Detection of a New and Unusual Isolate of *Plum pox virus* in Plum (*Prunus domestica*)

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ABSTRACT

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Plum pox virus (PPV) isolate 3174-01 was detected by triple-antibody sandwich enzyme-linked immunosorbent assay using the universal PPV monoclonal antibody (MAb) 5B as the secondary antibody, and by reverse-transcription polymerase chain reaction (RT-PCR) using primers that amplify a 243-bp fragment in the C-terminus of the coat protein coding region. The restriction sites *RsaI* and *AluI* were absent from this fragment, which is a feature unique to PPV-C isolates. The restriction sites in 3174-01 were replaced by GTAA/GTGA and GGCA, respectively. There was 95 to 99, 94, 91, and 92 to 94% identity of the 243-bp fragment of 3174-01 with the corresponding region of the strains C, D, EA, and M, respectively. Attempts to detect the virus by RT-PCR using strain C-specific primers in three different approaches were unsuccessful. All molecular techniques assessed in attempting to strain type isolate 3174-01 gave negative results, or results inconsistent for D or M in the case of P3-6K₁ restriction fragment length polymorphism analysis. Isolate 3174-01 reacted in Western blot assay with MAb 5B, with an estimated molecular mass of 32 kDa. No reaction was observed with D-, M-, EA-, or C-specific monoclonal antibodies in Western blot or enzyme-linked immunosorbent assay. The molecular and serological data seem to indicate that PPV isolate 3174-01 does not belong to any of the recognized strains of PPV.

Plum pox virus (PPV) causes plum pox or Sharka disease, which is considered the most devastating disease of stone fruits. Stone fruits infected by PPV include plum, peach, apricot, nectarine, sweet cherry, and sour cherry (5,14,16,17). The genome of PPV consists of approximately 10 kb (13) with a virus-encoded protein covalently linked to the 5' terminus of the genome, and a poly(A) tail at the 3' end. The complete nucleotide sequences of several PPV isolates have been determined (11,13,21). This has facilitated detailed analysis, comparison, and specific detection of the virus.

There are four recognized strains of PPV (D, M, EA, and C), which differ in pathogenicity, host range, efficiency of aphid transmission, and geographic distribution (3,15,18,20). Consequently, strain identification is important in the management or successful eradication of this disease. There are serological and molecular techniques that allow reliable identification and differentiation of PPV strains. Each strain appears to have unique epitopes that

have facilitated the development of monoclonal antibodies specific to strain D (2), M (1), C, and EA (14). Nucleic acid-based approaches to strain identification include: reverse-transcription polymerase chain reaction (RT-PCR) with strain-specific primers (15), RT-PCR with restriction fragment length polymorphism (RFLP) analysis (7,8,23), Heminested PCR with D- and M-specific primers (18), PCR-enzyme-linked immunosorbent assay (ELISA) (18,19), and integrated RT-PCR/nested PCR (20).

PPV recently was detected in the United States (12), and Canada (22). Attempts to eliminate the virus are ongoing and delimitation surveys are being conducted to determine the extent of spread and distribution of the virus. Strain typing is carried out when the virus is detected in a new location in Canada. This article describes attempts at strain typing an isolate of PPV detected in plum trees found growing on the property of a homeowner in the province of Ontario.

MATERIALS AND METHODS

Virus isolates. The virus isolate 3174-01 is a Canadian isolate of PPV detected in plum (*Prunus domestica*) in the garden of a residential property owner during the course of a PPV survey of *Prunus* spp. on homeowner properties in the Stoney Creek area, Ontario. The virus was mechanically sap-transmitted to *Nicotiana benthamiana* by triturating infected plum leaves in 0.01

M potassium phosphate buffer containing 0.02 M diethyldithiocarbamic acid (sodium salt), 0.04 M sodium thioglycolate, and 0.5% nicotine. Several known or well-characterized isolates of PPV were included in this study as positive controls. Isolates PPV-Fa (20-4-1) and PPV-Vu (20-13-1) are Canadian isolates of PPV detected in symptomless nectarine (cv. Fantasia) and in symptomatic clingstone peach (cv. Vulcan), respectively. These two isolates have been identified as D type (9,22). The isolate Q1902-01 (PPV Marcus, type member M strain) was obtained from F. Dosba, France; isolate Q2185-02, D type, was obtained from W. Jelkmann, Germany; PPV-C (a sweet cherry isolate) and PPV-EA (El Amar) were obtained as freeze-dried tissue samples from A. Myrta, Italy.

