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MOLECULAR SCREENING OF APPLE CULTIVARS FOR TWO SCAB RESISTANCE GENES IN ROMANIA

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Abstract

Apple scab, caused by Venturia inaequalis, is considered the most damage disease of the apple growing in Romania. Pyramiding multiple sources of quantitative resistance could be the best way to control the attack of this fungi. A set of forty apple cultivars from the apple gene bank in Pitesti, Romania was evaluated for the presence of two scab resistance genes: Rvi8 and Rvi11. Using marker OPB18, it was made the difference between 2 genes, Rvi2 and Rvi8, located in the same locus (Vr gene). For identification of Rvi11, the SCAR marker, K08, was used and seventeen cultivars amplified the 743 bp fragment that is linked with Rvi11 gene. The results on resistance of apple Romanian cultivars towards economically important disease is necessary for future breeding work and for establishing of new commercial orchards.

Key words*: breeding; genotypes; Rvi2; Rvi8; Rvi11.*

INTRODUCTION

SCAR markers were first developed and initially applied to study on resistance genes for downy mildew in lettuce by Paran and Michelmore (1993). A SCAR marker is a technique mediated by PCR, which identifies the genomic DNA fragment at a single locus, using a pair of specific oligonucleotides, of 15-30 bp, projected from nucleotide sequences derived from dominant polymorphic RAPD fragments.

To highlight some apple resistance genes, the most used markers associated were: AL-07, AM19, Vfc, OPL19, OPB12, OPB18, AD13, OPB12, K08, T06, Z13, 41A24T7, ARGH17 and for resistance to powdery mildew, the most used markers were: OPU02, EM M01, AT20, EMDM01, EM 02. Most of them (Al07, AM19, Vfc, OPL19, AD13, OPB12, AT20, EM M01) were used in the studies initiated at the Research Institute for Fruit Growing Pitești-Mărăcineni (Militaru et al., 2020; Iancu et al., 2022; Militaru et al., 2022; Iancu et al., 2023).

$OPL19_{433}$ was developed by cloning the 550 bp fragment from the RAPD marker $OPL19_{550}$, located near the *Rvi2* gene, at a distance of 2.5 cM (Gardiner et al. 1999a; Bus et al., 1999). The SCAR marker was also mapped to 1 cM (Bus et al., 2005b) close to *Rvi2* (*Vh2*) in differential host 2 ('TSR34T15'), but also close to the *Rvi8* gene (*Vh8*) in differential host 8 (*M. sieversii W193B*), being considered very useful for identifying varieties carrying the *Vr* gene (*Rvi2* or *Rvi8*).

To differentiate the carrier varieties of one of the genes *Rvi2* or *Rvi8*, Bus et al. (2005a), developed the OPB18 SCAR marker, which amplified in a population 'Royal Gala × *M. sieversii W193B*' only those hybrids carrying of the *Rvi8* gene.

The *Rvi11* gene was identified in *Malus baccata* by Dayton and Williams in 1968 and was mapped to LG-2 by Gessles et al. in 2006. Gygax et al. (2004) showed the presence of the *Rvi11* gene (old name *Vbj*) using the SCAR markers $K08_{743}$, $Z13_{773}$, $T06_{410}$ which were projected from the decamer predecessors $OPK08_{848}$, $OPZ13_{869}$ and $OPT06_{801}$.

Galli et al. (2010) revealed three transcribed putative resistance gene analogues (Resistance Gene Analogs) for resistance to *Venturia inaequalis* (*Vr2-A*, *Vr2-B* and *Vr2-C*) after isolation of Bacterial Artificial Chromosome

(BAC) spanning the *Rvi15* (*Vr2*) region and it sequenced, and Schouten et al. (2014) concluded that Vr2-C is the *Rvi15* (*Vr2*) gene that provides the resistance. Markers 41A24T7 and 43M10RP have cosegregated on the TIR-NBS-LRR domain of the *Vr2-B*. The Vr2-C region was flanked by the markers: 77G20RP (SSR), 21K14T7 (SNP), 8K11RP (SCAR) and GmTNL1 (SNP). Two other flanking markers ARGH17 (CAPS) and 48K16T7 (SCAR) cover the region of the *Vr2-A* gene. ARGH17 and GmTNL1 were mapped closest to both regions of the resistance locus, *Vr2-A* (0.3 cM) and *Vr2-C* (0.2 cM), respectively (Galli et al., 2010).

