

CONTROVERSIAL CONSIDERATIONS BETWEEN NUCLEOTIDE DIVERSITY OF THE 3' TERMINAL REGION OF *PLUM POX VIRUS* AND DIFFERENCES IN VIRAL SEROTYPES

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SUMMARY

Plum pox virus (PPV) is a potyvirus causing economic losses to stone fruit trees. In the present study we have analyzed more than 40 PPV isolates collected from different western, eastern, central European and north African countries. To preserve the originality of these isolates, the majority was maintained in GF305 peach (*Prunus persica*), some in apricot (*P. armeniaca*), plum (*P. domestica*, *P. insititia*), or ornamental (*P. tomentosa*) trees. Serological tests conducted in 2004, 2005 and 2006 with polyclonal antisera by Agdia (USA) validated the high rate of response with all PPV tested. Two primer pairs were designed to amplify 1200 nucleotides (nt) of the 3' region of the PPV genome spanning from the carboxy-terminal part of the Nib cistron to the carboxy-terminus of CP, and 500 nt starting from the conserved *EcoRI* site of the CP cistron to the polyA tail, respectively. Nucleotide sequences of these isolates were determined either from the amplified fragment or the recombinant plasmid cloned into *E. coli*. Two regions with variable nucleotides were found, the first 110 nt upstream the cleavage site, and the second in nucleotides encoding the cleavage site of NIa-Pro between the Nib and CP cistrons. Sequence homology among the viral isolates was very high. However, phylogenetic analysis of the sequences coding for CP indicated that diversity resided in the amino-terminal section. This finding was corroborated by the use of monoclonal antibodies to PPV D (Durviz, Spain) and PPV M (Agritest, Italy). We discuss the results of these analyses and, particularly, the significance of these nucleotide diversities.

Key words: Sharka, PPV, serology, sequencing, bioinformatics.

INTRODUCTION

Plum pox virus (PPV), a member of the genus *Potyvirus* which comprises more than 175 independent species, has devastating effects on the stone fruit industry. This virus was originally identified in Europe (Atanassov, 1932), but is now widely distributed in the Balkans and Central Europe and has inadvertently crossed the Atlantic Ocean through the movement of infected germplasm, being reported from Chile in 1993, then USA and Canada from 1999 (Gildow *et al.*, 2004).

More than two hundred complete or partial sequences of the PPV genome are available in NCBI Database and GenBank. Six well-characterized PPV strains are known, namely C (cherry), D (Dideron, French), EA (El Amar, Egyptian), M (Markus, Greek), Rec (commonly found in Central Europe) and W (Canadian) (James and Varga, 2005; Candresse and Cambra, 2006). All can be detected by polyclonal antisera but only some can be distinguished with monoclonal antibodies (Cambra *et al.*, 1994; Boscia *et al.*, 1997). The development of molecular tools aimed at the typing of PPV genomes has allowed the arrangement of viral isolates in different subtypes. Because the viral genome has a high consensual sequence in the 3' region, many studies have investigated this genome segment, allowing the detection of many forms of stellates. This 3' region that comprises the carboxy-terminus of Nib and the beginning of the core sequence of CP contains predominantly one of the lesser conserved sequence of the viral genome. The CP segment that harbours the DAG amino acid residue triplet, involved in aphid transmission (Atreya *et al.*, 1990), is suspected as encoding the viral epitopic sites (Cambra *et al.*, 2006). The main goal of the present study was to analyse the phylogenetic relationships of selected PPV isolates. Samples were collected from the field. Viral isolates maintained in woody perennials were utilized as the basic material for identifying possible links between isolates and their molecular sequences. Geographical origins, *Prunus* species sources, serological properties and sequencing data are the key-factors that we have explored for understanding the complex diversity of the PPV genome.

MATERIALS AND METHODS

Viral isolates. A collection of 40 PPV isolates sampled mostly from orchards of 12 different countries was used. Since these isolates elicit different reactions on GF305 peach (*Prunus persica*), *P. domestica*, *P. insititia*, *P. tomentosa* and *P. armeniaca*, symptoms were used as a basis for their selection. For instance, leaf distortion and vein clearing on peach and mosaic on *P. tomentosa*, apricot and plum represented the severe form of the disease. Some isolates that induced mild vein clearing or recovery reactions in peach trees, were analyzed (Table 1). The rest exhibited long-lasting symptoms on the leaves.

To standardize analytical assays, attempts were made to detect the same relative virus concentration in woody plant tissue. Therefore, plants were vernalized and samples were collected 3 weeks after bud-breaking.

Serological assays. Fresh leaves from infected woody hosts were collected, placed in an ice bag prior to grinding. Plant tissues for immunodetection were extracted in an appropriate buffer and the extracts were subjected to ELISA according to the protocols recommended by the manufacturers, relative to the use of a polyclonal antiserum (Agdia, USA) and to three monoclonal antibodies raised to PPV, PPV-D (Durviz,

Table 1. PPV isolates, their geographical origin, source, pathogenicity, and response to serological and molecular assays.

