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SELECȚIA VIRALĂ A STOCULUI PREBAZĂ-CANDIDAT A UNOR PORTALTOI ROMÂNEȘTI DE PRUN LA ICDP PITEȘTI-MĂRĂCINENI

VIRAL SELECTION OF PREBASIC - CANDIDATE STOCK OF SOME ROMANIAN ROOTSTOCKS AT RIFG PITEȘTI-MĂRĂCINENI

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Abstract

The viral evaluation of the Prebasic Candidate for 'Mirobolan dwarf', 'Mirobolan C5' and 'Mirodad 1' plum rootstock was carried out by the few methods. The biological method used *Cucumis sativus* grassy indicators monitored symptoms indicating the presence of *ApMV, PDV* and *PNRSV*. The serological ELISA method was applied for 2 consecutive years to identify the viruses: *ACLSV, ApMV, MLRSV, PPV, PDV* and *PNRSV*. For the *PPV* virus, was also applied AgriStrip immunochromatographic method. The presence of *ACLR* phytoplasma based by PCR using the primers P1/P7,5'-AAGAGTTTGATCCTGGCTCAGGATT-3',5'-CGTCCTTCATCGGCTCTT-3'. During the biological test, symptoms that could have been attributed to viral infections were observed to Mirobolan dwarf plant, the application of the DAS-ELISA test confirming the presence of an infection with the *PNRSV* virus.

Cuvinte cheie: teste virale, material Prebază, fitosanitar, virus, fitoplsma, test.
Key words: viral test, PreBasic material, phytosanitary, virus, phytoplasma, test.

1. Introduction

Candidate-Pre-Basic refers to plants that have undergone initial testing for viruses and diseases. This begins with selecting plants that have desired agronomic traits, are true to variety, and exhibit good visual health. The establishment of the "pest-free" status of Candidate-Pre-Basic plants is a very laborious process for a certification scheme, which starts with the Pre-Basic category and represents the upper level of clean biological material. This rigorous selection involves tests using various diagnostic methods, such as the use of biological indicators, the serological technique ELISA and molecular biology PCR, etc.

An extensive evaluation of major and minor pathogens, including viruses, is carried out using established standards, such as those of the EPPO (European and Mediterranean Plant Protection Organization). Viral evaluation is a complex process that must be based on the recommendations of specialized working groups, in this case the International Working Group on Fruit Tree Viruses Jelkmann, et al., (2004), EPPO standards, for the plum certification scheme EPPO STANDARDS [PM 4/30(1)]/2000 and for PM diagnosis [4/1(1)]/2004), PM [3/76(2)]/2021 and PM [7/62(3)]/2000 for ACLRV phytoplasma diagnosis and last but not least the actual legislation. At the moment, OM 784/2016; OM 119/2020, OM 40/2023 and OM 92/2025 are legislation for this field in Romania. These paper presents the viral evaluation of some Candidate-Pre-Basic plants, based on scientific and technical methods for establishing the virus-free status.

2. Material and methods

Biological material was represented by plum rootstocks: 'Mirobolan dwarf', 'Mirobolan C5' and 'Mirodad 1', creations of ICDP Pitești-Mărăcineni.

The working methods were:

The phytosanitary assessment was carried out based on the previously mentioned EPPO standards and the methodology published by Jelkmann, (2004);

- **Biological testing** was applied using the herbaceous biological indicator *Cucummis sativus* for the appearance of viral symptoms that could be produced by the viruses *Apple mosaic virus (ApMV); Prune dwarf virus (PDV); Prunus necrotic ringspot virus (PNRSV),* according to the recommendations of Jelkmann Wilhelm, 2004;

- **Serological test**-Enzyme Linked Immunosorbent Assay (DAS-ELISA) (Clark and Adams, 1977) was applied 2 consecutive years with a protocol recommended by the reagent manufacturer BIOREBA for

identification of *Apple chlorotic leaf spot viruses (ACLSV), Apple mosaic virus (ApMV), Plum pox virus (PPV), Prune dwarf virus (PDV), Prunus necrotic ringspot virus (PNRSV), Myrobolan latent ring spot virus (MLRSV)*;

- Immunochromatographic test - AGRISTRIP-BIOREBA protocol was applied for the identification of *Plum pox virus (PPV);*

- *Metoda de testare PCR* conform protocoalelor validate de EPPO PM [7/62(3)]/(2000) și EPPO (*Buletin,* [*48(3)]/(2004),* a fost aplicată pentru identificarea existenței phytoplasmei *Apricot chlorotic leaf roll (ACLR),* EPPO [PM 7/133] (1)/ (2018).

