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Serological and Molecular Typing of *Plum pox* Virus Isolates in the Transylvania, Romania

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Abstract

Plum pox virus (PPV) is considered the most destructive viral pathogen of stone fruits. Although PPV is widespread in all plum growing areas from Romania and causes serious yield losses, little is known about the virus variability. For this reason we investigated 100 PPV isolates collected from 13 different plum orchards in the Transylvania - Romania. PPV detection was made by DAS-ELISA and by IC-RT-PCR. PPV strains were serologically determined by TAS-ELISA using PPV-D and PPV-M specific monoclonal antibodies. Molecular strain typing was done by RT-PCR targeting three different genomic segments corresponding to (Cter)CP, (Cter)NIb/(Nter) CP and CI. RFLP analysis was used to distinguish the two major strains, D and M based on a RsaI polymorphism located in (Cter)CP. All PCR products targeting (Cter)CP and 10 PCR products spanning the (Cter)NIb/(Nter)CP were sequenced. All PPV isolates typed as PPV-M by serological analysis and by molecular differentiation in the genomic region corresponding to (C-ter)CP proved to be PPV recombinant (PPV-Rec) when the molecular analysis were performed in the region corresponding to NIb/CP. Sequencing confirmed a high similarity with different sequences of PPV-Rec previously reported. All these recombinant isolates share the same recombination breakpoint and conserve the DAG motif, which is considered essential for aphid transmission. Overall results provided that in Transylvania the predominant strain is PPV-D, followed by PPV-Rec which shares the CP gene with M strain and, therefore, it is serologically detected as PPV-M with M-specific monoclonal antibodies. In this big plum growing area, mixed infections (PPV-D+PPV-Rec), which might generate additional variation by recombination, are also frequent.

Keywords: Sharka, PPV strains, diagnosis, ELISA, RT-PCR, sequencing

Introduction

Plum pox or sharka is the most devastating disease of stone fruits. The disease is highly detrimental because it reduces the quality of the fruits and causes their premature dropping (Dunez and Sutic, 1988; Nemeth, 1994). Therefore, this disease is among the significant limiting factors for plum production (Stoev *et al*., 2004). Sharka has a Balkan origin and was described for the first time around 1917 in Bulgaria (Atanasoff, 1932). Since then, the disease has progressively spread to a large part of the European continent, around the Mediterranean basin and Middle East. Also, it has been found in America (Chile, Argentina, USA and Canada), as well as in Asia (India, China, Pakistan, Kazakhstan and Iran) (Capote *et al*., 2006; García and Cambra 2007). In Romania, sharka occurs in all plum-growing areas causing serious yield losses especially to sensitive cultivars (Minoiu, 1997; Zagrai *et al*., 2001; Sestras *et al*., 2007).

PPV strain identification is useful for controlling virus spreading. Breeding programmes are associated with epidemiological studies of PPV. For this reason, it is important to know the distribution of the virus and the different strains occurring (Pasquini and Barba, 1994).

Two major serologically distinguishable strains, PPV-D and PPV-M, are known (Kerlan and Dunez, 1976) which can be distinguished by strain-specific monoclonal antibodies (Cambra *et al*., 1994; Boscia *et al*., 1997). In addition both strains can be discriminated by RsaI polymorphism in the 243 bp DNA fragment amplified by P1 and P2 primers located at the C-terminus of PPV CP gene (Wetzel *et al*., 1991a) or by direct IC/RT-PCR typing using PD and PM specific oligonucleotides (Olmos *et al*., 1997).

A third major group including isolates resulted from recombination event between PPV-D and PPV-M was identified and denoted PPV-Rec (Glasa *et al*, 2002). This natural recombinant was reported in Albania, Bulgaria, Czech Republic, Germany, Hungary, Slovakia (Glasa *et al*, 2002; 2004) and Romania (Zagrai et. al., 2006, 2008). Two additional minor PPV groups are represented by geographically limited strains El Amar (PPV-EA) originally isolated from Egypt (Wetzel *et al*., 1991b), and Cherry (PPV-C) isolated from sour cherry in Moldavia (Kalashyan *et al*., 1994) and

from sweet cherry in southern Italy (Crescenzi *et al*., 1996) and Romania (Maxim *et al*., 2002).