Isolate	Country of origin	Source	Outward appearance	DAS- ELISA	RT-PCR
Austria	Austria	Peach GF305	Symptomless	++	+
Austria	Austria	" "	Symptomatic	+++	+
PT3-2	Romania	" "	Symptomatic	+++	+
PT3-2	Romania	" "	Symptomless	-	+
El Amar	Egypt	" "	Symptomless	++	+
El Amar	Egypt	" "	Symptomatic	+++	+
SR	Bulgaria	" "	Symptomatic	+++	+
C2-38a	Romania	" "	Symptomless	+++	+
4R	Romania	" "	Symptomatic	++	+
KV	Czech Rep	" "	Symptomatic	+++	+
Seo	Serbia	" "	Symptomless	+	+
Seo	Serbia	" "	Symptomatic	+++	+
Apr.Turk	Turkey	" "	Symptomless	++	+
Apr.Turk	Turkey	" "	Symptomatic	+++	+
Reg	Romania	" "	Symptomless	+	+
Reg	Romania	" "	Symptomatic	+++	+
C2-38	Romania	" "	Symptomless	+++	+
C2-38	Romania	" "	Symptomatic	+++	+
V7	Romania	" "	Symptomatic	+++	+
NC	Serbia	" "	Symptomatic	+++	+
606	Tunisia	" "	Symptomatic	+++	+
For	Tunisia	" "	Symptomatic	+++	+
M	Greece	" "	Symptomless	++	+
M	Greece	" "	Symptomatic	+++	+
M	Greece	<i>P. tomentosa</i>	Symptomatic	+++	+
P2-6	Romania	Peach GF305	Symptomatic	+++	+
Apg	Romania	" "	Symptomatic	+++	+
Fur.rotura	Romania	" "	Symptomatic	+++	+
Roy	Spain	" "	Symptomatic	+++	+
Diana	Romania	" "	Symptomatic	+++	+
Bn	Romania	" "	Symptomatic	+++	+
DF	France	" "	Symptomatic	+++	+
Bor	Slovakia	<i>P. armeniaca</i>	Symptomless	+++	+
SP	France	Peach GF305	Symptomatic	+++	+
NQ	Romania	" "	Symptomatic	+++	+
M-C6	Greece	<i>P. domestica</i>	Symptomatic	+++	+
Va	Romania	<i>P. insititia</i>	Symptomatic	+++	+
V6	Romania	Peach GF305	Symptomless	++	+
P40-2	Romania	" "	Symptomatic	+++	+
BN-6	Romania	" "	Symptomatic	+++	+
Pea	Romania	" "	Symptomatic	+++	+

(+++): Means of OD values higher than 2.0

(++): Means of OD values between 1.0 and 2.0

(+): Means of OD values between 0.5 and 1.0

(-): Backgrounds similar to the OD values of buffer.

Spain) and PPV-M (Agritest, Italy), respectively.

Total RNA extraction and amplification of cDNA.

100 mg of fresh leaf tissues from the previously tested plants were ground in liquid nitrogen and the resulting powder was treated for total RNA extraction. Using a quick protocol (kit Rneasy; Qiagen, Germany), approximately 30 to 40 µg of total RNA were produced, 5 µg of which were used as a template to amplify, via the one-step RT-PCR protocol (Qiagen, Germany), a fragment of 1.2 Kbp spanning the carboxy-terminus of N1b to the end of the CP cistron. These cDNAs were amplified using primer pairs Clafar and P1 (Ravelonandro *et al.*, 2007). Amplicons were checked in agarose gel electrophoresis, and purified by polyacrylamide gel electrophoresis. After elution, DNA was precipitated with ethanol and the pellet was suspended in water for direct sequencing or cloning in *E. coli* plasmid (Promega, USA).

Sequencing of amplified fragments and bioinformatics. To obtain molecular information about the selected PPV isolates, amplified cDNA matching the 3' region of the PPV genome was sequenced by the dye terminator technology (IBMP, France). Analysis with data baseline was conducted with information available in GenBank (PPV-Bor3, AY028309, PPV-D, X16415; PPV-M; Ravelonandro, 2006) and the results from the experiments were then added. Further analyses, such as sequence alignments, were done using the Multiple alignment

program of the bioinformatic Kodon software purchased from Applied Maths (Saint-Martens-Latem, Belgium). Multiple alignment was analyzed with the distance matrix program (UPGMA) and phylogenetic trees were generated.

RESULTS AND DISCUSSION

High recognition of different PPV isolates by the tested polyclonal antiserum. To verify the sensitivity and specificity of the polyclonal antiserum produced by Agdia (Elkhart, USA), challenge experiments were conducted in parallel with the universal monoclonal antibody (5B-IVIA) commercialized by Durviz (Valencia, Spain). The rationale for these experiments was the standardized testing of a wide range of PPV isolates collected from 12 different countries in Europe and North Africa. To preserve the native form of PPV in *Prunus*, we decided to sample fresh leaves from the presumably infected woody hosts. As expected, the OD values of the healthy plant controls matched those of negative controls and of the extraction buffer. As to the known PPV isolates such as D, M (Kerlan and Dunez, 1979), El Amar (Wetzel *et al.*, 1991) and Bor3 or Rec (Slovakia) (Glasa *et al.*, 2004), Table 1 shows the good sensitivity of the assays. As expected, the lower positive reaction of a few PPV isolates, notably those from the symptomless leaves (e.g those inoculated with PPV-Rec or

Table 2. Selected viral isolates for serological assays.