MATERIALS AND METHODS

Biological material studied

Forty apple cultivars ('Alex', 'Aura', 'Auriu de Cluj', 'Bistriţean', 'Cezar', 'Ciprian', 'Colmar', 'Colonade', 'Dacian', 'Dany', 'Delicios de Voineşti', 'Discoprim', 'Doina', 'Estival', 'Frumos de Voineti', 'Generos', 'Inedit', 'Iris', 'Irisem', 'Ionaprim', 'Luca', 'Nicol', 'Pomona', 'Precoce de Ardeal', 'Productiv de Cluj', 'Real', 'Rebra', 'Redix', 'Revidar', 'Remar', 'Remus', 'Romus 3', 'Romus 4', 'Romus 5', 'Rustic', 'Salva', 'Starkprim', 'Voinicel', 'Voinea', 'Valery'), which belong to the gene bank located at Research Institute for Fruit Growing Pitesti, Romania were used in the study.

Three leaf samples from each three trees per cultivar were collected. For the identification of the scab resistance genes, *Rvi8* and *Rvi11*, the following SCAR markers were applied: OPB18 and K08, respectively (Table 1).

'*Malus baccata jackii*' was used as a positive control for the K08 marker associated with the scab resistance gene *Rvi11*.

Chemical material

The DNA was extracted according to the recommended working method of the "Isolate II Plant DNA Kit" protocol. For the migration of the amplified fragments, a 3% agarose gel was prepared in TBE 1X buffer and subsequently stained with "RedSafe Staining" Nucleic Acid. The amplification of the reactions was performed using the "2x PCRBIO Taq Mix Red" kit from Biosystems.

PCR amplification

PCR amplification was performed using the "FastGene" analyzer. The PCR reaction was performed separately for each of the primer pairs, in a reaction volume of 15 µl, of which: 12 µl "2x PCRBIO Taq Mix Red"; 0.1 µl from every marker and 3 µl DNA (75 ng/µl) for both markers. The reaction conditions were: initial denaturation at 94°C for 3 min; 40 cycles of 1 min at 94°C, 1 min at Tm and 2 min at 72°C; final extension of 10 min at 72°C (65°C for K08, 55°C for OPB18).

Table 1. SCAR molecular markers for *Rvi8* and *Rvi11* genes

Gene	Name primer	Primer sequences	Fragment size (bp)	References
Rvi8 (Vh8)	OPB18	F: CCACAGCAGTCATTGGGA R: CCACAGCAGTGCATAAAC	628; **799**	Bus et al, 2005a
Rvi11(Vbj)	K08	F: GAACACTGGGCAAAGGAAAC R: TAAAAGCCACGTTCTCTCGC	**743;** 900	Gzgax et al., 2004

Evaluation of results

The fragments amplified following the PCR reaction were loaded in a volume of 10 µl for each sample, in the agarose gel and read with a high-quality "Uvitec Cambridge Essential" imaging system using UVITec1D analysis software. The duration of the sample migration was 4 hours at a voltage of 50 volts for gels with a concentration of 3% and a horizontal electrophoresis system "Wide Midi Horizontal Electrophoresis System" from Cleaver Scientific was performed.

Statistical analysis

Statistical analysis has been used to order varieties with polygenic characteristics into groups and subgroups. This grouping was made with the GeneAlex v software. 6.51b2. The genotype-phenotype correlation was calculated with the Pearson correlation coefficient, using Minitab18 software.

The statistical analysis of allelic polymorphism, taking into account only the dominant allele of the genes of interest, was expressed using the PIC index (Content of polymorphic

information), which takes into account the allelic frequency, being calculated using mathematical expression: 2f (1-f). Two statistical indices were used to quantify genetic diversity: the Shannon index and the Simpson index. The Shannon index was calculated with the mathematical formula: - $\sum_{i=1}^{n} {}^{n}/_{N} * ln\, n/N$ and the Simpson index with the formula: ($\frac{\sum_{i=1}^{n} n*(n-1)}{N*(n-1)}$), where: n represents the allele at the monolocus level, and N - the total number of alleles (Shannon et al., 1948; Simpson, 1960).

