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MOLECULAR IDENTIFICATION OF *PLUM POX VIRUS* ISOLATES FROM LITHUANIA AND UKRAINE

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Abstract

Plum pox virus (PPV) (genus *Potyvirus* of the family *Potyviridae*) causes devastating stone fruit disease known as sharka and can provoke a serious economic impact. It is known that PPV has four major strain groups – Dideron (D), Marcus (M), El-Amar (EA) and Cherry (C). The severity of crop losses often depends on the strain of the virus, so it is important to identify the most common strain of PPV in our investigation regions. Plum tree leaves with characteristic mottle symptoms were collected from stone-fruit orchards in Lithuania and Ukraine in 2007. In these samples, PPV was detected using ELISA test, immunosorbent electron microscopy and the reverse transcription and polymerase chain reaction (RT-PCR). After confirmation of the virus presence, strain specificity of PPV was determined by amplification of two genomic regions (243 bp sequence flanking the coat protein coding region and 836 bp sequence of the P3-6K$_1$ genomic region) in nested PCR reactions and RFLP analysis of these products with specific restriction endonucleases. In all analyzed samples from Lithuania and Ukraine presence only PPV-D strain of the virus was proved.

Key words: *Plum pox virus*, identification, strains, *Prunus domestica*, PPV-D, RT-PCR, RFLP.

Introduction

Plum pox or Sharka disease is considered most devastating disease of stone-fruits. The disease is caused by *Plum pox virus* (PPV), a member of the genus *Potyvirus* of the family *Potyviridae*. Potyviruses have a single–stranded plus-sense RNA genome of about 10 kb with a single open reading frame (ORF). The virus particles are c. 760 nm long and 20 nm in diameter /Kegler, Schade, 1971/. Stone-fruits infected by PPV include wild and cultivated *Prunus* spp.: plum (*Prunus domestica*), peach, apricot, almond, nectarine, sweet cherry and sour cherry. The disease has been spreading since the beginning of the 20th century from the Balkan countries throughout most of the European subcontinent and around the Mediterranean basin. PPV is now reported in most European countries, in parts of Asia, northern Africa and in South America and is

considered as quarantine pathogen in all continents. The disease outbreaks were reported in the USA /Levy et al., 2000/. The main source of the virus is infected trees or budwood /OEPP/EPPO, 1983/. From these, PPV is transmitted either by grafting or by aphids in a non-persistent stylet-borne manner to uninfected hosts.

According to serological, biological and other properties European PPV isolates can be divided into two major subgroups: PPV-M (Marcus) and PPV-D (Dideron) isolates. In nature, these isolates can show significant differences in their epidermiological properties and host range /Bousalem et al., 1994; Cambra et al., 1994; Quiot et al., 1995; Myrta et al., 1996; Dallot et al., 1998; Myrta et al., 2001/. Strains of PPV-D group are less aggressive than PPV-M and have limited host range. Two additional minor subgroups, including cherry infecting isolates PPV-C /Nemchinov et al., 1996; James, 2003/ and ElAmar isolates (PPV-EA) /Wetzel et al., 1991/, have also been confirmed.

The first data on the presence of PPV in Lithuania were obtained using ELISA tests during survey of plum tree samples from Kaunas region in 1995. Local Lithuanian isolate of PPV was transmitted to herbaceous plants in 1997. Lithuanian isolate of PPV appeared to be attributed to PPV-D group of strains, as was determined using monoclonal antibodies in ELISA tests conducted by Dr. Donato Boscia (de Bari, Italy) /Staniulis et al., 1998; Jackeviciene, Staniulis, 2003/. During survey of plum gardens in Lithuania for the presence of PPV (EPPO A2 quarantine pest) in 2000 the virus was found in 6 orchards and 9 private gardens.

Earlier, in 1966, Sharka disease has been detected for the first time in Ukraine in Chernivtsy region /Piskun, 1969/. In 1969, localized foci of the infection were registered in Chernivtsy, Lviv, Transcarpathian, Ternopil, Ivano-Frankivsks and Vinnitsya regions. In the beginning of 1993, areas of limited virus spread and virus-free areas have been determined. Chernivtsy, Lviv, Transcarpathian, Ternopil, Ivano-Frankivsks, Mykolayiv, Odessa and Vinnitsya regions were considered as the areas of limited virus spread, in which Sharka foci have been identified in 83 farms. All the remaining regions were shown to be virus-free. The measures taken in order to eradicate the virus disease were quite successful for Vinnitsya, Ivano-Frankivsks and Mykolayiv regions; however, the area of virus spread in other mentioned regions grew with every year despite the radical quarantine measures ensured /Ratushnyak, 2003/. In January of 2008, the hotbeds for *Plum pox virus* have been confirmed for 6 regions (Transcarpathian, Lviv, Ternopil, Chernivtsy, Odessa regions and the AR of Crimea) with the area totalling 4543,9 hectares /The quarantine state…, 2006/.

