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SCREENING-UL UNOR SOIURI ROMÂNEȘTI DE MĂR PENTRU GENELE DE REZISTENȚĂ LA RAPĂN

MOLECULAR SCREENING OF SOME ROMANIAN APPLE CULTIVARS FOR SCAB RESISTANCE GENES

Militaru Mădălina, Sturzeanu Monica, Iancu Adina*
Research Institute for Fruit Growing Pitesti, Romania
*Corresponding author: Iancu Adina, email: aiancu@icdp.ro

Abstract

Apple scab, incided by the fungus *Venturia inaequalis* (Cke.) Wint., is a devastating disease of apple reported from almost all apple producing Romanian areas, which causes up to 70% losses of production. Molecular markers were used for detection of scab resistance genes in 22 old and introduced apple cultivars ('Romus 3', 'Romus 5', 'Rebra', 'Rustic', 'Nicol', 'Colmar', 'Colonade', registered by Research Institute for Fruit Growing Pitesti; 'Generos', 'Iris', 'Irisem', 'Luca', 'Ciprian', 'Cezar', 'Remar', 'Valery', 'Real', registered by Research Station for Fruit Growing Voinesti, Dambovita; 'Aura', 'Starkprim', 'Ionaprim', 'Bistritean', registered by Research Station for Fruit Growing Bistrita and old cultivars: 'Domnesc', 'Cretesc'). The presence of scab resistance genes were detected using the molecular markers: AL-07 (SCAR), AM19 (SCAR), VfC for *Rvi6* (*Vf)* gene, AD13 (SCAR) for *Rvi4* (*Vr1)* gene, OPL19 (SCAR) for *Rvi2 (Vh2)* and *Rvi8 (Vh8)* genes and OPB12 (STS) for *Rvi5 (Vm)* gene. The *Rvi6* gene was detected in 17 cultivars from different breeding center. The marker AD13 presents in genome of 8 cultivars, such as 'Romus 3', 'Romus 5', 'Generos', 'Iris', 'Irisem', 'Cezar', 'Remar', 'Aura'. The *Rvi5* gene was revealed in 3 cultivars ('Nicol', 'Generos', 'Irisem'), only.

Cuvinte cheie: *Malus x domestica*, marker, ameliorare.
Keywords: *Malus x domestica*, marker, breeding.

1. Introduction

Apples (*Malus x domestica* Borkh.) are one of the most important horticultural crop grown in temperate areas and most commonly consumed fruits in the world (Ferree and Warrington, 2003; Muneer et al., 2017). Apple is host to a wide range of pests and diseases and most of the commercial cultivars are susceptible. Scab disease, caused by the ascomycete fungus *Venturia inaequalis* (Cke) Wint. (anamorph: *Spilocaea pomi* Fries), negatively affects fruit size and quality, due to blemishes and poor ripening. Managing apple scab requires numerous treatments during the growing season and, unfortunately, the pathogen has already developed resistance to many classes of fungicides.

Several resistance genes were identified from wild cultivars or other progenies. An important source of resistance to scab is *Vf* gene (renamed *Rvi6*), derived from *Malus floribunda* 821 (Bus et al., 2009). It has been used extensively as an important source of scab resistance in commercial cultivars (Crosby et al., 1992), but a new scab race, designated race 6, overcomes *Vf* resistance, although the resistance of *M. floribunda* 821 itself remains effective (Parisi et al., 1993). It is suggested that *M. floribunda* 821 contains an additional gene for hypersensitivity (*Vfh*), which was lost early in breeding efforts (Parisi and Lespinasse, 1996). The *Rvi4* (old named *Vh4* = *Vx* = *Vr1*) gene was identified in the F_2 derivative $TSR_{33}T_{239}$ of Russian apple (Vincent et al., 2011). The *Rvi2* (old named *Vh2* = *Vr-A*) and *Rvi8 (Vh8)* genes are closely linked, if not allelic, but are clearly separate genes. A gene-for-gene (GfG) relationship was demonstrated for *Rvi8* gene from *Malus sieversii* originated from the Tarbagatai mountain range in Kazahhstan (Vincent et al., 2011). Another important source of resistance to scab is *Rvi5* (*Vm*) gene derived from the accession *Malus x atrosanguinea* 804 and *M. x micromalus* 245-38 (Dayton and Williams, 1970).