RESULTS AND DISCUSSIONS

Rvi8. The SCAR markers OPB18 and OPB19 are indicators for *Rvi8* and *Rvi2* genes. Iancu et al. (2022, 2023) using marker OPL19 reported an allele size of 433 bp for *Vr* gene (*Rvi2* or *Rvi8*) for the for twenty cultivars: 'Alex', 'Bistrițean', 'Cezar', 'Ciprian', 'Dany', 'Discoprim', 'Delicios de Voinești', 'Estival', 'Jonaprim', 'Luca', 'Pomona', 'Romus 3', 'Romus 4', 'Romus 5', 'Redix', 'Remar', 'Salva', 'Starkprim', 'Voinicel', 'Voinea'.

The segregation of the OPB18 marker with the gene locus made the difference between the *Rvi8* and *Rvi2* genes, producing the amplification of the 799 bp fragment associated with the dominant allele of the *Rvi8* gene, only. This gene did not segregate into the apple cultivars studied, and, in order to confirm our results, other two cultivars 'Verzișoare' and 'Pionier' were, supplementary, introduced, as control. The OPL18 marker amplified the 799 bp fragment corresponding to the dominant allele of the *Rvi8* gene for both control cultivars (Figure 1).

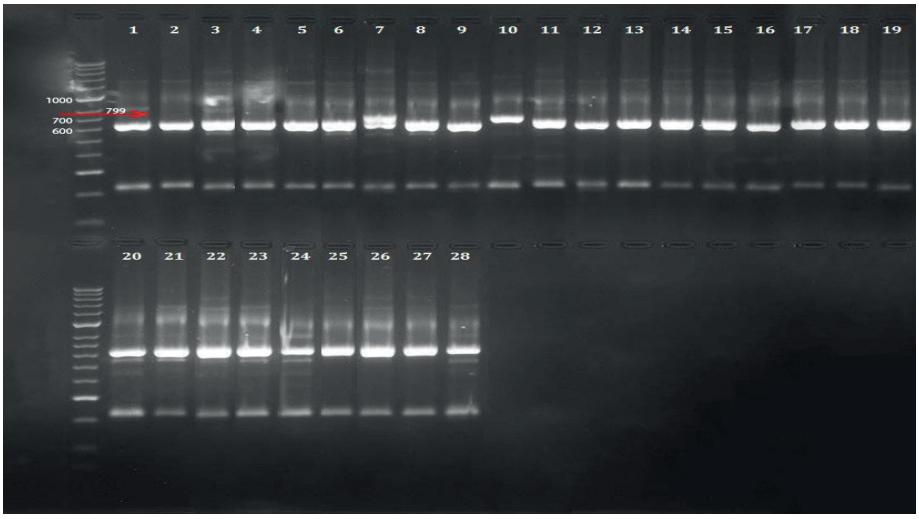

Figure 1. Electrophoretic profile performed with the OPB18 marker for genotypes in which specific amplifications of the Vr locus were obtained with the OPL19 marker:
1. 'Romus 2', 2. 'Romus 3', 3. 'Romus 4', 4. 'Romus 5', 5. 'Florina', 6. 'Prima', 7. 'Pionier', 8. 'Starkrimson', 9. 'Creţesc', 10. 'Verzișoare', 11. 'Parmen d'or', 12. 'Estival', 13. 'Bistrițean', 14. 'Dany', 15. 'Luca', 16. 'Ciprian', 17. 'Salva', 18. 'Ionaprim', 19. 'Starkprim', 20. 'Delicios de Voinești', 21. 'Redix', 22. 'Alex', 23. 'Voinicel', 24. 'Voinea', 25. 'Discoprim', 26. 'Cezar', 27. 'Pomona', 28. 'Remar'

Rvi11. The codominant marker K08 was segregated into 19 cultivars ('Alex', 'Bistrițean', 'Colonade', 'Ciprian', 'Colmar', 'Dany', 'Dacian', 'Estival', 'Generos', 'Irisem', 'Nicol', 'Pomona', 'Precoce de Ardeal', 'Productiv de Cluj', 'Rebra', 'Romus 3', 'Remus', 'Redix', 'Remar') and *'Malus baccata jackii',* as positive control. This marker made the difference between heterozygous and homozygous genotypes by amplifying the 743 bp and 900 bp, respectively: 11 were heterozygous with both dominant and recessive alleles, and 8 were homozygous with dominant alleles (Figure 2).