- **PCR test** according to the protocols validated by EPPO PM [7/62(3)]/(2000) and EPPO (Bulletin, [48(3)]/(2004), was applied to identify the existence of the Apricot chlorotic leaf roll (ACLR) phytoplasma, EPPO [PM 7/133] (1)/ (2018).

DNA isolation

DNA was isolated using the SDS extraction protocol recommended by the "Isolate II Plant DNA kit" and optimized with 1.5% beta-mercaptoethanol to prevent oxidation of phenols and other plant metabolites.

Conventional PCR protocol implementation

To improve the sensitivity of the PCR reaction, which may result in non-specific amplifications, was used the Nested PCR technique (two-step reaction or nested PCR). The use of two pairs of primers (external and internal) reduces the possibility of amplifying the wrong fragment. So, if the sequence amplified by the external pair of primers is non-specific, the probability that the target gene will be amplified by the internal pair of primers is very low. In the present study, the P1/P7 set was used as external primers, and the R16F2n/R16R2 set was used as internal primers. Both pairs of universal primers amplify the entire 16S rRNA gene between the 16S and 23S sequences and are recommended by EPPO (Table 1). The second universal primer pair P1A/P7A was also introduced used in many research studies but which does not have formal EPPO recognition as a validated method.

Amplification of PCR products for primer pairs P1/P7 and P1A/P7A was performed in a volume of 20 µl for each sample, consisting of: 3 µl DNA; 0.15 µl of each primer (0.75 µM in final volume); 12 µl "MyTaq™ Red DNA Polymerase" and 4.9 µl water. The reaction conditions were: initial denaturation at 94°C for 2 minutes; 35 cycles with: denaturation at 94°C for 2 minutes, annealing at 55°C for 2 minutes and extension at 72°C for 2 minutes; final extension at 72°C for 10 minutes.

The nested PCR reaction involved pipetting 2 µl of PCR product amplified with the primer pair P1/P7 over a volume of 16 µl consisting of: 0.2 µl of each internal primer (1 µM in the final volume), 12 µl "MyTaq™ Red DNA Polymerase" and 3.8 µl ultrapure water. The reaction conditions differed from those established for the first step by the shorter times configured for denaturation (94°C for 30 s), annealing (55°C for 1 min) and elongation (72°C for 1.5 min).

PCR product identification

The migration of PCR products was performed using a horizontal electrophoresis system, at a 3% agarose gel concentration, in TBE buffer (1 X). The image on the gel was saved digitally using a vertical transilluminator (Essential V6, Uvitech Cambridge), equipped with a camera and additional software.

3. Results and discussion

Biological testing

Observations carried out on indicator plants of *Cucummis sativus* showed the appearance of color changes that could be attributed to viral symptoms (Fig. 1).

Serological test

Serological testing DAS-ELISA, applied in 2023, for *ACLSV*, the cut-off value of 0.538, for *ApMV* the cut off was 0.583, for *PPV* 0.630 cut off, *PDV* 0.602 cut off and *MLRSV* with a cut off of 0.505. For 'Mirobolan dwarf' plant P8 (Table 2), where they observed the changes that could be attributed to viral infections, it was confirmed by the serological test that it was an infection with the *PNRSV* virus. In this sample, the absorbance value was 0.711 compared to the reference cut-off of 0.595. In the 'Mirobolan C5' plants (Table 3) and 'Mirodad 1' (Table 4), no absorbance values were recorded in the tested samples that exceeded the mentioned cut-off values.

Testing by the DAS-ELISA serological method, applied in 2024, the cut-off value was 0.660 *(ACLSV*), 0.508 (*ApMV*), 0.652 (*PPV*), 0.557 (*PDV*), 0.602 (*PNRSV*) and 0.631 (*MLRSV*). No positive samples were recorded for the 29 samples remaining in the viral evaluation process, of which: 9 'Mirobolan dwarf' samples (Table 5), 10 'Mirobolan C5' samples (Table 6) and 10 'Mirodad 1' samples (Table 7).