For durable resistance, new cultivars need to have several resistance genes incorporated. By traditional breeding, different sources of resistance can be introduced into one genotype.

The aim of this study was to screen 22 apple cultivars for the presence of five scab resistance genes employing a polymorphic sequence characterized amplified regions (SCAR) and sequence-tagged sites (STS) markers.

2. Material and methods

In our experiment, 22 apple cultivars were used for molecular screening. Two of all cultivars, 'Domnesc' and 'Cretesc', are known as old and scab susceptible cultivars (Table 1).

Young leaves from each cultivar were collected in plastic bags direct from an orchard at Genetic and Breeding Department of Research Institute for Fruit Growing Pitești, Romania. Total DNA was extracted from each cultivar using the kit extraction Isolate II Plant DNA (Bioline). Multi-locus genotyping of five apple scab resistance genes (*Rvi2, Rvi4, Rvi5, Rvi6, Rvi8*) was employed in all the cultivars using five SCAR markers and one STS marker (table 2).

The apple fresh grinded with liquid nitrogen was distributed into a tube of 2mL (100 mg) and kept at -80^{0}C. The amplification reaction of DNA was carried out using the MyTaq^{TM} Red Mix in 0.2-mL tubes with 25µL final reaction volume and 18-20 ng of genomic DNA. Amplifications were performed in a Termocycler PCR FastGene Ultra Cycler Gradient. The amplification was performed after the following programs: AL07 and AM19 markers (initial denaturation step at 95^{0}C for 1 min., followed by 35 cycles of 1 min at 94^{0}C, 1 min at 60^{0}C, 2 min. at 72^{0}C and final extension 10 min at 72^{0}C); VfC marker (initial denaturation step at 94^{0}C for 4 min., followed by 30 cycles of 1 min at 94^{0}C, 1 min at 58^{0}C, 1 min at 72^{0}C and final extension 7 min at 72^{0}C); AD13 marker (initial denaturation step at 94^{0}C for 2.45 min., followed by 30 cycles of 1 min at 94^{0}C, 3 min at 58^{0}C, 2 min at 72^{0}C, followed by 1 cycle of 1 min at 94^{0}C, 3 min at 58^{0}C, 10 min at 72^{0}C); OPL19 marker (initial denaturation step at 94^{0}C for 2.45 min., followed by 40 cycles of 55 s at 94^{0}C, 55 s at 55^{0}C, 1.39 min at 72^{0}C and final extension 10 min at 72^{0}C); OPB12 marker (initial denaturation step at 94^{0}C for 2.20 min., followed by 35 cycles of 30 s at 94^{0}C, 1 min at 55^{0}C, 1 min at 72^{0}C and final extension 8 min at 72^{0}C).

The amplified fragments were resolved in 2% agarose gel in a 10x TBE buffer, stained with midori green advance DNA Stain and visualization to combine Essential-V6WL26MX UV. The bands on the gels were transformed in binary data by being scored as ”+” for the presence and ”-” for absence of alleles for each cultivar per marker.

3. Results and discussions

Molecular markers linked to apple scab resistance would accelerate breeding by making it possible to select resistant individuals, regardless of the growth stage, interaction between resistance genes, source of inoculum or environmental conditions (Cheng et al., 1998).

To confirm the presence of *Rvi6* (*Vf*) gene were used three markers: AL07, AM 19 and VfC. Marker AL07 produced two bands of 823 bp represents the recessive *vf* allele and of 570 bp represents the dominant allele *Vf*, so, it can be used for identification of homozygous and heterozygous genotypes. For all studied cultivars, primers for this marker were detected in 17 cultivars. It was absent in susceptible cultivars like: 'Nicol', 'Generos', 'Irisem', 'Domnesc' and 'Crețesc' (Figure 1, Table 3).