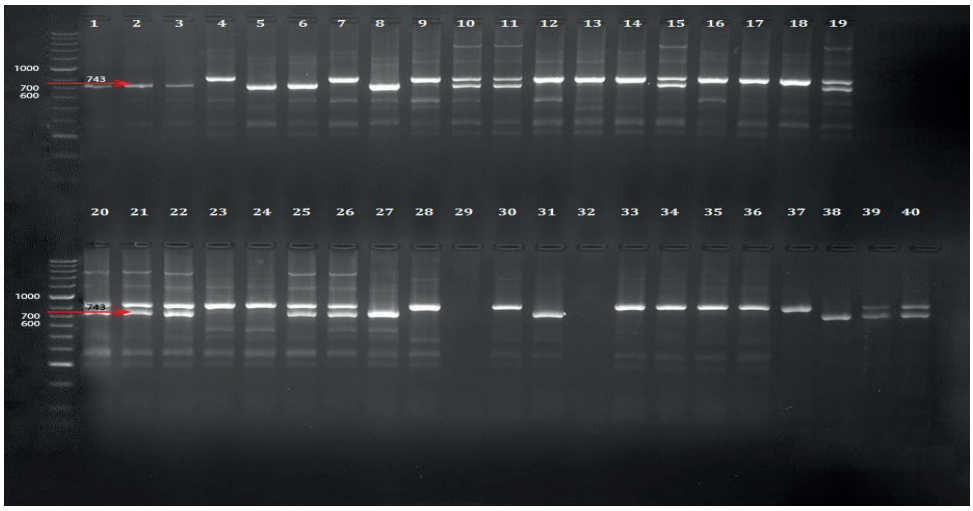

Figure 2. Electrophoretic profile released with the K08 marker:
1. 'Estival', 2. 'Rebra', 3. 'Bistriţean', 4. 'Aura', 5. 'Romus 3', 6. 'Dany', 7. 'Romus 4', 8. 'Productiv de Cluj', 9. 'Luca', 10. 'Ciprian', 11. 'Irisem', 12. 'Slava', 13. 'Ionaprim', 14. 'Rustic', 15. 'Precoce de Ardeal', 16. 'Iris', 17. 'Starkprim', 18. 'Auriu de Cluj', 19. 'Generos', 20. 'Colonade', 21.'Nicol', 22. 'Colmar', 23. 'Delicios de Voineşti', 24. 'Romus 5', 25. 'Remus', 26. Redix'', 27. 'Alex', 28. 'Doina', 29. 'Voinicel', 30. 'Inedit', 31. 'Dacian', 32. 'Voinea', 33. 'Valery', 34. 'Real', 35. 'Discoprim', 36. 'Cezar', 37. 'Frumos de Voineşti', 38. 'Pomona', 39. 'Revidar', 40. 'Remar'

By cumulating the present with previous results, we obtained a scab resistance genetic profile for all forty apple cultivars (Table 2). The data showed different scab gene accumulations, such as: tetragenic *(Rvi2+Rvi4+Rvi6+Rvi11)*, trygenic *(Rvi2+Rvi4+Rvi6*; *Rvi2+Rvi4+Rvi11*; *Rvi2+Rvi6+Rvi11)*, digenic *(Rvi2+Rvi6*; *Rvi6+Rvi11; Rvi5+Rvi11)*, monogenic (*Rvi2*; *Rvi6*; *Rvi11).* For two cultivars ('Frumos de Voinesti' and 'Auriu de Cluj') which did not show any scab resistance genes.

Table 2. Scab resistance genetic profile

Cultivar	*Rvi8*[a] OPB18	*Rvi11*[a] K08	*Rvi2*[b] OPL19	Genetic profile[a,b]
Estival	.-	+	+	*Rvi2+Rvi4 +Rvi11*
Rebra	-	+	-	*Rvi6+Rvi11*
Bistriţean	.-	+	+	*Rvi2+Rvi6 +Rvi11*
Aura	-	-	-	*Rvi6*
Romus 3	.	+	+	*Rvi2+Rvi6+Rvi4+Rvi11*
Dany	.	+	+	*Rvi2+Rvi6 +Rvi11*
Romus 5	.	-	+	*Rvi2+Rvi6+Rvi4*
Productiv de Cluj	-	+	-	*Rvi11*
Luca	-	-	+	*Rvi2+Rvi6*
Ciprian	-	+	+	*Rvi2+Rvi6 +Rvi11*
Irisem	-	+	-	*Rvi5+ Rvi11*
Salva	-	-	+	*Rvi2+Rvi6*
Ionaprim	-	-	+	*Rvi2+Rvi6*
Rustic	-	-	-	*Rvi6*
Precoce de Ardeal	-	+	-	*Rvi11*
Iris	-	-	-	*Rvi6*
Starkprim	-	-	+	*Rvi2+Rvi6*
Auriu de Cluj	-	-	-	-
Generos	-	+	-	*Rvi5+ Rvi11*
Colonade	-	+	-	*Rvi6+Rvi11*
Nicol	-	+	-	*Rvi5+ Rvi11*
Colmar	-	+	-	*Rvi6+Rvi11*
Delicios de Voineşti	-	-	+	*Rvi2*
Romus 4	.	-	+	*Rvi2+Rvi6*