The objective of this investigation was to determine strain specificity of PPV isolates collected from stone-fruit gardens in Lithuania and Ukraine.

Materials and Methods

For preliminary investigation of the presence of PPV stone-fruit tree leaf samples were collected from Lithuania (Kaunas, Varėna, Trakai, Šakiai and Jonava regions), and from Ukraine (Kiev, Odessa, Mironovka and Transcarpatia regions). Collected field samples were assayed by DAS-ELISA with a PPV polyclonal antibodies (BIOREBA, Switzerland or LOEWE, Germany), according to described procedures /Clark, Adams, 1977/ and immunosorbent electron microscopy (ISEM) /Derrick, 1973; Brlansky,

Derrick, 1979/. Electron microscopic preparations were examined with JEOL JEM-100S transmission electron microscope.

Location and number of samples from stone-fruit trees in Lithuania and Ukraine collected for preliminary detection of PPV infection by DAS-ELISA and ISEM are presented in Table 1.

Table 1. Locations and number of samples tested for detection of PPV by DAS-ELISA and ISEM collected in June 2007

Lithuania		Ukraine	
Tree species and location	No. of samples	Tree species and location	No. of samples
Garden plum (*Prunus domestica* L.) – Šakiai reg., Patašynė	5	Garden plum, Kiev, Novosilki	5
Garden plum – Trakai reg., Trakai	1	Garden plum, Kiev, Zuliany	2
Garden plum – Varėna reg., Perloja	1	Garden plum, Mironovka	2
Garden plum – Kaunas reg., Ringaudai	4	Garden plum, Transcarpatia reg.	3
Garden plum – Jonava reg., Pėdžiai	6	Garden plum, Odessa reg.	1
		Cherry plum (*Prunus cerasifera Ehrh.*), Kiev	2
		Apricot tree (*Armeniaca vulgaris* Lam.), Mironovka	3
		Peach (*Persica vulgaris* Mill.), Odessa reg., Iljichevsk	12

For the general PPV detection RT-PCR of a 243 bp fragment flanking the coat protein (CP) coding region was carried out using the primers P1 and P2 according to /Wetzel et al., 1991/ – P1: 5'–3' ACC GAG ACC ACT ACA CTC CC and P2: 5'–3' CAG ACT ACA GCC TCG CCA GA. The conditions used for the cycling reaction were as follows: 30 min at 42^{o} C; 3 min at 93^{o} C, followed by 40 cycles of 30 sec at 94^{o} C, 30 sec at 54^{o} C, 30 sec at 72^{o} C with a final extension of 5 min at 72^{o} C /Sertkaya et al., 2003/. For the general detection of PPV and strain identification in RT-PCR/nested PCR reactions primers and reaction conditions were used as described by Szemes /Szemes et al., 2001/. For restriction fragment length polymorphism (RFLP) analysis the amplified products (243bp) were incubated with *Rsa*I and *Alu*I restriction endonucleases /Sertkaya et al., 2003/.

Results and Discussion

DAS-ELISA tests conducted at the Institute of Botany with 17 garden plum leaf samples from Lithuania and 30 samples of stone fruits from Ukraine (13 garden plums, 3 apricot, 2 cherry plum and 12 peach) PPV was detected only in 8 plum trees samples. All PPV positive plum leaf samples had rather characteristic for PPV mottling or slight mosaic symptoms. Negative results received in ELISA tests for the presence of PPV in apricot and peach samples could be explained by absence of characteristic for PPV

symptoms. Presence of PPV in 8 ELISA positive plum samples was confirmed by ISEM detecting typical for Potyvirus filamentous virus particles (Table 2).