Isolate	Country of origin	Monoclonal anti-D	Monoclonal anti-M	Polyclonal antiserum
SR	Bulgaria	-	+	+++
4R	Romania	+	-	++
KV	Czech Rep.	-	+	+++
NC	Serbia	+	-	+++
NQ	Romania	-	+	+++
DF	France	+	-	+++
606	Tunisia	+	-	+++
Apg	Romania	+	-	+++
Bor	Slovakia	-	+	+++
Di	Romania	+	-	+++
For	Tunisia	+	-	+++
M	Greece	-	+	+++
Pea	Romania	-	+	+++
Reg	Romania	+	-	+++
Roy	Spain	+	-	+++
Seo	Serbia	-	+	+++
Bn	Romania	+	-	+++
SP	France	-	+	+++
V7	Romania	-	-	+++
Va	Romania	-	-	+++

Seo) may undoubtedly reflect the specific biological reaction of peach GF305, the same as for the symptomless plant inoculated with PPV-PT3-2 where virus infection was only confirmed by RT-PCR.

With reference to the variations of the relative quantity of virus detected in *Prunus* species, we decided to verify these inconsistent observations through the possible difference in specific epitopes. Using PPV-D and PPV-M (Kerlan and Dunez, 1979) as references, we verified the apparent diversity of 18 selected PPV isolates that were assayed with monoclonal antibodies to PPV-D (Cambra *et al.*, 1994) and PPV-M (Boscia *et al.*, 1997). If 16 PPV isolates were serotypically identified by this way, the absence of reaction of PPV-Va and PPV-V7 with both monoclonal antibodies led us to consider that these two isolates are different and no conclusive classification could be drawn from these serological tests (Table 2). The hypothesis that these negative results could be due to an uneven distribution of these isolates in their host, was not supported by the fact that a clear-cut positive response was obtained with the polyclonal antiserum and RT-PCR.

Sequences of the 3' region and alignment. To better characterize the PPV isolates under study, we have determined the nucleotide sequences of the 3' region of the genome of 18 selected isolates. Following extraction of total RNA from infected tissue, cDNAs were produced and the segment comprising the carboxy-terminus of the Nib and the CP cistrons was investigated for variations. In particular, through the analysis of a viral segment of 407 nt (Fig.1-4), we verified whether these isolates were substantially different and pointed out what variation would occur. A multiple alignment was built following the state-based and the distance-based methods. Eighteen major spots appeared as discriminating in the first 110 nt encoding the carboxy-terminus part of Nib.

Five significant changes reflected by the following transversions were identified: (i) position -92nt, where the T mutation of PPV-M to A of PPV-D is not silent for the amino acid residue "serine" is mutated to "threonine"; (ii) transversion at position -65nt, where the A of PPV-M mutated to C of PPV-D is silent, because both sequences encode an "arginine"; (iii) transversion at position -51nt, where the A of PPV-M mutated to T of PPV-D is silent because both amino acid residues encode "alanine", except for PPV-Roy belonging to strain D which encodes a "valine"; (iv) transversion at position -26/25, where the CT dinucleotides of PPV-M mutated to GG of PPV-D is not silent because the amino acid residue "isoleucine" is mutated to "glycine"; (v) transversion at position -21nt, which is not silent because the C mutation of PPV-M to G of PPV-D corresponds to the change of "aspartic acid" to "glutamic acid". Strikingly, these last 45 nt of Nib that encode the carboxy terminus portion is among the least conservative seg-

ment of the 3' region. In parallel with the cited transversions, a few substitutions occurred: (i) at position -45, A (PPV-M) to G (PPV-D) that led the change of an "aspartic acid" to "asparagine" residues; (ii) at position -38, G (PPV-M) to A (PPV-D) that led the change of an "valine" to "isoleucine"; (iii) at position -29, mutation A to G that induced variation of "serine" to "aspartic acid".

As reported by Glasa *et al.* (2004), the heptapeptide cassette (SNVIHQ for PPV-M) and (SNVVVHQ for PPV-D) that corresponds to the cleavage site of Nia-pro is consensually conserved. Fig.1 shows that the rare divergence can occur at the third nucleotide encoding the "glutamine" residue. This can be observed with PPV-V7 whose Nib cistron ends unexpectedly with a G, rather than an A as it is common for PPV-D genome. So what would it be the incidence of this mutation?