Primers for AM19 and VfC markers led to amplication of 526 bp (Figure 2, Table 3), respectively 286, 484 and 646 bp (Figure 3, Table 3) in 17 cultivars like: 'Romus 3', 'Romus 5', 'Rebra', 'Rustic', 'Colmar', 'Colonade', 'Iris', 'Luca', 'Ciprian', 'Cezar', 'Valery', 'Real', 'Remar', 'Aura', Starkprim', 'Jonaprim', 'Bistrițean'. The molecular marker AM19 proved to be highly useful because of its ability to distinguish resistant and susceptible cultivars on the basis of presence or absence of single band of gel.

To confirm *Rvi4* gene was used AD13 marker which amplified two fragments 950 bp and 1,200 bp only in 8 cultivars: 'Romus 3', 'Romus 5', 'Generos', 'Iris', 'Irisem', 'Cezar', 'Remar', 'Aura' (Figure 4, Table 3).

Marker OPL19 amplified two fragments: 433 bp and 1,200 bp (figure 5, table 3) in 13 cultivars ('Romus 3', 'Romus 5', 'Luca', 'Ciprian', 'Cezar', 'Real', 'Remar', 'Aura', 'Starkprim', 'Jonaprim', 'Bistrițean', 'Domnesc', 'Crețesc'). Two of them ('Domnesc' and 'Crețesc' cvs.) are very susceptible to apple scab in the field.

Marker OPB12 amplified 850 bp (figure 6, table 3) in only three cultivars: 'Nicol', 'Generos' and 'Irisem', susceptible and resistant to apple scab. Because OPL19 and OPB12 markers amplicon in both, susceptible and resistant genotypes, hence they cannot be use to distinguish between resistant and susceptible ones.

4. Conclusions

Romania has a large variety of apple cultivars released in different breeding programs for resistance to apple scab. The phenotypic evaluation of scab apple resistance is necessary to be complete with molecular screening using gene specific markers.

An examination of 22 *Malus x domestica* cultivars, including phenotypic susceptible and resistant cultivars, showed that they contained different PCR products. In our study, the frequencies of the

detected resistance alleles were about 59% for *Rvi2* and *Rvi8*, 36% for *Rvi4*, 13.5% for *Rvi5* and 77% for *Rvi6*.

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Tables and Figures

Table 1. Origin of Romanian apple cultivars

Cultivar	Parentage	Field phenotypic resistance to apple scab
Romus 3	unknown	resistant
Romus 5	Romus 3 x Prima	resistant
Rebra	Prima x Florina	moderately resistant
Rustic	Florina x Pionier	moderately resistant
Nicol	Mc Intosh Wijcik x Pionier	susceptible
Colmar	Mc Intosh Wijcik x Florina	resistant
Colonade	Florina x Mc Intosh Wijcik	resistant
Generos	(Parmain d'or x *M. kaido*) x (Jonathan x V53-39-2[a]) x (Frumos de Voinesti x V60-6-51[a])	low susceptible
Iris	gamma radiation (8000 ϒ) of Prima seeds (o.p.)	resistant
Irisem	Starkrimson x Prima	low susceptible
Luca	Champion x Prima	resistant
Ciprian	Prima x Starkrimson	resistant
Cezar	Prima x Starkrimson	resistant
Remar	gamma radiation (5000 ϒ) of Prima seeds (o.p.)	resistant
Valery	Goldenspur x Florina	resistant
Real	gamma radiation (8000 ϒ) of Prima seeds (o.p.)	resistant
Aura	Prima x BN 33/39	resistant
Starkprim	Starkrimson x Prima	resistant
Jonaprim	Jonathan x Prima	resistant
Bistrițean	Starkrimson x Prima	resistant
Crețesc	unknown	high susceptible
Domnesc	unknown	high susceptible