Table 2. Plum leaf samples from Lithuania and Ukraine positive for PPV in ELISA and ISEM tests

Location of PPV positive plum (*Prunus domestica*) samples	Number of samples tested/infected	
	DAS-ELISA	ISEM
Lithuania, Šakiai reg., Patašynė	5/1	1/1
Lithuania, Jonava reg., Pėdžiai	6/1	1/1
Ukraine, Kiev reg., Novosilki	5/2	2/2
Ukraine, Kiev reg., Zuliany	2/1	1/1
Ukraine, Transcarpatia reg.	3/3	3/3

RT-PCR analysis of DAS-ELISA and ISEM positive plum tree leaf samples and corresponding virus isolates in pea (*Pisum sativum*) plants confirmed presence of PPV. Expected product size of 465 bp was received (Figure 1). Presence of PPV in other samples from the same locations was confirmed in RT-PCR reactions as well (data not shown).

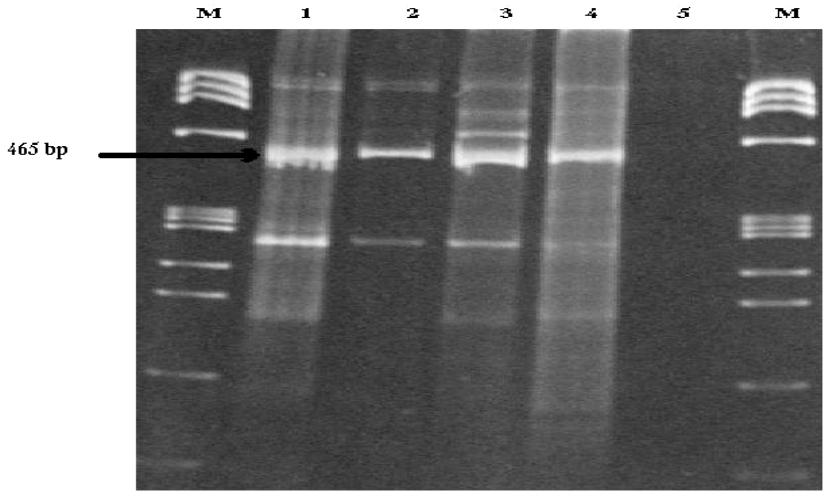

Figure 1. First step RT-PCR results after electrophoresis in 5% polyacrilamide gel. M line: size marker Gene ruler 50 bp; 1–3 lines: samples from plums from Ukraine; 4 line: sample from Lithuania plum; 5 line: water control

In separate parallel experiments conducted at the Department of Virology of Kiev University similar results were obtained. PCR amplification of samples' material using the same pair of primers (P1 and P2) allowed obtaining of clear amplicon bands of expected length 243 bp. (Figure 2).

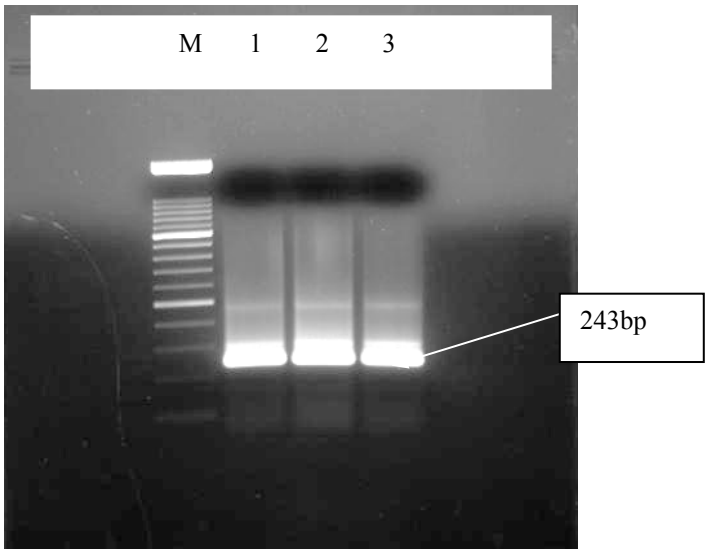

Figure 2. Agarose gel electrophoresis of PCR products amplified employing P1 and P2 primers: 1 – positive control; 2 – plum sample (Uzhgorod district); 3 – plum sample (Khust district); M – nucleotide size ladder (Roche 100 bp ladder)

In the second (nested) PCR amplification step with mixture of specific primers for simultaneous detection of PPV-D, PPV-M and PPV-EA strains presence only PPV-D was confirmed. The size of the reaction product on the gel was 159 bp indicating the amplification only of PPV-D strain (Figure 3).