The last PPV strain described is Winona (PPV-W) from Canada (James and Varga, 2004), which is genetically distinct from all other viral strains known to date (James and Varga, 2005).

The objective of our study was to provide new data about PPV strains occurring in the Transylvania.

Materials and methods

PPV isolates

One hundred PPV isolates were collected from 13 different plum orchards in the Transylvania area. Sampling was initially based on typical PPV symptoms and virus infection was confirmed by serological and molecular testing.

Serological and molecular detection

Serological diagnosis was made by DAS-ELISA (Clark and Adams, 1977) using a commercial polyclonal antiserum (Bioreba, Switzerland) according to the manufacturer's instructions. Molecular detection was made by IC-RT-PCR using the pair of primers P1/P2 and trapping with the above polyclonal antiserum. Qiagen one-step kit (Qiagen, Germany) was used for RT-PCR.

Strain differentiation

Serological differentiation was made by TAS-ELISA using the PPV-D and PPV-M specific monoclonal antibodies (Durviz, Spain) according to Cambra *et al*'s methods. (2004).

Molecular strain typing was done by RT-PCR targeting three genomic regions corresponding to: (i) (Cter) CP, using P1/PD and P1/PM pair of primers that distinguish PPV-D and PPV-M, respectively; (ii) (Cter) NIb/(Nter) CP, using mD5/mM3 pair of primers (Subr *et al*., 2004) that detect natural recombinants between D and M (PPV-Rec); (iii) CI, using CIf/CID or CIf/CIM primer sets (Glasa *et al*., 2002) to confirm the presence of PPV-Rec. Aliquots of PCR products corresponding to (Cter)CP were subjected to RFLP analysis to distinguish D strains from M strains based on RsaI polymorphism located in this genomic area. To confirm the molecular variability of the sampled PPV isolates, amplified DNAs purified by Wizard SV Gel and PCR Clean-Up System (Promega, USA), were then sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The samples were run on the ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA). The alignment of nucleotides from all PCR products corresponding to (Cter)CP and ten amplified fragments spanning (Cter) NIb/(Nter) CP region was done using the BioEdit package version 5.0.9 (Hall, 1999). Obtained sequences were then compared with those available in NCBI Data Base and GeneBank. A phylogenetic tree was constructed with the Mega 3.1 programme using Minimum Evolution method Jukes-Cantor model (Bootstrap value 10 000).

Results and discussion

Tab. 1. shows the differentiation of PPV isolates by TAS-ELISA using D and M monoclonal antibodies and by RT-PCR using PD and PM specific primers. All isolates reacted positively to at least one of the two mono-

Tab. 1. Serological and molecular detection and differentiation of 100 PPV isolates from 13 orchards of the Transylvania, Romania

Orchard No.	DAS / TAS-ELISA				IC-/RT-PCR (P1/P2 and P1/PD or PM)				RFLP ***Rsa*I**		
	PPV poly	PPV-D	PPV-M	PPV D +M	PPV poly	PPV-D	PPV-M	PPV D+M	PPV-D	PPV-M	PPV D+M
1	10	6	2	2	10	6	2	2	6	2	2
2	10	3	7	0	10	3	7	0	3	7	0
3	10	4	5	1	10	4	4	2	4	4	2
4	10	7	3	0	10	7	2	1	7	2	1
5	3	1	0	2	3	0	0	3	0	0	3
6	7	7	0	0	7	7	0	0	7	0	0
7	10	7	2	1	10	5	2	3	5	2	3
8	10	8	2	0	10	8	1	1	8	1	1
9	5	5	0	0	5	5	0	0	5	0	0
10	5	5	0	0	5	5	0	0	5	0	0
11	10	10	0	0	10	10	0	0	10	0	0
12	5	5	0	0	5	5	0	0	5	0	0
13	5	5	0	0	5	5	0	0	5	0	0
TOTAL	100	73	21	6	100	70	18	12	70	18	12
%	100%	73%	21%	6%	100%	70%	18%	12%	70%	18%	12%