Serological responses and phylogenetic analyses. To verify the possible relationships between these molecular variations and the difference in the serogrouping of PPV, we chose to restrict our investigation to the CP cistron. Multiple alignment of the 17 sequences of the uncharacterized PPV isolates in parallel with those of PPV-D, -M and -Bor3 (Rec), revealed a highly conserved sequences in these first 45 nt encoding the amino terminal region (Fig. 2). If a few transversions can occur at two sites of the amino terminus part of the CP cistron, notably at position 21 (mutation of T of PPV-M to G of PPV-D) and position 30 where the nucleotide is represented by T, G or C, all variations would lead to a silent mutation. However, the sequences comprising nucleotides at position 46 to 189 showed significant variations. Fig. 3 shows a few stretches of variances (20). Considering these 20 variable sites that range from 1 nt (positions 47, 51, 54, 91, 99,105, 120, 123, 126, 133, 150, 165, 168, 176 and 186), 2 nt (positions 116-117, 188-189), 3nt (positions 171-173) to 4 nt (positions 73-76, 80-83), substitutions and transversions of this cistron portion that encodes an hydrophobic domain may lead to the change of specific epitopes.

Apart from molecular considerations, Salamon and Palkovics (2002) pointed out the potential use of these divergent sites for PPV detection and Cambra *et al.* (2006) emphasized the diagnostic role of these sites based on the lack of recognition of isolate PPV B1298 by the monoclonal antibody to PPV-M. In fact, PPV B1298 genome has a 135 nt deletion from position 91 to 226.

Fig. 4 shows that the extensive analysis of CP, from nucleotides 190 to 291, contains 16 variable sites (positions 190, 192, 198-199, 200-203, 206, 210-211, 220-222, 228, 253, 261, 263-264, 266, 269, 276-278, 281, and 284). Even if an epitope consists of 6 amino acid residues or less, taking together the deletions, substitutions and transversions detected in the CP segment in nt positions 47 to 228, it seems plausible to conclude that this is determinant gene segment encoding epitopes in-

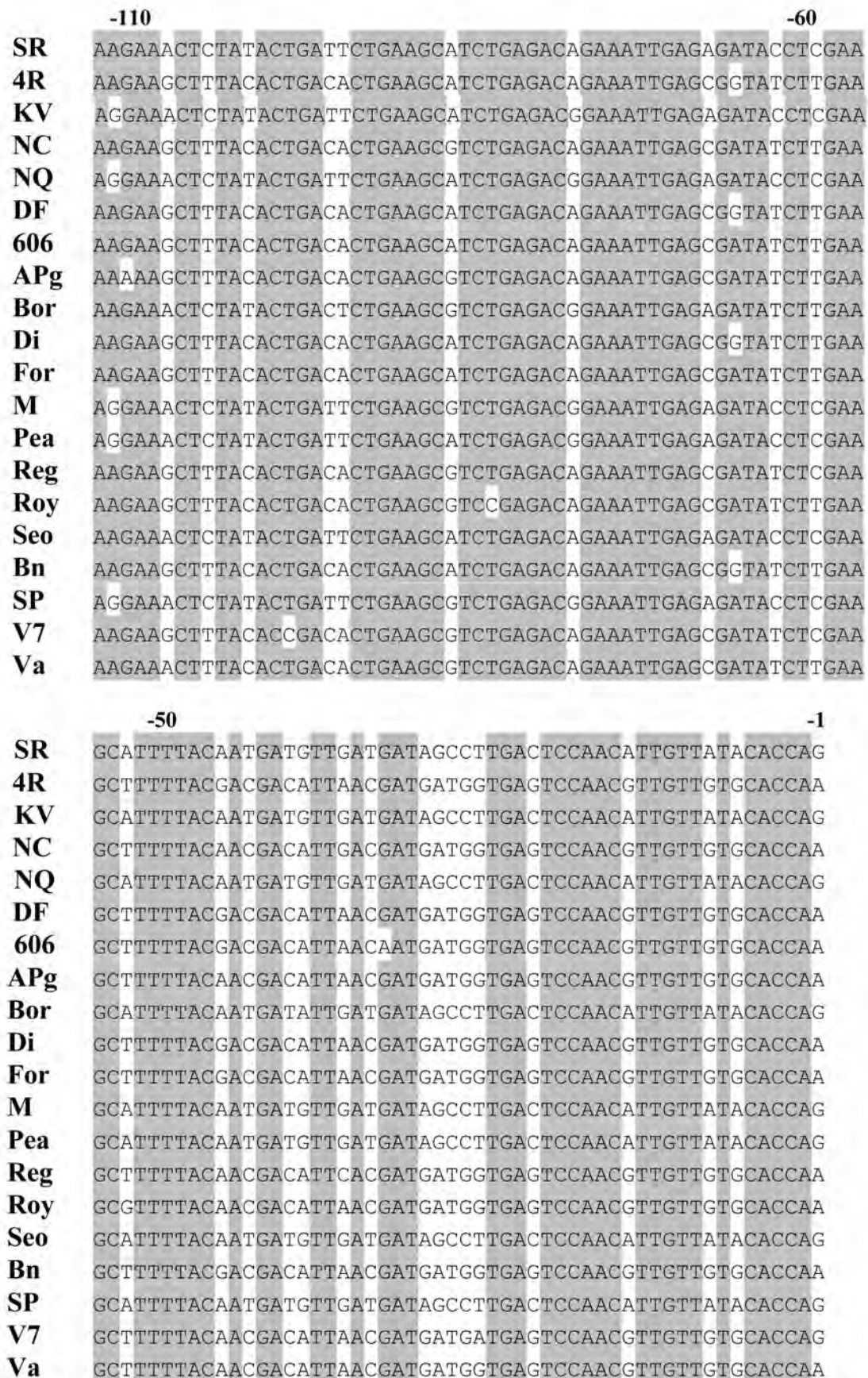


Fig. 1. Sequence alignment of the 110 nucleotides encoding the carboxy terminus of the Nib cystron. The position of the nucleotide at the COOH portion is indicated by the numbers. Consensus blocks are shaded.