[a] Unknown parentage
o.p. = open-pollinated

Table 2. Primers used for amplification of scab resistant genes

Gene	Name/ type marker	Primer sequence (5'→3')	Fragment size (bp)	References
Rvi6 (Vf)	AL07/ SCAR	F: TGGAAGAGAGATCCAGAAAGTG R: CATCCCTCCACAAATGCC	570; 823	Khajuria *și colab.*, 2014 Tartarini *și colab.*, 1999
Rvi6 (Vf)	AM19/ SCAR	F: CGTAGAACGGAATTTGACAGTG R: GACAAAGGGCTTAAGTGCTCC	526	Khajuria *și colab.*, 2014 Tartarini *și colab.*, 1999
Rvi6 (Vf)	VfC/ SCAR	F: GGTTTCCAAAGTCCAATTCC R: CGTTAGCATTTTGACTTGAC	286; 484; 646	Afumian și colab., 2004
Rvi4 (Vr1,Vh4, Vx)	AD13/ SCAR	F: GGTTCCTCTGTAAAGCTAG R: GGTTCCTCTGCCCAACAA	950; 1200	Boudichevskaia și colab., 2006
Rvi2 (Vh2) Rvi8 (Vh8)	OPL19 / SCAR	F: ACCTGCACTACAATCTTCACTAATC R: GACTCGTTTCCACTGAGGATATTTG	433; 1200	Bus și colab., 2005 a
Rvi5 (Vm)	OPB12 / STS	F: CCTTGACGCAGCTT R: CCTTGACGCATCTACG	687	Cheng și colab., 1998

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Table 3. Results of the molecular screening of the Romanian apple cultivars for scab resistance using molecular markers

Cultivar	*Rvi2*	*Rvi4*	*Rvi5*	*Rvi6*			*Rvi8*
	OPL19	AD13	OPB12	AL07	AM19	VfC	OPL19
Romus 3	+	+	-	+	+	+	+
Romus 5	+	+	-	+	+	+	+
Rebra	-	-	-	+	+	+	-
Rustic	-	-	-	+	+	+	-
Nicol	-	-	+	-	-	-	-
Colmar	-	-	-	+	+	+	-
Colonade	-	-	-	+	+	+	-
Generos	-	+	+	-	-	-	-
Iris	-	+	-	+	+	+	-
Irisem	-	+	+	-	-	-	-
Luca	+	-	-	+	+	+	+
Ciprian	+	-	-	+	+	+	+
Cezar	+	+	-	+	+	+	+
Remar	+	+	-	+	+	+	+
Valery	+	-	-	+	+	+	-
Real	+	-	-	+	+	+	+
Aura	+	+	-	+	+	+	+
Starkprim	+	-	-	+	+	+	+
Jonaprim	+	-	-	+	+	+	+
Bistrițean	+	-	-	+	+	+	+
Crețesc	+	-	-	-	-	-	+
Domnesc	+	-	-	-	-	-	+

+/- = the presence/absence of the respective amplified allelic fragment linked to each resistance gene (*Rvi2*, *Rvi4, Rvi5, Rvi6, Rvi8)* in each cultivar