The Immunochromatographic method applied to identify *PPV* did not reveal positive results; during the evaluations performed, only one red line could be seen on the strips respectively the control line (Photo 4).

Molecular testing

Following amplification with the primer pair P1/P7, amplifications exceeding 1500 bp were obtained for samples P1, P2, P3, P4, P15 and P16 and which could correspond to the fragment of approximately 1784 bp, specific to the 16S rRNA/23S rRNA regions (Figure 1). The Nested PCR reaction increased the specificity of the reaction, allowing the identification of 5 positive samples (P1, P2, P3, P4 and P16), as a result of the amplification of the fragment of 1239 bp, associated with the 16S rRNA/23S rRNA region. The lack of this amplicon in sample P15 showed that it had a non-specific amplification with the primer pair P1/P7 (Fig. 2). For samples P1, P2, P3, P4, P10, P11, P15 and P16, the primer pair P1A/P7A amplified fragments that can be associated with the 1759 bp, assigned to the 16S rRNA gene region (Fig. 3).

***Electrophoretic profile - primer pair P1/P7*.**

In this profile appear the amplified fragments, exceeding 1500 bp for samples P1, P2, P3, P4, P15 and P16, but for confirmation of the 16S rRNA gene, it is recommended to use the Nested PCR technique using the internal primer pair R162nF/R162nR2.

***Electrophoretic profile - primer pair P1A/P7A*.**

Amplifications that can be associated with the fragment corresponding to the 1759 bp are indicated for samples P1, P2, P3, P4, P10, P11, P15 and P16, the fragment being assigned to the 16S rRNA/23S rRNA region (16Sr-23Sr Group).

Electrophoretic profile - primer pair R16F2n/R16R2.

Nested PCR increased the specificity of the method, allowing the identification of 5 positive samples (P1, P2, P3 – 'Mirobolan dwarf' and P4, P16 – 'Mirobolan C5'), as a result of the amplification of the fragment corresponding to 1239 bp. For samples P10, P11 and P15 it was shown that the amplifications obtained with the external primer pairs were non-specific.

4. Conclusions

The viral testing methods used each other reconfirmed the results obtained, the ELISA test demonstrated the viral existence of *PNRSV* appeared from the biological test; all 3 tests applied validated the absence of *PPV*.

Phytoplasma *ACLR* was identified by molecular method to 3 plants from 'Mirobolan dwarf' and 2 plants from 'Mirobolan C5'.

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Tables and Figures

Table 1. Primers used for the 16S/23S gene region of ribosomal RNA

Primer	Primer sequence	Amplified fragment	References
P1/P7	F: AAGAGTTTGATCCTGGCTCAGGATT; R: CGTCCTTCATCGGCTCTT	1784 bp	Deng & Hiruki, 1991; Schneider și colab., 1995
P1A/P7A	F: ACGCTGGCGGCGCGCCTAATAC; R: CCTTCATCGGCTCTTAGTGC	1759 bp	Lee și colab., 2004
R16F2n / R16R2	F: GAAACGACTGCTAAGACTGG; R: TGACGGGCGGTGTGTACAAACCCCG	1239 bp	Lee și colab., 1993; Gundersen și Lee, 1996

Table 2. Results of viral evaluation of Candidate-Pre-Basic plants by DAS-ELISA method for Mirobolan dwarf - 2023

No.	Samples	Viruses											
		ACLSV		*ApMV*		*PPV*		*PDV*		*PNRSV*		*MLRSV*	
		Absorbance value	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*
1	Mirobolan dwarf p1	0.304	0.538	0.308	*0.583*	0.300	0.630	0.296	0.602	0.316	0.595	0.267	0,503
2	Mirobolan dwarf p2	0.280		0,258		0.298		0.294		0.259		0.275	
3	Mirobolan dwarf p3	0.258		0.256		0.303		0.290		0.282		0.234	
4	Mirobolan dwarf p4	0.335		0.251		0.309		0.299		0.294		0.224	
5	Mirobolan dwarf p5	0.342		0.259		0.307		0.297		0.293		0.234	
6	Mirobolan dwarf p6	0.362		0.265		0.313		0.306		0.291		0.227	
7	Mirobolan dwarf p7	0.368		0.266		0.286		0.309		0.264		0.248	
8	Mirobolan dwarf p8	0.245		0.253		0.302		0.299		**0.711**		0.235	
9	Mirobolan dwarf p9	0.254		0.270		0.301		0.272		0.264		0.266	
10	Mirobolan dwarf p10	0.239		0.251		0.283		0.259		0.272		0.253	