	1	40
SR	GCTGATGAAAGGGAGGACGATGAAGAAGTTGATGCAGGAAGACCT	
4R	GCTGACGAAAGAGAAGACGAGGAGGAAGTTGATGCAGGCAAGCCG	
KV	GCTGATGAAGAGGAAGACGATGAAGAAGTGGATGCGGGAAGACCT	
NC	GCTGACGAAAGAGAAGACGAGGAAGAAGTCGACGCAGGCAAGCCG	
NQ	GCTGATGAAGAGGAGGACGATGAAGAAGTGGATGCAGGAAGACCT	
DF	GCTGACGAAAGAGAAGACGAGGAGGAAGTTGATGCAGGCAAGCCG	
606	GCTGACGAAAGAGAAGACGAGGAGGAAGTTGATGCAGGCAAGCCG	
APg	GCTGACGAAAGAGAAGACGAGGAGGAAGTTGATGCAGGCAAGCCG	
Bor	GCTGATGAAAAGGAGGACGATGAAGAAGTCGATGCAGGAAAACCC	
Di	GCTGACGAAAGAGAAGACGAGGAGGAAGTTGATGCAGGCAAGCCG	
For	GCTGACGAAAGAGAAGACGAGGAGGAAGTTGATGCAGGCAAGCCG	
M	GCTGATGAAGAGGAGGATGATGAAGAAGTGGATGCAGGAAGACCT	
Pea	GCTGATGAAGAGGAGGACGATGAAGAAGTGGATGCAGGAAGACCT	
Reg	GCTGACGAAAGAGAAGACGAGGAGGAAGTTGATGCAGGCAAGCCG	
Roy	GCTGACGAAAAGAAGACGAGGAGGAAGTTGACGCAGGCAAGCCG	
Seo	GCTGATGAAAGGGAGGACGATGAAGAAGTTGATGCAGGAAGACCT	
Bn	GCTGACGAAAGAGAAGACGAGGAGGAAGTTGATGCAGGCAAGCCG	
SP	GCTGATGAAGAGGAGGACGATGAAGAAGTGGATGCAGGAAGACCC	
V7	GCTGACGAAAGAGAAGACGAGGAGGAAGTTGATGCAGGCAAGCCG	
Va	GCTGACGAAAGAGAAGACGAGGAGGAAGTTGATGCAGGCAAGCCA	
	50	90
SR	ACTGTGGTAACTGCACCGGCAGCAACTGTGACAACAACTCAACCA	
4R	ATTGTAGTTACTGCACCGGCAGCAACTAGCCCAATACTTCAACCA	
KV	ACTGTGGTAACTGCAGCGGCAGCAACTGTGGCAACGACTCAACCC	
NC	GTTGTAGTTACTGCACCGGCAGCAACTAGCCCAATACTTCAACCA	
NQ	ACTGTGGTAACTGCACCGGCAGCAACTGTGGAAACGACTCAACCA	
DF	ATTGTAGTTACTGCACCGGCAGCAACTAGCCCAATACTTCAACCA	
606	ATTGTAGTCACTGCACCGGCAGCAACTAGCCCAATACTTCAACCA	
APg	ATTGTAGTTACTGCACCGGCAGCAACTAGCCCAATACTTCAACCA	
Bor	ACTGTAGTAACTGCACCGGCAGCAACTGTGGCAACAACTCAACCA	
Di	ATTGTAGTTACTGCACCGGCAGCAACTAGCCCAATACTTCAACCA	
For	ATTGTAGTCACTGCACCGGCAGTAACTAGCCCAATACTTCAACCA	
M	ACTGTGGTAACTGCACCGGCAGCAACTGTGGCAACGACTCAACCA	
Pea	ACTGTGGTAACTGCACCGGCAGCAACTGTGGAAACGACTCAACCA	
Reg	ATTGTAGTTACTGCACCGGCAGCAACTAGCCCAATACTTCAACCA	
Roy	ATTGTAGTTACTGCACCGGCAGCAACCAGCCCAATACTTCAACCA	
Seo	ACTGTGGTAACTGCACCGGCAGCAACTGTGACAACAACTCAACCA	
Bn	ATTGTAGTTACTGCACCGGCAGCAACTAGCCCAATACTTCAACCA	
SP	ACTGTGGTAACTGCACCGGCAGCAACTGTGGCAACGACTCAACCA	
V7	ATTGTAGTTACTGCACCGGCAGCAACTAGCCCAATACTTCAACCA	
Va	ATTGTAGTTACTGCACCGGCAGCAACTAGCCCAATACTTCAACCA	

Fig. 2. Sequence alignment of the first 90 nucleotides encoding the amino terminus of CP. The position of the nucleotide at the NH₂ portion is indicated by the numbers. Consensus blocks are shaded.