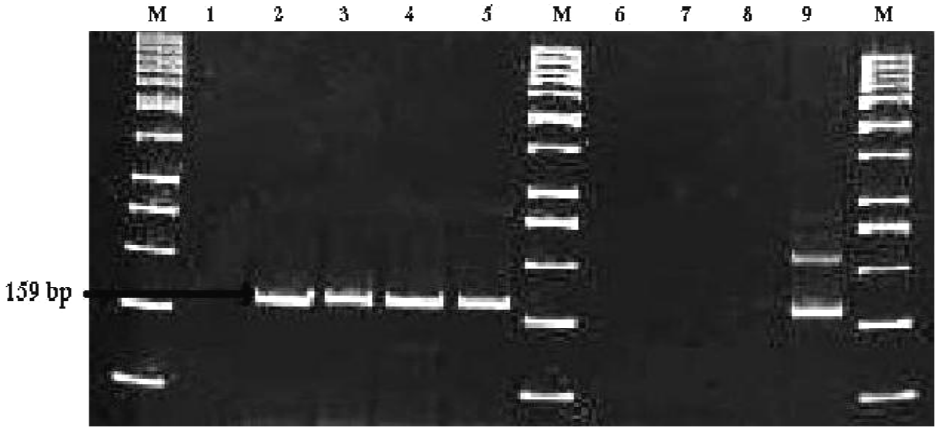

Figure 3. Second step: nested PCR results after electrophoresis on 5% polyacrilamide gel. M line: size marker Gene ruler 50 bp; 1 and 6 lines: water control; 2–5 lines: PCR products amplified by using mixture of specific primers (2–4 lines: samples from plums from Ukraine; 5 line: sample from Lithuania plum), 7–9 lines: sample from Lithuania amplified individually with strain specific primers (7 line: PPV-EA; 8 line: PPV-M; 9 line: PPV-D)

RFLP analysis of RT-PCR 243 bp product using *Alu*I and *Rsa*I restriction endonucleases allowed discrimination between strain groups, because PPV-M does not contain the *Rsa*I recognition site. Results of RFLP analysis confirmed that plum PPV isolates from Lithuania and Ukraine are members of PPV-D strain group (Figure 4). The same results were obtained with *Asn*I and *Eco*RI endonucleases in RFLP analysis of amplified 836 bp product from P3-6K1 genomic region (data not shown).

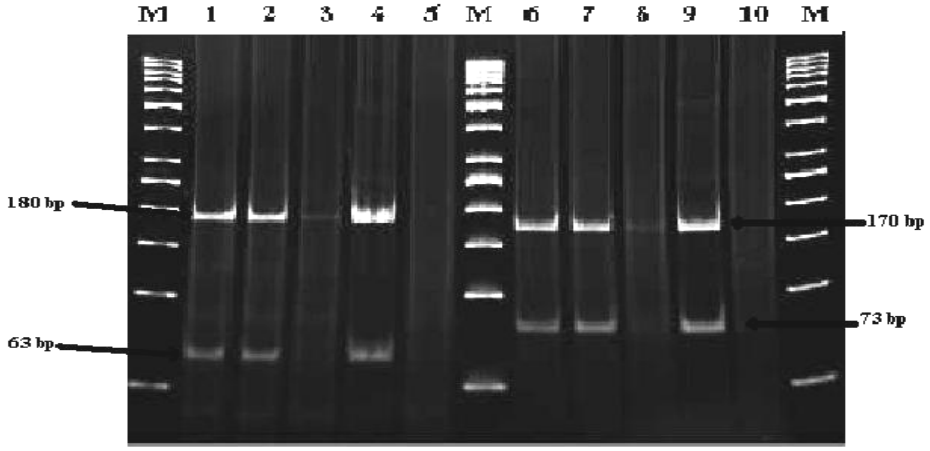

Figure 4. Results of RFLP analysis of 243 bp fragment with *Alu*I and *Rsa*I after electrophoresis in 5% polyacrilamide gel. M line: size marker Gene ruler 50 bp; 1–4 lines: restriction with *Alu*I (1–3 lines: samples of plums from Ukraine, 4 line: sample from Lithuania), 5 and 10 lines: water control, 6–9 lines: restriction with *Rsa*I (6–8 lines: samples of plums from Ukraine, 9 line: sample from Lithuania)

To double-check the identity of PCR amplification products for their compliance with the expected genetic nucleotide sequence encoding structural protein of *Plum pox virus*, the amplicons from *P. domestica* sample material have been subjected to sequencing, and the outcomes were further aligned against corresponding virus sequences available in NCBI GenBank database. The search for similar genetic sequences deposited in GenBank revealed that the PCR amplification products have been nearly identical to the known gene sequences for coat protein gene of *Plum pox virus*, strain D, isolated by Teycheney et al. (sequence code X16415.1) /Teycheney et al., 1989/. The amplicons obtained from PPV-infected *P. domestica* samples collected in Ukraine were characterized by almost 99% homology to corresponding PPV strain D sequences. The alignment of obtained sequences as compared to PPV strain D sequences deposited in GenBank is represented in Figure 5.