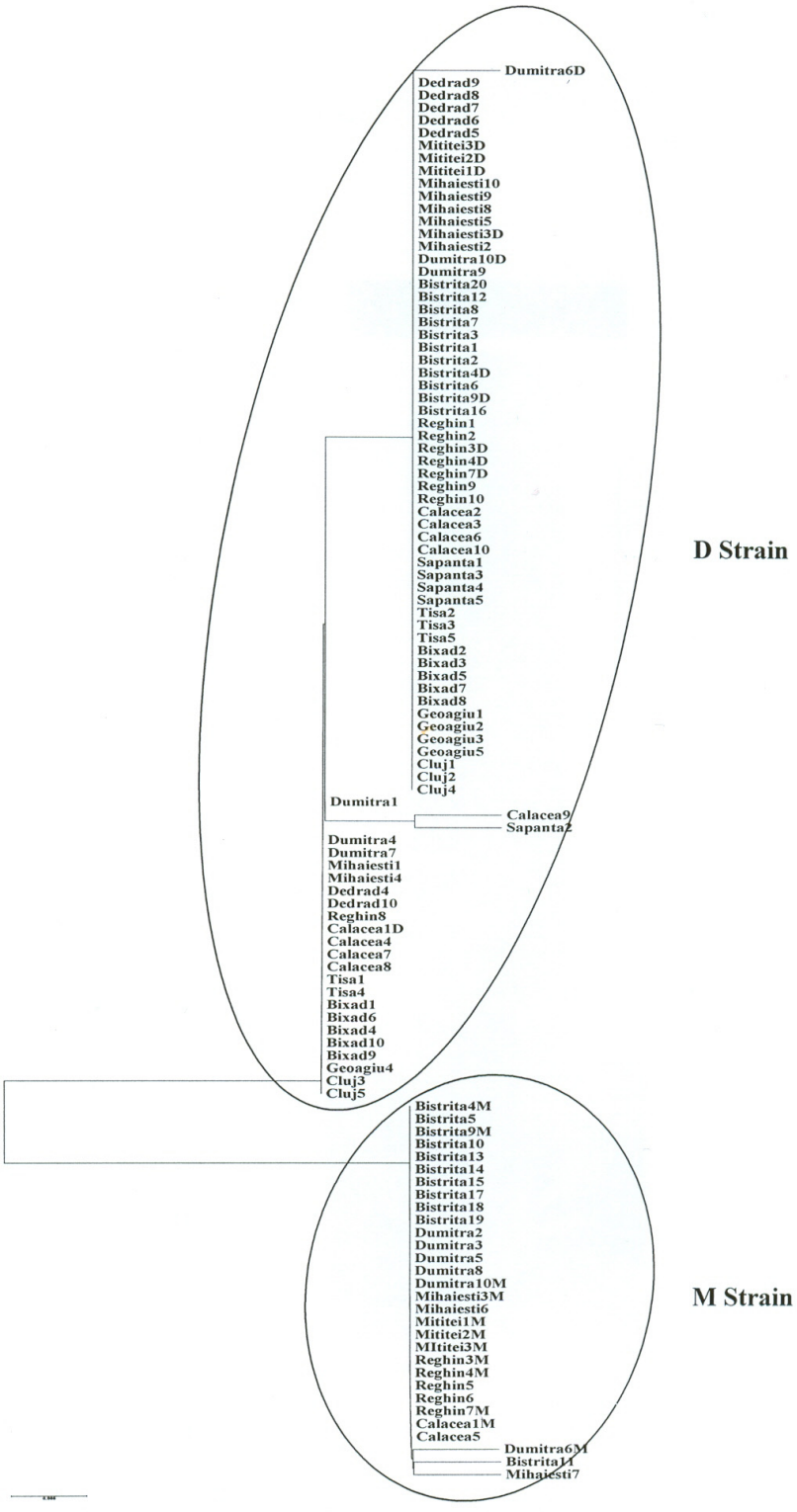

Fig. 1. The phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to C-terminus of PPV coat protein. The isolates Bistrita 4, Bistrita 9, Dumitra 6, Dumitra 10, Mihaiesti 3, Mititei 1, Mititei 2, Mititei 3, Reghin 3, Reghin 4, Reghin 7 and Calacea 1 represent mixed infection and they were sequenced both for PPV-D and PPV-M.

clonal antibodies as well as PPV-D or/and PPV-M specific primers. Using TAS-ELISA, 73 of 100 isolates tested (73%) were identified as PPV-D, 21 (21%) as PPV-M and 6 (6%) revealed the presence of a mixed infection involving D and M strains. The RT-PCR analyses confirmed that 70 isolates were PPV-D type and 18 isolates PPV-M. RT-PCR detected six more mixed infection. RFLP analysis confirmed these results based on the presence of the RsaI polymorphism in PPV-D strain.

The phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to PPV (C-ter)CP confirmed the clear-cut splitting of the two major groups D and M in this genomic area (Fig. 1). Sequences from Romanian PPV isolates were 98-100 % identical to sequences from the NCBI Data Base.

Using the primer pair (mD5/mM3) targeting (Cter) NIb/(Nter)CP region, was observed that all PPV isolates typed as PPV-M were in fact PPV-Rec. Using specific primers to distinguish the two strains D and M in CI region were detected only fragments belonging to PPV-D. That confirmed the presence of PPV-Rec (Tab. 2).