	100	130
SR	GCTCCAGTGATACAACCCGCACCCCAAACCACAGCACCAATGTTCAAC	
4R	CCTCCAGTCATACAGCCTGCACCCCGGACTACGGCGCCAATGCTCAAC	
KV	GCTCCAGTGATACAACCTGCACCCCAAACCACAGCACCAATGTTCAAC	
NC	CCTCCAGTCATACAGCCTGCACCCCGGACTACGGCGCCAATGTTCAGC	
NQ	GCTCCAGTGATACAACCTGCACCCCAAACCACAGCACCAATGTTCAAC	
DF	CCTCCAGTCATACAGCCTGCACCCCGGACTACGGCGCCAATGCTCAAC	
606	CCTCCAGTCATACAGCCTACACCCCGGGCTACGGCGCCAACGCTCAAC	
Apg	CCTCCAGTCATACAGACCGCACCCCGGACTACGGCGCCAATGTTCAAC	
Bor	GCTCCAGTGATACAACCTGCAATTCAAACCACAACACCAATGTTCAAC	
Di	CCTCCAGTCATACAGCCTGCACCCCGGACTACGGCGCCAATGCTCAAC	
For	CCTCCAGTCATACAGCCTACACCCCGGGCTACGGCGCCAATGCTCAAC	
M	GCTCCAGTGATACAACCTGCACCCCAAACCACAGCACCAATGTTCAAC	
Pea	GCTCCAGTGATACAACCTGCACCCCAAACCACAGCACCAATGTTCAAC	
Reg	CCTCCAGTCATACAGCCTGCACCCCGAACCACGGCGCCAATGTTCAAC	
Roy	CCCCCAGTCATACAGCCTGCACTCCGGACTACGGCGCCAATGCTCAAC	
Seo	GCTCCAGTGATACAACCCGCACCCCAAACCACAGCACCAATGTTCAAC	
Bn	CCTCCAGTCATACAGCCTGCACCCCGGACTACGGCGCCAATGCTCAAC	
SP	GCCCCAGTGATACAACCTGCACCCCAAACCACAGCACCAATGTTCAAC	
V7	CCTCCAGTCATACAGCCTGCACCCCGGACCACGGCGCCAATGTTCAAC	
Va	CCTCCAGTCATACAGCCTGCACCCCGGACTACGGCGCCAATGCTCAAC	
	140	180
SR	CCTATTTTCACTCCAGCAACAACCTCAACCTGCGGTAAGACCAGTTCTCCA	
4R	CCCATTTTCACGCCAGCAACAACCTCAACCAGCAACAAAACCAGTTTCACAG	
KV	CCCATTTTCACTCCAGCAACAACCTCAGCCTGCGATAAGACCAGTTTCTCCA	
NC	CCCATTTTACGCCAGCAACAACCTCAACCAGCAACAAAACCAGTTTCACAG	
NQ	CCCATTTTCACCCCAGCAACAACCTCAGCCCGCGGTAAGACCAGTTCTCCA	
DF	CCCATTTTCACGCCAGCAACAACCTCAACCAGCAACAAAACCAGTTTCACAG	
606	CCCATTTTCACGCCAGCAACAACCTCAACCAGCAACAAAACCAGTTTCACAG	
Apg	CCCATTTTACGCCAGCTACAACCTCAACCAGCAACAAAACCAGTTTCACAG	
Bor	CCCATTTTCACTCCAGCAACAACCTCAGCCTGCGATAAGACCAGTTTCTCCA	
Di	CCCATTTTCACGCCAGCAACAACCTCAACCAGCAACAAAACCAGTTTCACAG	
For	CCCATTTTCACGCCAGCAACAACCTCAACCAGCAACAAAACCAGTTTCACAG	
M	CCCATTTTCACTCCAGCAACAACCTCAGCCTGCGGTAAGACCAGTTCTCCA	
Pea	CCCATTTTCACCCCAGCAACAACCTCAGCCTGCGGTAAGACCAGTTCTCCA	
Reg	CCCATTTTCACGCCAGCAACAACCTCAACCAGCAACAAAACCAGTTTCACAG	
Roy	CCCATTTTCACGCCAGCAACAACCTCAACCAGCAACAAAACCAGTTTCACGG	
Seo	CCTATTTTCACTCCAGCAACAACCTCAGCCTGCGGTAAGACCAGTTTCTCCA	
Bn	CTCATTTTCACGCCAGCAACAACCTCAACCAGCAACAAAACCAGTTTCACAG	
SP	CCCATTTTCACTCCAGCAACAACCTCAGCCTGCGATAAGACCAGTTTCTCCA	
V7	CCCATTTTCACGCCAGCAACAACCTCAACCAGCAACAGAACCAGTTTCACAG	
Va	CCCATTTTCACGCCAGCAACAACCTCAACCAGCAACAAAACCAGTTTCACAG	

Fig. 3. Sequence alignment of the following 98 nucleotides encoding the NH₂ part of CP (from nucleotide 91 to 189). The position of the nucleotide is indicated by the numbers. Consensus blocks are shaded.