Table 3. Results of viral evaluation of Candidate-Pre-Basic plants by DAS-ELISA method for Mirobolan C5 - 2023

No	Samples	Viruses											
		ACLSV		*ApMV*		*PPV*		*PDV*		*PNRSV*		*MLRSV*	
		Absorbance value	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*
1	Mirobolan C5 p1	0.245	0.538	0.267	*0.583*	0.249	0.630	0.242	0.602	0.242	0.595	0.259	0,503
2	Mirobolan C5 p2	0.254		0.275		0.262		0.277		0.270		0.266	
3	Mirobolan C5 p3	0.239		0.234		0.248		0.239		0.249		0.282	
4	Mirobolan C5 p4	0.239		0.224		0.259		0.249		0.246		0.265	
5	Mirobolan C5 p5	0,230		0.234		0.262		0.239		0.238		0.270	
6	Mirobolan C5 p6	0.248		0.227		0.265		0.251		0.254		0.248	
7	Mirobolan C5 p7	0.267		0.248		0.254		0.167		0.264		0.300	
8	Mirobolan C5 p8	0.250		0.235		0.248		0.263		0.258		0.267	
9	Mirobolan C5 p9	0.245		0.266		0.250		0.266		0.268		0.259	
10	Mirobolan C5 p10	0.265		0.253		0.245		0.253		0.252		0.265	

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Table 4. Results of viral evaluation of Candidate-Pre-Basic plants by DAS-ELISA method for Mirodad 1 - 2023

No.	Samples	Viruses											
		ACLSV		*ApMV*		*PPV*		*PDV*		*PNRSV*		*MLRSV*	
		Absorbance value	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*
1	Mirodad 1 p1	0.275	0.538	0.168	*0.583*	0.236	0.630	0.250	0.602	0.240	0.595	0.242	0,503
2	Mirodad 1 p2	0.266		0.248		0.242		0.217		0.262		0.215	
3	Mirodad 1p3	0.253		0.206		0.240		0.230		0.240		0.261	
4	Mirodad 1 p4	0.267		0.192		0.261		0.229		0.231		0.232	
5	Mirodad 1 p5	0.234		0.208		0.236		0.231		0.230		0.207	
6	Mirodad 1 p6	0.224		0.190		0.222		0.242		0.232		0.239	
7	Mirodad 1 p7	0.234		0.240		0.236		0.261		0.251		0.291	
8	Mirodad 1 p8	0.262		0.209		0.172		0.262		0.242		0.208	
9	Mirodad 1 p9	0.250		0.241		0.216		0.216		0.200		0.242	
10	Mirodad 1 p10	0.253		0.197		0.189		0.253		0.242		0.254	

Table 5. Results of viral evaluation of Candidate-Pre-Basic plants by DAS-ELISA method for Mirobolan dwarf - 2024

No	Samples	Viruses											
		ACLSV		*ApMV*		*PPV*		*PDV*		*PNRSV*		*MLRSV*	
		Absorbance value	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*
1	Mirobolan dwarf p1	0.225	0,660	0.206	0,508	0.222	0,652	0.224	0,557	0.281	0,602	0.239	0,631
2	Mirobolan dwarf p2	0.216		0.192		0.224		0.236		0.303		0.228	
3	Mirobolan dwarf p3	0.222		0.210		0.236		0.263		0.299		0.291	
4	Mirobolan dwarf p4	0.336		0.233		0.263		0.250		0.305		0.332	
5	Mirobolan dwarf p5	0.262		0.221		0.250		0.254		0.228		0.253	
6	Mirobolan dwarf p6	0.349		0.233		0.312		0.214		0.281		0.282	
7	Mirobolan dwarf p7	0.358		0.254		0.403		0.245		0.391		0.287	
8	Mirobolan dwarf p9	0.397		0.214		0.385		0.222		0.370		0.206	
9	Mirobolan dwarf p10	0.335		0.245		0.280		0.215		0.358		0.223	