To verify whether the recombination breakpoint position suspected to occur in the (Cter)NIb/ (Nter)CP region corresponds with those PPV-Rec previously reported by Glasa *et al*. (2002, 2004), ten PCR products spanning this genomic section were sequenced (Fig. 2). Multiple sequence alignment showed that the recombination breakpoint is located in the region corresponding to (Cter)NIb at the nucleotide position 8450. The DAG motif that is considered as essential for aphid transmission was present in all PPV-Rec isolates analyzed. As expected, this site was located downstream the recombination breakpoint. Based on comparative alignment, sequencing revealed a high similarity (98-99%) with different sequences of PPV-Rec available in GeneBank. All these recombinant isolates shared the same recombination breakpoint.

Tab. 2. Results of serological and molecular typing based on different targeted regions of the genome of PPV isolates selected from 13 orchards of the Transylvania, Romania

Plots no.	Isolate	PPV strain identified by			
		TAS-ELISA	RT-PCR		
			(C-ter) CP P1-PD/PM	(C-ter) NIb /(N-ter) CP(a) mD5/mM3	CI CIf –CID/CIM
1	Bistrita 1	D	D	-	D
	Bistrita 2	D	D	-	D
	Bistrita 3	D	D	-	D
	Bistrita 4	D+M	D+M	Rec	D
	Bistrita 5	M	M	Rec	D
	Bistrita 6	D	D	-	D
	Bistrita 7	D	D	-	D
	Bistrita 8	D	D	-	D
	Bistrita 9	D+M	D+M	Rec	D
2	Bistrita 10	M	M	Rec	D
	Bistrita 11	M	M	Rec	D
	Bistrita 12	D	D	-	D
	Bistrita 13	M	M	Rec	D
	Bistrita 14	M	M	Rec	D
	Bistrita 15	M	M	Rec	D
	Bistrita 16	D	D	-	D
	Bistrita 17	M	M	Rec	D
	Bistrita 18	M	M	Rec	D
	Bistrita 19	M	M	Rec	D
	Bistrita 20	D	D	-	D
3	Dumitra 1	D	D	-	D
	Dumitra 2	M	M	Rec	D
	Dumitra 3	M	M	Rec	D
	Dumitra 4	D	D	-	D
	Dumitra 5	M	M	Rec	D
	Dumitra 6	D+M	D+M	Rec	D
	Dumitra 7	D	D	-	D
	Dumitra 8	M	M	Rec	D
	Dumitra 9	D	D	-	D
	Dumitra 10	M	D+M	Rec	D