Remus	-	+	-	*Rvi11*
Redix	-	+	+	*Rvi2+Rvi6+Rvi4+Rvi11*
Alex	-	+	+	*Rvi2+Rvi6 +Rvi11*
Doina	-	-	-	*Rvi6*
Voinicel	-	-	+	*Rvi2+Rvi6+Rvi4*
Inedit	-	-	-	*Rvi6*
Dacian	-	+	-	*Rvi6+Rvi11*
Voinea	+		-	*Rvi2+Rvi6*
Valery	-	-	-	*Rvi6*
Real	-	-	-	*Rvi6*
Discoprim	-	-	+	*Rvi2+Rvi6+Rvi4*
Cezar	-	-	+	*Rvi2+Rvi6+Rvi4*
Frumos de Voineşti	-	-	-	-
Pomona	-	+	+	*Rvi2+Rvi6+Rvi4+Rvi11*
Revidar	-	-	-	*Rvi6+Rvi11*
Remar	-	+	+	*Rvi2+Rvi6+Rvi4+Rvi11*

[a]Molecular screening conducted in this study
[b]Iancu et al., 2023

In figure 3, using the statistical method PCoA analysis (standard covariance) with the help GeneAlex v software. 6.51b2, it can observe the distribution of cultivars which carried dominant alleles for scab resistance in groups and subgroups.

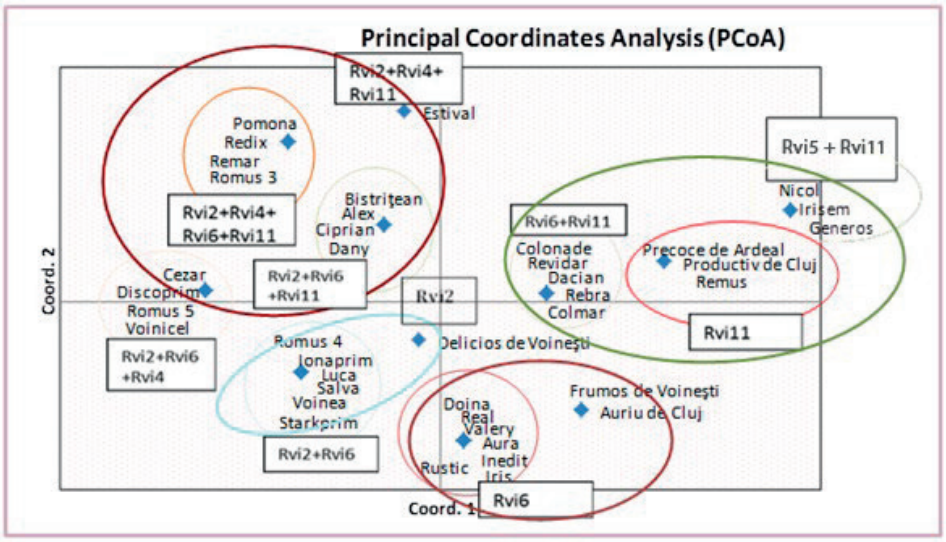

Figure 3. Distribution of cultivars based on R genes screening

Correlating phenotypic data presenting by Militaru et al. (2022) with our genotype results, we conclude that five of the monogenic varieties (‘Aura’, ‘Inedit’, ‘Iris’, ‘Valery, ‘Real’) have a scab resistant response, while the varieties ‘Doina’ and ‘Rustic’, with the same monogenic characteristic (*Rvi6* gene), show a moderate response.

Some varieties with trigenic characteristics from the subgroup “*Rvi2+Rvi6+Rvi11*” (’Bistritean’ and ’Ciprian’) are scab resistant, while ’Dany’ and ’Alex’ varieties, for same subgroup, manifest a phenotypic response of moderate resistance.

’Colmar’, ’Colonade’, ’Revidar’ and ’Dacian’ of the subgroup “*Rvi6+Rvi11*” are resistant to apple scab, while the variety ‘Rebra’ of the same subgroup shows a “moderate resistance”.

