In the present study, this gene was identified in four Kazakh local cultivars ('Ainur', 'Maksat', 'Kamila', and 'Talgarskoe').

'Aport' is the most used cultivar in Kazakh apple breeding as a donor of the taste qualities of fruits and frost resistance, and 'Aport' has the scab resistance genes *Rvi2* + *Rvi8* and *Rvi11* [61]. The results of our previous research on 'Aport' *x M. sieversii* scion–rootstock combinations showed resistance to powdery mildew and scab at the beginning of fruiting over 3 years [62].

Based on foliar resistance reactions, apple *R* genes can be grouped into three predominant resistance classes exhibiting distinctive resistance responses: the classical hypersensitive response (HR), in which fungal growth is normally terminated very rapidly on penetration, e.g., conditioned by *Rvi4*, *Rvi5* [63], *Rvi7*, *Rvi10*, *Rvi15* [17,64], and *Rvi16* [17]; responses involving limited subcuticular growth inducing stellate necrosis, e.g., conditioned by *Rvi2*, *Rvi3*, *Rvi8* [17,33], *Rvi9*, *Rvi11* (stellate necrosis/chlorosis), *and Rvi13* [17]; and chlorosis, often accompanied by limited sporulation and therefore providing only partial resistance, e.g., conditioned by *Rvi6*, *Rvi12*, *Rvi14*, and *Rvi17* [17]. *Rvi1* is considered ineffective as a resistance gene because the complementary race (1) is predominant in the European *V. inaequalis* population [65], which explains the highly susceptible status of cv. 'Golden Delicious'. On the other hand, resistance genes in our study such as *Rvi2*, *Rvi4*, *Rvi6*, and *Rvi9* are still useful for breeding, but their use is recommended only in extended pyramids of $\geq$3 resistance genes [66].

Our molecular studies suggest that most cultivars in the three study regions possess resistance genes, including 'Maksat' (*Rvi15*), 'Kamila' (*Rvi2/Rvi8*, *Rvi11*, *Rvi14*, *Rvi15*), 'Diana' (*Rvi2/Rvi8*, *Rvi5*, *Rvi11*), 'Saltanat' (*Rvi2/Rvi8*, *Rvi4*, *Rvi9*, *Rvi5*, *Rvi11*), 'Korey' (*Rvi2/Rvi8*, *Rvi11*, *Rvi14*), 'Mutsu' (*Rvi2/Rvi8*, *Rvi11*), 'Talgarskoye' (*Rvi2/Rvi8*, *Rvi11*, *Rvi14*, *Rvi15*), 'Williams Pride' (*Rvi2/Rvi8*, *Rvi5*, *Rvi6*, *Rvi11*, *Rvi14*), 'Piros' (*Rvi9*, *Rvi14*, *Rvi15*), 'Honeycrisp' (*Rvi4*, *Rvi9*), 'SuperChief' (*Rvi2/Rvi8*, *Rvi4*, *Rvi5*, *Rvi11*, *Rvi14*), SQ159 (Natyra) (*Rvi2/Rvi8*, *Rvi6*, *Rvi11*, *Rvi15*), 'Modi' (*Rvi2/Rvi8*, *Rvi4*, *Rvi6*, *Rvi9*, *Rvi11*), and 'Prima' (*Rvi2/Rvi8*, *Rvi2*, *Rvi6*). Only in *Malus sieversii*, 'Golden Delicious', 'Tulpan', 'Nikola', 'Jonagold', 'Babuskino', and 'Golden Resistant' were we not able to identify any previously studied resistance gene. These genotypes may have another unstudied resistance gene.

Many breeding programs worldwide are aiming at breeding for durable disease resistance against apple scab [67]. Until now, most of the scab-resistant cultivars that have been released carry only *Rvi6* [66]. However, the value of the resistance mediated by *Rvi6* is weakened by the occurrence of the *avrRvi6* races of the pathogen in Europe [65] and the US [10], which can break the resistance of *Rvi6*. Therefore, the use of single *R* genes of durable resistance is not effective in the long term, which suggests a combination of different *R* genes for new cultivars, as carried out in pyramidization breeding programs [3]. Several *Rvi* genes described to date, including *Rvi5*, *Rvi11*, *Rvi12*, *Rvi14*, and *Rvi15*, confer durable resistance to scab; therefore, they are of special interest for resistance breeding [66]. The obtained data are important for identifying new donors for optimizing key stages of the breeding process for long-term resistance to the pathogen by pyramiding target genes.