	190	230
SR	ATTTTCAGGGACCACACCGCAGTCTTTTGGAGTTTATGGAAATGAGGAT	
4R	GTGCCAGGACCTCAACTGCAAACCTTTTGGAACATATGGTAATGAGGAT	
KV	ATTTTCAGGGGGCCACACCGCAGTCTTTTGGAGTTTATGGAAATGAGGAT	
NC	ATGTC AAGACCTCAACTACAACTTTTGGAACACATGGTAATGAGGAT	
NQ	ATTTTCAGGGGGCCAAACCGCGGTCTTTTGGAGTTTATGGAAATGAAGAC	
DF	GTGCCAGGACCTCAACTGCAAACCTTTTGGAACATATGGTAATGAGGAT	
606	GTGTCAGGACCTCAACTGCAAACCTTTTGGAACATATGGTAATGAGGAT	
APg	GTGTCAGGACCTCAACTGCAAACCTTTTGGAACACATGGTAATGAGGAT	
Bor	ATTTTCAGGGGGCCACACCGCAGTCTTTTGGAGTTTATGGAAATGAGGAT	
Di	GTGCCAGGACCTCAACTGCAAACCTTTTGGAACATATGGTAATGAGGAT	
For	GTGTCAGGACCTCAACTGCAAACCTTTTGGAACATATGGTAATGAGGAT	
M	ATTTTCAGGGGGCCAAACCGCGGTCTTTTGGAGTTTATGGAAATGAAGAC	
Pea	ATTTTCAGGGGGCCAAACCGCGGTCTTTTGGAGTTTATGGAAATGAAGAC	
Reg	GTGTCAGGACCTCAACTGCAAACCTTTTGGAACACATGGTAATGAGGAT	
Roy	GTGTCAGGACCTCAACTGCAAACCTTTTGGAACACATGGTAATGAGGAT	
Seo	ATTTTCAGGGGGCCACACCGCAGTCTTTTGGAGTTTATGGAAATGAGGAT	
Bn	GTGTCAGGACCTCAACTGCAAACCTTTTGGAACATATGGTAATGAGGAT	
SP	ATTTTCAGGGGGCCACACCGCAGTCTTTTGGAGTTTATGGAAATGAGGAT	
V7	GTGTCAGGACCTCAACTGCAAACCTTTTGGAACATATGGTAATGAGGAT	
Va	GTGTCAGGATCTCAACTGCAAACCTTTTGGAACACATGGTAATGAGGAT	
	240	290
SR	GCATCACCTAGCACCTCAAACACTTTGGTGAACACAGGAAGGGATAGGGACG	
4R	GCATCACCTAGCAACTCAAACCGCGCTAGTCAACACAAACAGAGACAGGGACG	
KV	GCATCACCTAGCACCTCAAACACTTTGGTGAACACAGGAAGGGATAGGGACG	
NC	GCCTCACCTAGCAACTCAAACCGCGCTAGTCAACACAAACAGAGACAGGGACG	
NQ	GCATCACCTAGCACCTCAAACACTTTGGTGAATACAGGAAGGGATAGGGACG	
DF	GCATCACCTAGCAACTCAAACCGCGCTAGTCAACACAAACAGAGACAGGGACG	
606	GCATCACCTAGCAACTCAAACCGCGCTAGTCAACACAAACAGAGACAGGGACG	
APg	GCCTCACCTAGCAACTCGAACCGCGCTAGTCAACACAAACAGAGACAGGGACG	
Bor	GCATCACCTAGCACCTCAAACACTTTGGTGAACACAGGAAGGGATAGGGACG	
Di	GCATCACCTAGCAACTCAAACCGCGCTAGTCAACACAAACAGAGACAGGGACG	
For	GCATCACCTAGCAACTCAAACCGCGCTAGTCAACACAAACAGAGACAGGGACG	
M	GCATCACCTAGCACCTCAAACACTTTGGTGAATACAGGAAGGGATAGGGACG	
Pea	GCATCACCTAGCACCTCAAACACTTTGGTGAATACAGGAAGGGATAGGGACG	
Reg	GCCTCACCTAGCAACTCAAACCGCGCTAGTCAACACAAACAGAGACAGGGACG	
Roy	GCATCACCTAGCAACTCAAACCGCGCTAGTCAACACAAACAGAGACAGGGACG	
Seo	GCATCACCTAGCACCTCAAACACTTTGGTGAACACAGGAAGGGATAGGGACG	
Bn	GCCTCACCTAGCAACTCAAACCGCGCTAGTCAACACAAACAGAGACAGGGACG	
SP	GCATCACCTAGCACCTCAAACACTTTGGTGAACACAGGAAGGGATAGGGACG	
V7	GCATCACCTAGCAACTCAAACCGCGCTAGTCAACACAAACAGAGACAGGGACG	
Va	GCCTCACCTAGCAACTCAAACCGCGCTAGTTAACACAAACAGAGACAGGGACG	

Fig. 4. Sequence alignment of the 101 nucleotides (from nt 190 to 291) following the former 189 nucleotides encoding the NH₂ portion of CP. The position of the nucleotide is indicated by the numbers. Consensus blocks are shaded.

volved in the recognition of PPV.