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Table 6. Results of viral evaluation of Candidate-Pre-Basic plants by DAS-ELISA method for Mirobolan C5- 2024

No	Samples	Viruses											
		ACLSV		*ApMV*		*PPV*		*PDV*		*PNRSV*		*MLRSV*	
		Absorbance value	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*
1	Mirobolan C5 p1	0.339	0.660	0.323	*0.508*	0.235	0.652	0.277	0.557	0.300	0.602	0.323	0,631
2	Mirobolan C5 p2	0.328		0.337		0.297		0.293		0.243		0.237	
3	Mirobolan C5 p3	0.291		0.266		0.235		0.265		0.297		0.366	
4	Mirobolan C5 p4	0.232		0.320		0.281		0.321		0.360		0.320	
5	Mirobolan C5 p5	0.253		0.313		0.252		0.299		0.336		0.213	
6	Mirobolan C5 p6	0.325		0.223		0.273		0.305		0.333		0.320	
7	Mirobolan C5 p7	0.316		0.327		0.243		0.223		0.327		0.320	
8	Mirobolan C5 p8	0.232		0.262		0.365		0.257		0.262		0.215	
9	Mirobolan C5 p9	0.346		0.322		0.325		0.367		0.300		0.212	
10	Mirobolan C5 p10	0.265		0.323		0.290		0.386		0.343		0.273	

Table 7. Results of viral evaluation of Candidate-Pre-Basic plants by DAS-ELISA method for Mirodad 1- 2024

No	Samples	Viruses											
		ACLSV		*ApMV*		*PPV*		*PDV*		*PNRSV*		*MLRSV*	
		Absorbance value	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*	*Absorbance value*	*Cut-off*
1	Mirodad 1 p1	0.345	0.660	0.260	*0.508*	0.242	0.652	0.242	0.557	0.244	0.602	0.209	0,631
2	Mirodad 1 p2	0.263		0.205		0.202		0.277		0.271		0.263	
3	Mirodad 1p3	0.230		0.224		0.218		0.239		0.250		0.271	
4	Mirodad 1 p4	0.209		0.222		0.229		0.249		0.250		0.256	
5	Mirodad 1 p5	0,273		0.235		0.263		0.239		0.278		0.210	
6	Mirodad 1 p6	0.258		0.327		0.265		0.251		0.254		0.280	
7	Mirodad 1 p7	0.255		0.230		0.274		0.167		0.254		0.309	
8	Mirodad 1 p8	0.251		0.230		0.235		0.263		0.267		0.235	
9	Mirodad 1 p9	0.205		0,231		0.234		0.266		0.250		0.354	
10	Mirodad 1 p10	0.273		0.284		0.232		0.253		0.245		0.269	

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Table 8. PCR test results

No	Sampes	Phytoplasma 16S rRNA/23S rRNA (16Sr-23Sr Grup)		
		P1/P7	P1A/P7A	Nested PCR P1/P7 R162nF/R16R2
1	Mirobolan Dwarf	+	+	+
2	Mirobolan Dwarf	+	+	+
3	Mirobolan Dwarf	+	+	+
4	Mirobolan C5	+	+	+
5	Mirobolan C5	-	-	-
6	Mirobolan C5	-	-	-
7	Mirodad 1	-	-	-
8	Mirodad 1	-	-	-
9	Mirodad 1	-	-	-
10	Mirodad1	-	+	-
11	Mirodad1	-	+	-
12	Mirodad1	-	-	-
13	Mirobolan C5	-	-	-
14	Mirobolan C5	-	-	-
15	Mirobolan C5	+	+	-
16	Mirobolan Dwarf	+	+	+
17	Mirobolan Dwarf	-	-	-
18	Mirobolan Dwarf	-	-	-