4	Mihaiesti 1	D	D	-	D
	Mihaiesti 2	D	D	-	D
	Mihaiesti 3	M	D+M	Rec	D
	Mihaiesti 4	D	D	-	D
	Mihaiesti 5	D	D	-	D
	Mihaiesti 6	M	M	Rec	D
	Mihaiesti 7	M	M	Rec	D
	Mihaiesti 8	D	D	-	D
	Mihaiesti 9	D	D	-	D
	Mihaiesti 10	D	D	-	D
5	Mititei 1	D+M	D+M	Rec	D
	Mititei 2	D	D+M	Rec	D
	Mititei 3	D+M	D+M	Rec	D
6	Dedrad 4	D	D	-	D
	Dedrad 5	D	D	-	D
	Dedrad 6	D	D	-	D
	Dedrad 7	D	D	-	D
	Dedrad 8	D	D	-	D
	Dedrad 9	D	D	-	D
	Dedrad 10	D	D	-	D
7	Reghin 1	D	D	-	D
	Reghin 2	D	D	-	D
	Reghin 3	D+M	D+M	Rec	D
	Reghin 4	D	D+M	Rec	D
	Reghin 5	M	M	Rec	D
	Reghin 6	M	M	Rec	D
	Reghin 7	D	D+M	Rec	D
	Reghin 8	D	D	-	D
	Reghin 9	D	D	-	D
	Reghin 10	D	D	-	D
8	Calacea 1	M	D+M	Rec	D
	Calacea 2	D	D	-	D
	Calacea 3	D	D	-	D
	Calacea 4	D	D	-	D
	Calacea 5	M	M	Rec	D
	Calacea 6	D	D	-	D
	Calacea 7	D	D	-	D
	Calacea 8	D	D	-	D
	Calacea 9	D	D	-	D
	Calacea 10	D	D	-	D
9	Sapanta 1	D	D	-	D
	Sapanta 2	D	D	-	D
	Sapanta 3	D	D	-	D
	Sapanta 4	D	D	-	D
	Sapanta 5	D	D	-	D
10	Tisa 6	D	D	-	D
	Tisa 7	D	D	-	D
	Tisa 8	D	D	-	D
	Tisa 9	D	D	-	D
	Tisa 10	D	D	-	D

270

11	Bixad 1	D	D	-	D
	Bixad 2	D	D	-	D
	Bixad 3	D	D	-	D
	Bixad 4	D	D	-	D
	Bixad 5	D	D	-	D
	Bixad 6	D	D	-	D
	Bixad 7	D	D	-	D
	Bixad 8	D	D	-	D
	Bixad 9	D	D	-	D
	Bixad 10	D	D	-	D
12	Geoagiu 1	D	D	-	D
	Geoagiu 2	D	D	-	D
	Geoagiu 3	D	D	-	D
	Geoagiu 4	D	D	-	D
	Geoagiu 5	D	D	-	D
13	Cluj 6	D	D	-	D
	Cluj 7	D	D	-	D
	Cluj 8	D	D	-	D
	Cluj 9	D	D	-	D
	Cluj 10	D	D	-	D

(a)Only the isolates identified as PPV-M in the region corresponding to (C-ter)CP were tested