Also, varieties with digenic characteristics (’Ionaprim’, ’Starkprim’, ’Luca’, ’Voinea’ and ’Romus 4') from the subgroup "*Rvi2+Rvi6*" are scab resistant, instead, the ‘Salva’ variety, from the same subgroup, shows a phenotypic response as ”moderate resistance”.

The varieties with monogenic characteristics 'Precoce de Ardeal' and 'Productiv de Cluj' (*Rvi11*) have a moderate resistant to apple scab, while 'Remus' (*Rvi11*) and 'Delicious de Voinesti' (*Rvi2*) are scab susceptible.

The disease susceptibility response positions the 'Redix' cultivar in the category of plants known as GPI (Genotype-Phenotype Incongruence) as the genotyping result is unexpected, this having a polygenic character: *Rvi2+Rvi4+Rvi6+Rvi11*. The gene combination "*Rvi2+Rvi11*" is not enough to produce an immune response to the attack of the pathogen, *Venturia inaequalis*. So, the varieties 'Nicol', 'Generos' and 'Irisem' are susceptible to this disease. Using as statistical method the correlation, Pearson coefficient correlated with Minitab18 software, the combination of genes "*Rvi2+Rvi4+Rvi11*" is in perfect correlation with the phenotypic expression manifested by 'Estival' cultivar.

Significant results were also obtained for the combinations of genes "*Rvi2+Rvi6+Rvi11*" and "*Rvi2+Rvi4+Rvi6+Rvi11*" (Figure 4).

	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
C13	0.031	-0.093	-0.217	0.450	0.015	-0.221	-0.190	-0.202	0.372	0.238	0.277
	0.850	**0.568**	0.179	0.004	**0.927**	0.170	0.241	0.212	0.018	0.139	0.083

Figure 4. Pearson correlation between genotypes and phenotypes:
C2 (Rvi2+Rvi4+Rvi6+Rvi11: 'Pomona', 'Redix', 'Remar', 'Romus 3'); C3 (Rvi2+Rvi6+Rvi11: 'Alex', 'Bistriţean', 'Ciprian', 'Dany'); C4 (Rvi2+Rvi4+Rvi6: 'Cezar', 'Discoprim', 'Romus 5', 'Voinicel'); C5 (Rvi5+Rvi11: 'Generos', 'Irisem', 'Nicol'); C6 (Rvi2+Rvi4+Rvi11: 'Estival'); C7 (Rvi2+Rvi6: 'Ionaprim', 'Luca', 'Romus 4', 'Salva', 'Starkprim', 'Voinea'); C8 (Rvi6+Rvi11: 'Colonade', 'Colmar', 'Dacian', 'Rebra', 'Revidar'); C9 (Rvi6: 'Aura', 'Doina', 'Inedit', 'Iris', 'Real', 'Rustic', 'Valery'); C10 (Rvi2: 'Delicios de Voineşti'); C11 (Rvi11:' Precoce de Ardeal', 'Productiv de Cluj', 'Remus'); C12 (-); C13 (phenotipic expression)

Genetic diversity and allelic polymorphism were calculated only for the dominant allele of interest genes. The results showed moderately informative polymorphism and low genetic diversity (Table 3). The small value for genetic diversity is explained by the fact that the same gene has been highlighted in several varieties.

Table 3. Statistical analysis of allelic polymorphism and genetic diversity

PIC value>0.25 (moderately informative)	Shannon Index H > 3 (high genetic diversity)	Simson Index (D) D=0 (infinite diversity) D=1 (lack of diversity)	Simpson diversity index (1-D) value Є [0.1]
0.333542	1.294129	0.2494129	0.759944

CONCLUSIONS

In Romania, the major problem of apple production is scab caused by fungal pathogen, *Venturia inaequalis*. In order to solve this problem, the most Romanian breeding programs included releasing of new cultivars with scab resistance, as major objective. So, the present data complete the information about using of Romanian bred cultivars as a source of diverse functional alleles involved in quantitative resistance against *V. inaequalis* strains. Using K08 marker, it has been established that 19 apple cultivars carriers of *Rvi11* gene and, using OPB18 marker, is not obtained amplified for *Rvi8*. Genetic diversity of the dominant characteristic for the gene of interest is reduced and allelic polymorphism has a moderately informative value. Segregation of the genes of interest into descendants allowed for validation of the identity of the genitors.

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