As presented in Figure 5, the comparison of PCR-generated amplification products with GenBank PPV sequences demonstrated nearly 99% homology with the coat protein gene sequences for *Plum pox virus*, strain D (sequence code X16415.1). Based on this, we may conclude that plantings of stone fruit cultures in Transcarpathian region are infected by strain D of *Plum pox virus*.

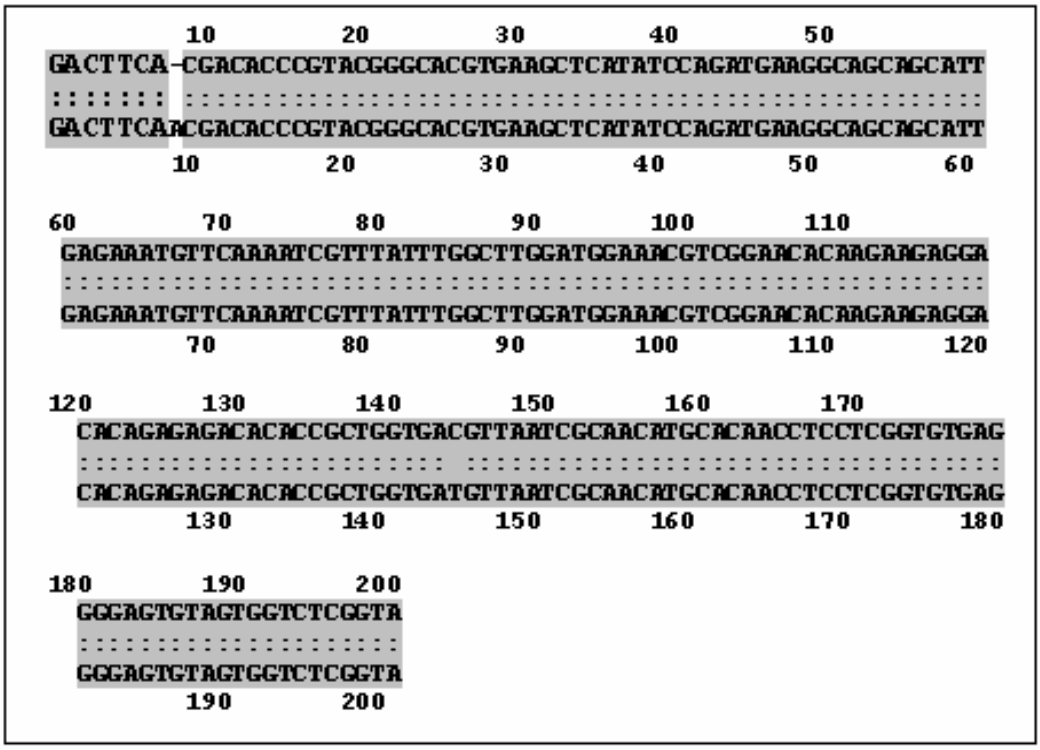

Figure 5. Comparison of amplified nucleotide sequence, obtained from PPV-infected *P. domestica* with the PPV strain D sequence X16415.1 retrieved from GenBank database

The results obtained using several different identification methods clearly indicate that in Lithuania and Ukraine detected Plum pox virus isolates should be attributed to PPV-D group of strains. In Lithuania, natural sources of PPV infection are not known. PPV-D strain group isolates cause milder symptoms, thus virus infection can easily be overlooked in grafting or propagating material and introduced. Having in mind that in central or south-eastern part of Europe both major strain groups (PPV-M and PPV-D) are distributed some expectation to detect presence of PPV-M in Ukraine could not be rejected. Discussing negative results for PPV in apricot and peach samples it should be noticed that too small quantity of samples was delivered and analyzed, besides they had not very characteristic PPV symptoms.

Conclusions

1. The presence of *Plum pox virus* in plum tree samples from Lithuania (Šakiai and Jonava regions) and Ukraine (Kiev and Transkarpatia regions) was confirmed by DAS-ELISA, ISEM and RT-PCR methods.

2. RT-PCR, sequencing and RFLP analysis have confirmed that in all plum leaf samples from Lithuania and Ukraine, the detected PPV isolates belong to PPV-D strain group.

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