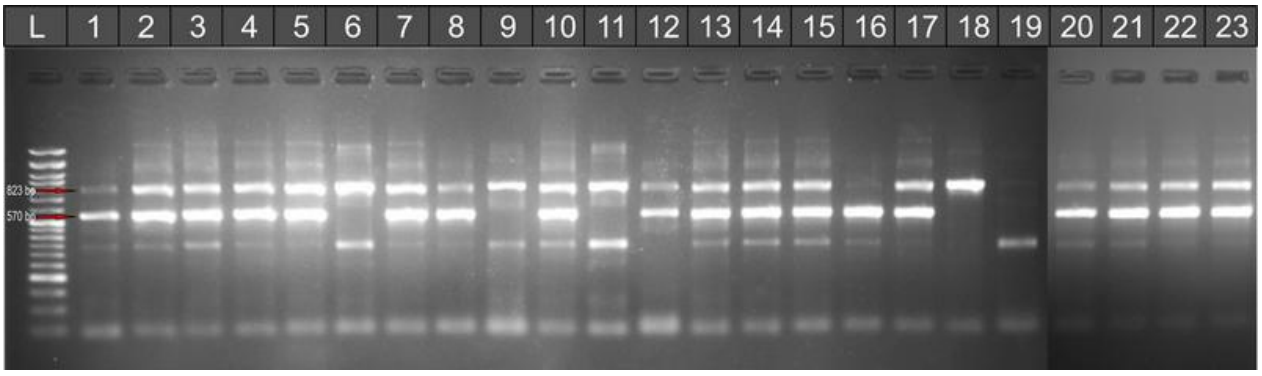

Fig. 1. Amplification profile of the marker AL07
(L–ladder 50 pb, 1-'Florina', 2-'Romus 3', 3-'Romus 5', 4-'Rebra', 5-'Rustic', 6-'Nicol', 7-'Colmar', 8-'Colonade', 9-'Generos', 10 - 'Iris', 11 - 'Irisem', 12 - 'Luca', 13 - 'Ciprian', 14-'Aura', 15-'Starkprim', 16-'Jonaprim', 17-'Bistrițean', 18-'Domnesc', 19-'Cretesc', 20-'Cezar', 21-'Remar', 22-'Valery', 23-'Real')

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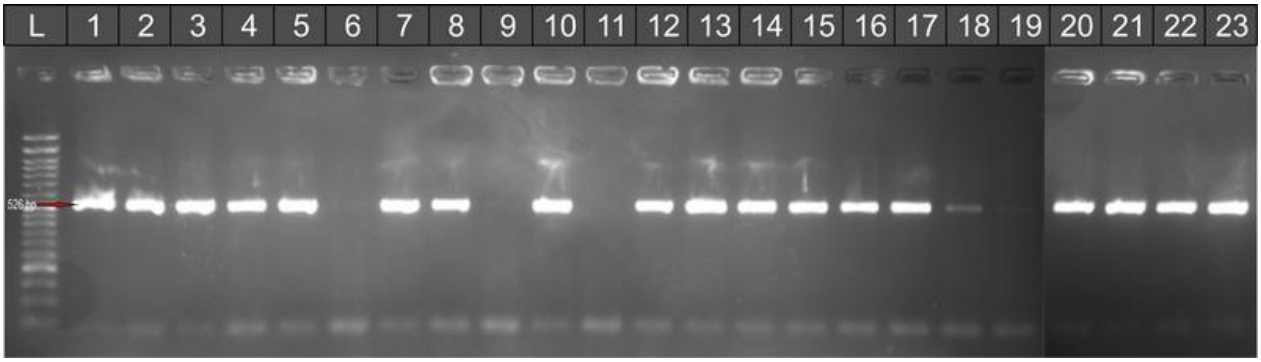

Fig. 2. Amplification profile of the marker AM19
(L–ladder 50 pb, 1-'Florina', 2-'Romus 3', 3-'Romus 5', 4-'Rebra', 5-'Rustic', 6-'Nicol', 7-'Colmar', 8-'Colonade', 9-'Generos', 10 - 'Iris', 11 - 'Irisem', 12 - 'Luca', 13 - 'Ciprian', 14-'Aura', 15-'Starkprim', 16-'Jonaprim', 17-'Bistrițean', 18-'Domnesc', 19-'Cretesc', 20-'Cezar', 21-'Remar', 22-'Valery', 23-'Real')