Fig. 1. Biological test on *Cucummis sativus* indicator – symptoms

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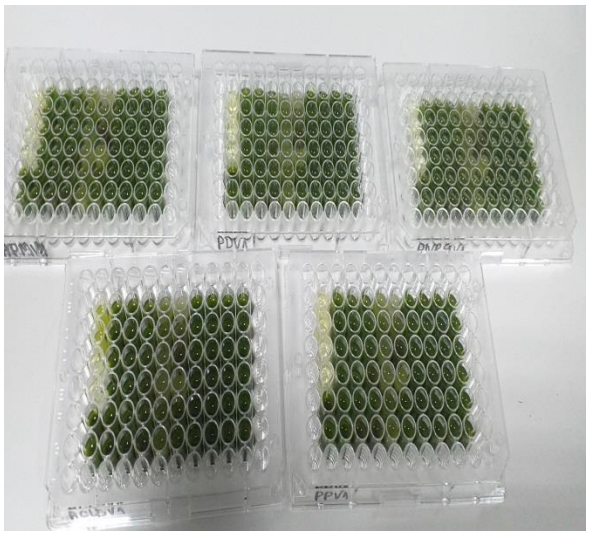

Fig. 2. Nunc MaxiSorp plates containing supernatant (plant extract)

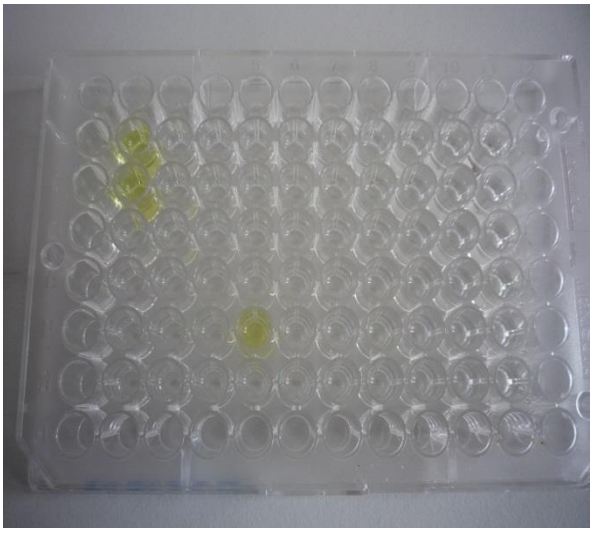

Fig. 3. Positive reaction yellow color indicating the presence of *PNRSV* virus

Fig. 4. AGRISTRIP test results applied for *PPV* identification

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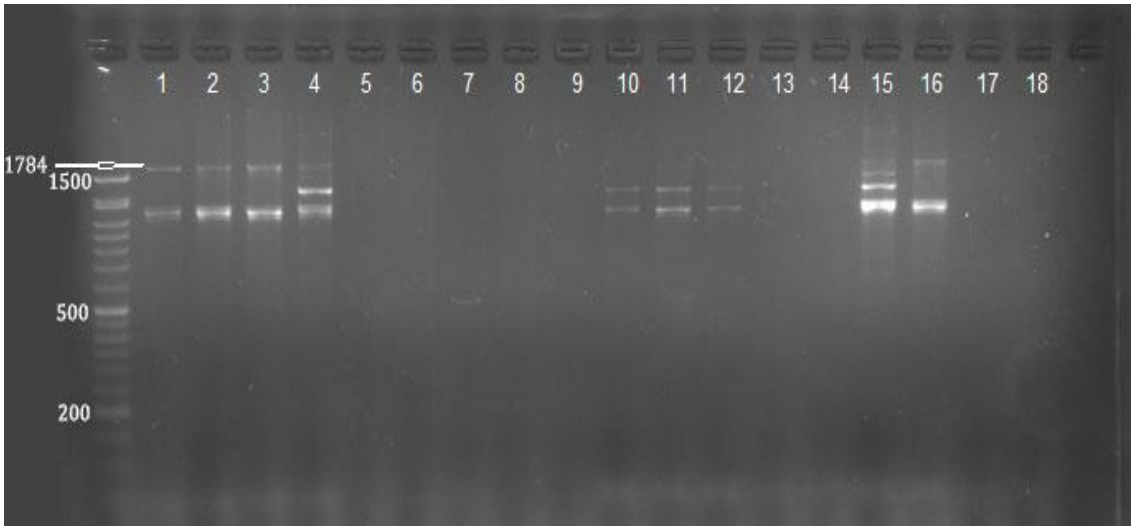