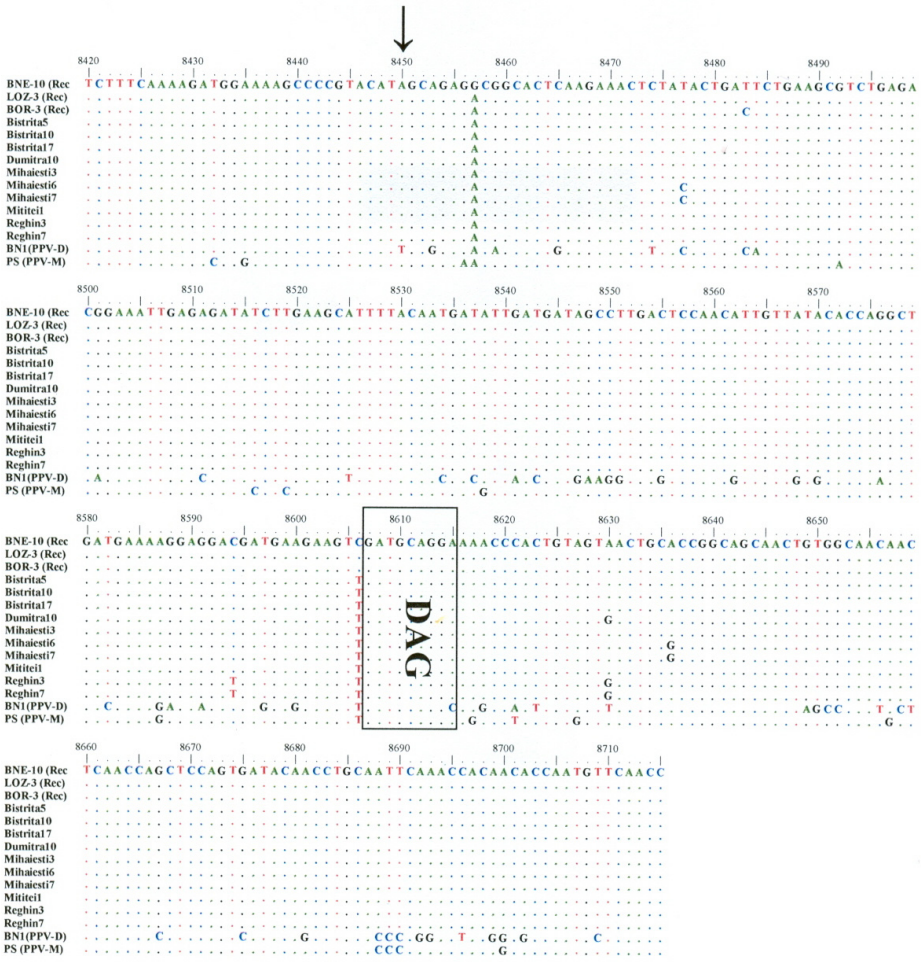

Fig. 2. Multiple alignment of recombinant sequences (NIb/CP) of ten Romanian isolates (Bistrita 5, Bistrita 10, Bistrita 17, Dumitra 10, Mihaiesti 3, Mihaiesti 6, Mihaiesti 7, Mititei 1, Reghin 3, Reghin 7) and three isolates [BNE-10 (accession number AF450311), LOZ-3 (accession number AF450312), BOR–3 (accession number AY028309)] previously reported.

The typing of PPV isolates from Transylvania showed that PPV-D is the predominant strain, followed by PPV-Rec which shares the CP gene with M strain and, therefore, it is serologically detected as PPV-M with M-specific monoclonal antibodies. The mixed infections (D+Rec),

Fig. 3. Relative frequency of PPV strains in Transylvania.

which might generate additional genetic variations by recombination, are also frequent (Fig. 3).

Conclusions

The serological and molecular typing of PPV isolates from Transylvania provided that PPV-D is the prevalent strain, followed by PPV-Rec. In this important plum growing area, mixed infections (PPV-D+PPV-Rec), are also frequent.

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