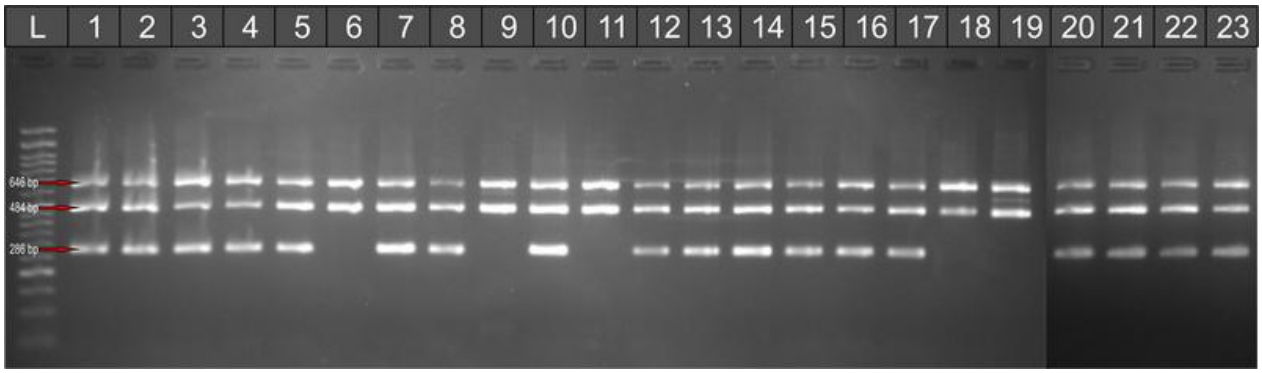

Fig. 3. Amplification profile of the marker VfC
(L–ladder 50 pb, 1-'Florina', 2-'Romus 3', 3-'Romus 5', 4-'Rebra', 5-'Rustic', 6-'Nicol', 7-'Colmar', 8-'Colonade', 9-'Generos', 10 - 'Iris', 11 - 'Irisem', 12 - 'Luca', 13 - 'Ciprian', 14-'Aura', 15-'Starkprim', 16-'Jonaprim', 17-'Bistrițean', 18-'Domnesc', 19-'Cretesc', 20-'Cezar', 21-'Remar', 22-'Valery', 23-'Real')

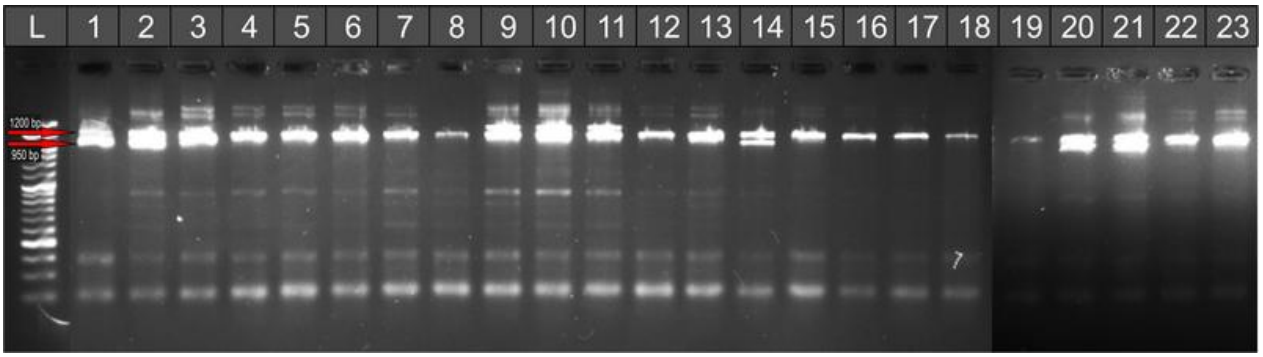

Fig. 4. Amplification profile of the marker AD13
(L–ladder 50 pb, 1-'Florina', 2-'Romus 3', 3-'Romus 5', 4-'Rebra', 5-'Rustic', 6-'Nicol', 7-'Colmar', 8-'Colonade', 9-'Generos', 10 - 'Iris', 11 - 'Irisem', 12 - 'Luca', 13 - 'Ciprian', 14-'Aura', 15-'Starkprim', 16-'Jonaprim', 17-'Bistrițean', 18-'Domnesc', 19-'Cretesc', 20-'Cezar', 21-'Remar', 22-'Valery', 23-'Real')