With reference to the failure of the two commercial monoclonal antibodies to D and M types to recognize PPV-V7 and PPV-Va, we analyzed the multiple alignment that was built following the state-based and the distance-based methods. This phylogenetic exercise allowed to show that PPV-V7 and PPV-Va belong to strain D (Fig. 5). The derived amino acid sequences (not shown) confirmed that the subclades obtained revealed some links between PPV isolates having the same geographical origin. Fig. 5 shows that the molecular phylogeny follows a division between the selected isolates belonging to D and M types. A typical example is given by the PPV-Pea and PPV-NQ isolates that come from different *Prunus* species (i.e. *P. persica* for PPV-Pea and *P. domestica* for PPV-NQ) thus representing an example of Romanian isolates co-existing in the same area and replicating in different hosts. Likewise, PPV-SR (Bulgaria) and PPV-Seo (Serbia), came both from the Balkans but were originally collected from peach and apricot trees.

With reference to the same geographical context [PPV-606 and PPV-For from Tunisia (Boulila *et al.*, 2004); PPV-Reg and PP-V7; PPV-Apg and PPV-Va from Romania], some heterogeneity occurred in the genome and the analysis of sequence alignments disclosed that they diverged in the amino terminus section of CP. Strikingly, this heterogeneity at the NH₂ terminus is rare in strain D but appears to be consistent within the geographical divergence, e.g. Tunisian versus Romanian isolates. However, the larger heterogeneity of a few predicted sites in this CP amino terminus allows to distinguish D and M, the two major PPV serotypes (Fig. 5).

The challenge remains on the detection of the epitopic divergence between PPV-M and PPV-Rec strains since the restricted numbers of nucleotide variations between CP-M and CP-Rec render complex such identification. Extensive work must be conducted to establish whether these amino acid residues can really represent the signature motifs of these targeted specific epitopes, or to extend the exploration to other part of the CP cistron.

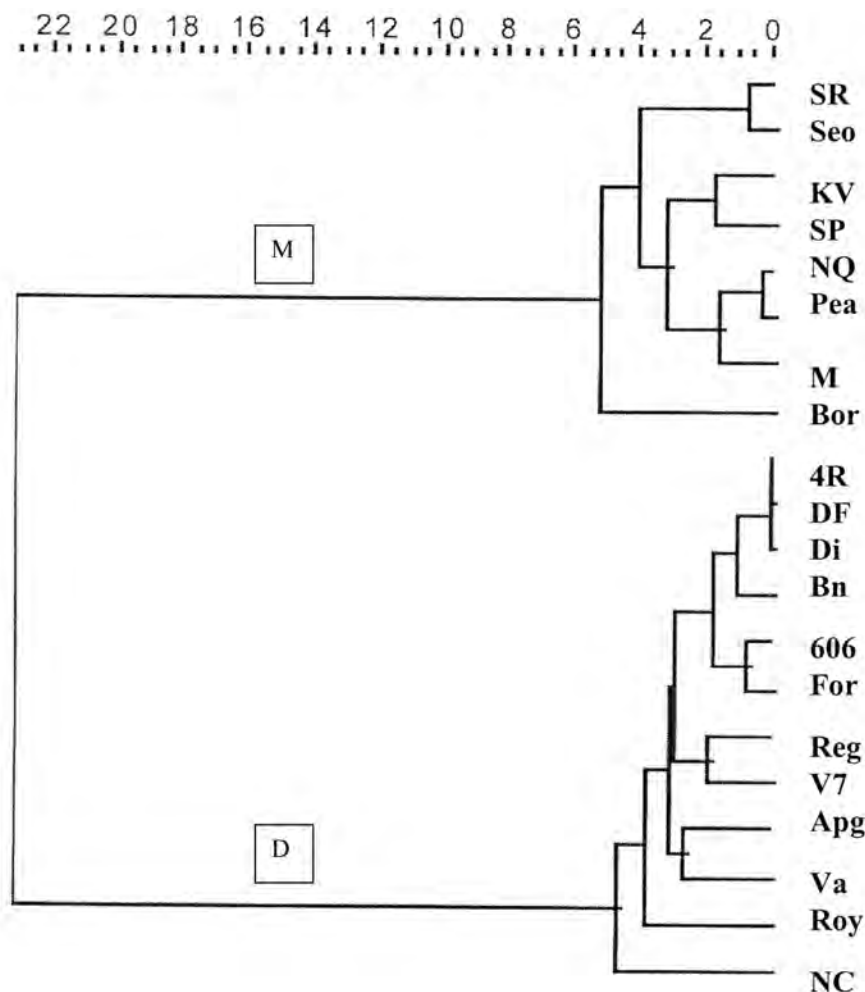


Fig. 5. Phylogenetic tree generated by the distance matrix program, UPGMA, branch lengths are proportional to the relative mutational distance.

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