5. Conclusions

This study monitored the incidence of apple scab in three regions in the south and southeast of Kazakhstan. Using field evaluation and molecular analysis, this study sought to identify the apple genotypes most resistant to scab among Kazakh and foreign cultivars. The promising gene sources *Rvi2/Rvi8*, *Rvi2/Rvi4/Rvi9/Rvi11*, *Rvi5*, *Rvi11*, *Rvi14*, and *Rvi15* were identified for molecular screening.

It has been established that 24 apple cultivars are carriers of the *Rvi2/Rvi8* gene, i.e., 'Ainur', 'Aigul', 'Kamila', 'Diana', 'Saltanat', 'Korey', 'Mutsu', 'Landsberger Renette', 'Star Crimson', 'Stark's Earliest', 'Talgarskoye', 'Williams Pride', 'Gala', 'Idared', 'Pink Lady', 'Braeburn', 'Jeromine', 'SuperChief', SQ159 (Natyra), 'Fuji', 'Pinova', 'Modi', 'Voskhod', and 'Prima'. Apple cultivars such as 'Ainur', 'Aigul', 'Kandil Sinap', 'Stark's Earliest', 'Fuji', 'Pinova', and 'Prima' were found to be carriers of the *Rvi2* gene using the marker CH05e03. *Rvi4* gene carriers were detected in 14 apple cultivars: 'Zaman', 'Saltanat', 'Kandil Sinap', 'Granny Smith', 'Stark's Earliest', 'Gala', 'Pink Lady', 'Braeburn', 'Honeycrisp', 'Jeromine', 'SuperChief', 'Fuji', 'Pinova', and 'Modi'. Thirteen apple cultivars were found to be *Rvi9* gene carriers, i.e., 'Zaman', 'Saltanat', 'Granny Smith', 'Star Crimson', 'Stark's Earliest', 'Pestrushka', 'Pink Lady', 'Piros', 'Braeburn', 'Honeycrisp', 'Fuji', 'Modi', and 'Quinte'. However, the *Rvi11* gene was detected only in the 'Modi' cultivar. The *Rvi5* gene was found in 'Diana', 'Danalyk', 'Saltanat', 'Stark's Earliest', 'Williams Pride', 'Idared', 'Jeromine', 'SuperChief', and 'Deljonca'. The resistance gene *Rvi6* was detected in six cultivars, i.e., 'Danalyk', 'Williams Pride', SQ159 (Natyra), 'Santana', 'Modi', and 'Prima', using the AL07 and AM19 markers. The *Rvi11* resistance gene was detected in 20 cultivars: 'Kamila', 'Diana', 'Saltanat', 'Kandil Sinap', 'Granny Smith', 'Korey', 'Mutsu', 'Star Crimson', 'Stark's Earliest', 'Talgarskoye', 'Williams Pride', 'Pestrushka', 'Idared', 'Jeromine', 'SuperChief', 'Red Topaz', SQ159 (Natyra), 'Pinova', 'Voskhod', and 'Red Delicious'. The *Rvi14* resistance gene was detected in 14 cultivars, i.e., 'Aigul', 'Zaman', 'Kamila', 'Korey', 'Stark's Earliest', 'Talgarskoye', 'Williams Pride', 'Pestrushka', 'Idared', 'Pink Lady', 'Piros', 'Jeromine', 'SuperChief', and 'Red Topaz'. The *Rvi15* resistance gene was detected in 14 cultivars, i.e., 'Ainur', 'Maksat', 'Kamila', 'Kandil Sinap', 'Stark's Earliest', 'Talgarskoye', 'Gala', 'Piros', 'Braeburn', 'Wilton's Star', 'Red Topaz', SQ159 (Natyra), 'Deljonca', and 'Pinova'.

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