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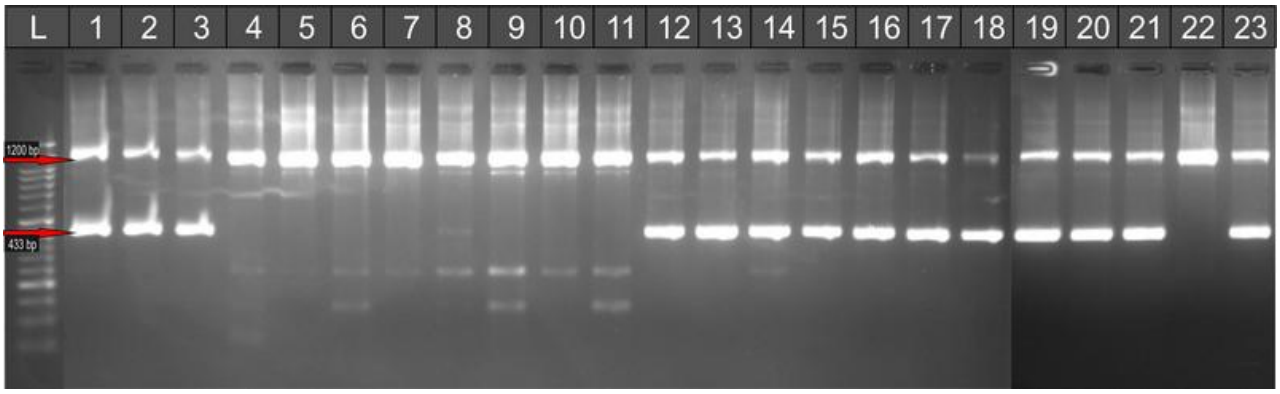

Fig. 5. Amplification profile of the marker OPL19
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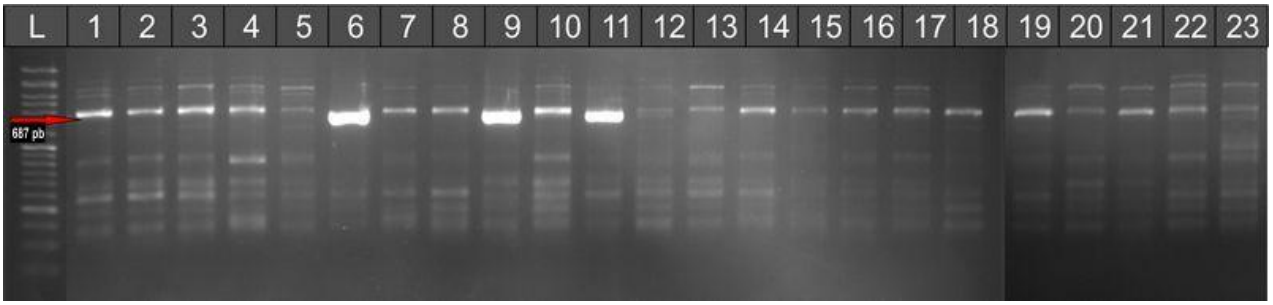

Fig. 6. Amplification profile of the marker OPB12
(L–ladder 50 pb, 1-'Florina', 2-'Romus 3', 3-'Romus 5', 4-'Rebra', 5-'Rustic', 6-'Nicol', 7-'Colmar', 8-'Colonade', 9-'Generos', 10 - 'Iris', 11 - 'Irisem', 12 - 'Luca', 13 - 'Ciprian', 14-'Aura', 15-'Starkprim', 16-'Jonaprim', 17-'Bistrițean', 18-'Domnesc', 19-'Cretesc', 20-'Cezar', 21-'Remar', 22-'Valery', 23-'Real')