Triple antibody sandwich ELISA. Triple-antibody sandwich (TAS)-ELISA was carried out as described by Clark and Adams (4). The secondary antibody included the following PPV-derived monoclonal antibodies (MAbs): (i) 5B (a universal PPV MAb obtained from the REAL Durviz TAS ELISA kit), (ii) 4D (D-specific MAb obtained from M. Cambra, Valencia, Spain), (iii) AL (M-specific MAb), (iv) EA24 (El Amar-specific MAb), and (v) AC (C-specific MAb). All MAbs were used at a 1:1000 dilution. The antibodies MAB-AL, MAB-EA24, and MAB-AC were kindly provided by A. Myrta, Bari, Italy. The instructions in the Durviz kit were followed except for (i) use of a modified grinding buffer (phosphate-buffered saline [PBS]-Tween, 0.2% skim milk powder, and 2% polyvinylpyrrolidone [PVP]) and (ii) inclusion of a blocking step using PBS-Tween with 0.2% skim milk powder for 30 min at room temperature. Approximately 0.5 g of leaf tissue was triturated in the grinding buffer using a 1:10 ratio (wt/vol). The homogenate was added to microtiter plates (200 µl/well) that then were stored overnight at 4°C. The monoclonal antibody and conjugate were used at a 1:1000 dilution and stored at 37°C for 3 h and 4°C for 16 h, respectively. The substrate nitrophenyl phosphate was used at 0.6 mg/ml. Samples with absorbance values (at 405 nm) greater than three times the absorbance of the healthy controls were considered positive.

Total RNA extraction. Total RNA was extracted from healthy and infected tissue (100 mg) using the QIAGEN RNeasy Total RNA kit (QIAGEN, Chatsworth, CA). The

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extraction procedure used was essentially as described by the supplier except for the following. The tissue was ground in RiboLyser tubes using the HYBAID RiboLyser Cell Disrupter (InterScience Inc., Markham, Ontario, Canada). QIAGEN's RLT buffer (650 or 500 µl) was added to each tube, to which was added 100 mg of woody or herbaceous tissue, respectively; the sample was triturated using the RiboLyser Cell Disrupter. The tubes were centrifuged at 5,000 × g for 5 min, the supernatant collected and added to QIAGEN's QIASHredder spin columns, and the procedure completed as described by the kit's supplier (QIAGEN).

RT-PCR. Approximately 4 µg of total RNA in 5 µl of diethylpyrocarbonate-treated double-distilled (depc) H₂O was combined with 2 µl of antisense P1 primer (approximately 20 pmol) and incubated at 75°C for 5 min. RT was carried out in a 20-µl volume by adding 13 µl of RT mix. The RT mix consisted of 4 µl of 5× First Strand buffer (Gibco BRL, Life Technologies, Burlington, Ontario), 2 µl of 0.1 M dithiothreitol (DTT), 1 µl of 10 mM dNTP Mix, 0.5 µl of RNase Block (Stratagene, La Jolla, CA), and 1 µl of SUPERScript II (Gibco BRL), and was adjusted up to a final volume of 13 µl with depc H₂O. The PCR reaction mix consisted of 2 µl of 10 mM dNTP mix, 3 µl of 50 mM MgCl₂, 1 µl of sense primer (P2, 12 pmol), 1 µl of antisense primer (P1, 12 pmol), 1 µl of *Taq* DNA polymerase (Gibco BRL), 1 µl of *Taq* Extender PCR Additive (Stratagene), and 10 µl of 10× *Taq* Extender buffer, adjusted to a final volume of 80 µl with depc H₂O. The final volume of each reaction was 100 µl (20 + 80). The final reaction (10 µl) was assessed by agarose gel electrophoresis.