Fig. 5. Electrophoretic profile - primer pair P1/P7

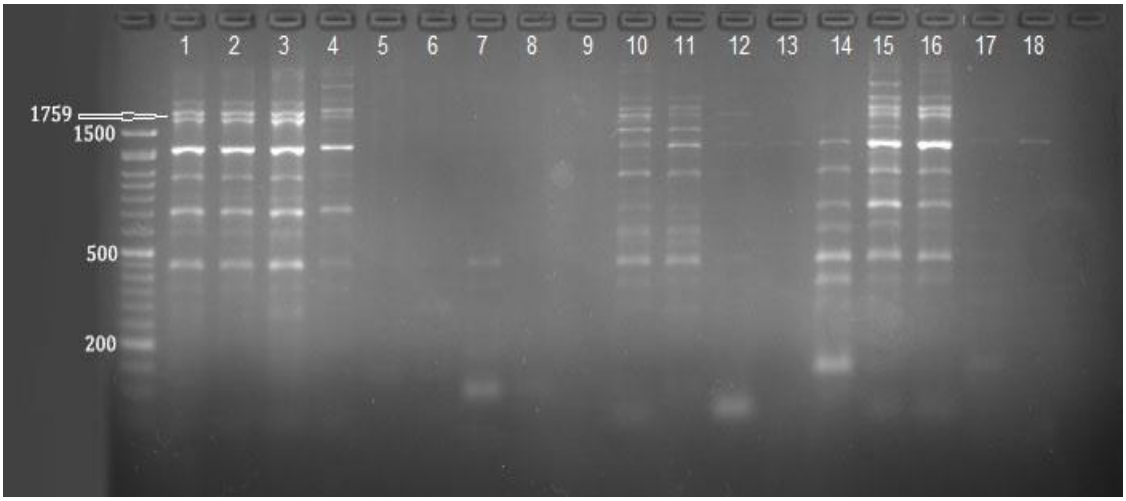

Fig. 6. Electrophoretic profile - primer pair *P1A/P7A*

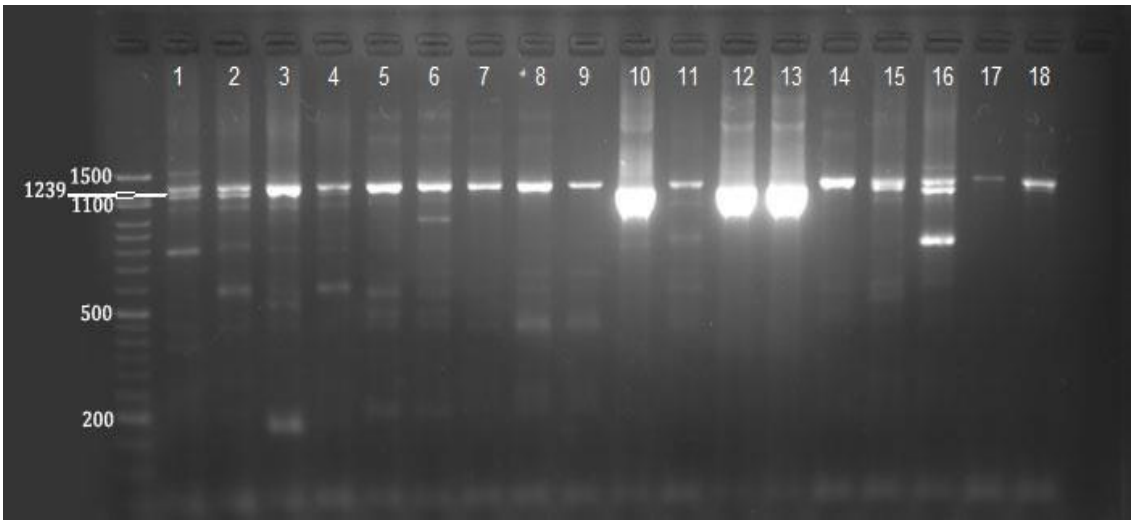

Fig. 7. Electrophoretic profile - primer pair *R16F2n/R16R2*