Heminested PCR. Heminested PCR using D- and M-specific sense primers combined with the P1 PPV antisense primer were used for strain typing (18).

RFLP analysis. Three different regions of the virus genome were targeted for strain typing using RFLP analysis of RT-PCR-amplified fragments. These included (i) the coat protein (CP) coding region (23), (ii) the replicase (NIb) gene region (8), and (iii) the P3-6K₁ genomic region (7).

When amplification of 3174-01 was successful, agarose gel-purified cDNA was quantified by spectrophotometry, then subjected to restriction endonuclease digestion.

Strain-specific RT-PCR. RT-PCR specific for strain C isolates of PPV was carried out (15). Both sets of primers were assessed; HsoC-1/CsoC-1 amplifies a 259-

bp fragment, and HsoC-2/CsoC-2 amplifies a 193-bp fragment with sour cherry isolates of PPV.

The integrated RT-PCR/nested PCR technique (20) also was used in attempting to determine the PPV strain to which isolate 3174-01 belongs.

Western blot analysis. Leaf tissue from healthy and infected plants was triturated

Fig. 1. Reverse-transcription polymerase chain reaction and restriction fragment length polymorphism showing amplification but no digestion of the *Plum pox virus* 3174-01-associated 243-bp fragment (23). The fragments were treated with the restriction endonucleases *AluI* (lanes 1–6) and *RsaI* (lanes 8–13); lane 7 is blank. Lane M, 100-bp DNA ladder; lanes 1 and 8, Q2185-02 (D); lanes 2 and 9, Q1902-01 (M); lanes 3–6 and 10–13, 3174-01 in plum. The arrow indicates the position of the 243-bp fragment.

Fig. 2. Heminested polymerase chain reaction using D-specific (lanes 1–5) and M-specific primers (lanes 6–11) for the amplification of a 193-bp fragment (18). Lane 6 is blank. Lane M, 100-bp DNA ladder; lanes 1 and 7, healthy GF305; lanes 2 and 8, Q2185-02 (D); lanes 3 and 9, Q1902-01 (M); lanes 4, 5, 10, and 11, 3174-01 in plum. The arrow indicates the position of the 193-bp fragment.

Table 1. Average absorbance values ($n = 11$, 405 nm) of triple-antibody sandwich enzyme-linked immunosorbent assay for isolate 3174-01 and control samples using the universal *Plum pox virus* (PPV)-specific monoclonal antibody (MAb) 5B, and the D-, M-, EA-, and C-specific MAbs^a

Samples	Detecting antibody ^b				
	5B	4D	AL	EA24	AC
3174-01 Plum	0.174	0.024	0.018	0.032	0.027
3174-01 <i>Nicotiana benthamiana</i>	0.483	0.033	0.024	0.024	0.139
PPV-D <i>N. benthamiana</i>	1.09	0.301	0.020	0.023	0.102
PPV-M <i>N. benthamiana</i>	0.808	0.032	0.240	0.019	0.031
PPV-EA <i>N. benthamiana</i>	0.623	0.027	0.021	0.560	0.142
PPV-C <i>N. benthamiana</i>	0.702	0.027	0.021	0.022	1.62
Healthy <i>N. benthamiana</i>	0.044	0.026	0.020	0.026	0.200
Healthy Shiro Plum	0.044	0.031	0.022	0.021	0.034

^a Readings were taken 1 h after addition of the substrate.

^b 4D = D-specific MAb, AL = M-specific MAb, EA24 = El Amar-specific MAb, and AC = C-specific MAb. Positive values ($>3\times$ absorbance of healthy control) are in bold.

in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.02 M diethyldithiocarbamic acid, sodium salt, 0.04 M sodium thioglycolate, and 0.5% nicotine. Proteins were resolved under denaturing conditions by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (10), and electroblotted using a Semi-Dry Transfer Unit (Bio-Rad, Mississauga, Ontario, Canada) onto nitrocellulose membranes (0.45- μ m pore size; Schleicher & Schuell, Keene, NH). Prestained markers (Bio-Rad) were used to assess transfer of the proteins from the gel to the membrane. Blots were screened with the MABs described above. All MABs were used at a 1:1000 dilution. Goat anti-mouse alkaline phosphatase conjugate (1:2000; Bio-Rad) was used as the

secondary antibody, with NBT-BCIP substrates used as recommended by the supplier (Bio-Rad). The blocking buffer consisted of 0.05 M Tris, 0.15 M sodium chloride, pH 7.4, containing either 1% bovine serum albumin or 2% skimmed milk powder to minimize spurious cross-reactions (24).

Cloning of complementary DNA. Complementary (c)DNA was generated by RT-PCR using *Taq* DNA polymerase as described above. Amplified fragments were identified and isolated by agarose gel electrophoresis with ethidium bromide staining. The appropriate bands were excised and purified using QIAGEN's MiniElute Gel Extraction kit (catalog #28604; QIAGEN Inc., Mississauga, Ontario, Canada). Gel-purified PCR products were ligated into the pCR^(R)2.1 TOPO Vector and cloned using the TOPO TA Cloning Kit, as directed by the supplier (Invitrogen, Carlsbad, CA).

Sequencing and sequence analysis. Sequencing was carried out as described by James and Upton (9). Nucleotide sequence data were compiled and analyzed using the

Staden Program package (6) and database searches were performed with BLAST (blastn) programs at the National Centre for Biotechnology Information (NCBI, National Institutes of Health).

RESULTS

TAS-ELISA. Isolate 3174-01 was detected by the universal PPV MAB 5B in both plum and *Nicotiana benthamiana* (Table 1). None of the strain-specific MABs reacted with 3174-01. They did, however, react with the appropriate positive control virus (Table 1).

RT-PCR analysis. A 243-bp fragment was amplified for all samples of 3174-01 tested, using the P1 and P2 primers (Fig. 1). A fragment of similar size was observed for the positive PPV controls, including isolates of D, M, EA, and C (Fig. 1; lane 9 shows the M fragment). No amplification was observed with any of the healthy controls included in this study.

Heminested PCR. The D-specific primer detected isolate Q2185-02 (Fig. 2, lane 2) and did not detect Q1902-01 (Fig. 2, lane 3) or isolate 3174-01 (Fig. 2, lanes

Fig. 3. Reverse-transcription polymerase chain reaction and restriction fragment length polymorphism of the *Plum pox virus* P3-6K₁ 836-bp fragment (7). The fragments were treated with the restriction endonuclease *AsnI*. Lane M, 100-bp DNA ladder; lane 1, Q2185-02 (D); lane 2, Q1902-01 (M); lanes 3 and 4, 3174-01 in *Nicotiana benthamiana*. The arrow indicates the position of the 836-bp fragment.

Fig. 4. Reverse-transcription polymerase chain reaction and restriction fragment length polymorphism of the *Plum pox virus* P3-6K₁ 836-bp fragment (7). The fragments were treated with the restriction endonuclease *DdeI*. Lane M, 100-bp DNA ladder; lane 1, Q2185-02 (D); lane 2, Q1902-01 (M); lanes 3 and 4, 3174-01 in *Nicotiana benthamiana*. The arrow indicates the position of the 836-bp fragment.

Fig. 5. Reverse-transcription polymerase chain reaction using the *Plum pox virus* C (PPV-C)-specific primers HsoC-1/CsoC-1 to amplify a PPV-C specific fragment of 259 bp (15). Lane M, 100-bp DNA ladder; lane 1, healthy GF305; lane 2, Q2185-02 (D); lane 3, Q1902-01 (M); lane 4, 20-4-1 (D); lane 5, PPV-C (sweet cherry isolate); lanes 6–8, isolate 3174-01. The arrow indicates the position of a band estimated at 300 bp.

Fig. 6. Reverse-transcription polymerase chain reaction using the *Plum pox virus* (PPV-C)-specific primers HsoC-2/CsoC-2 to amplify a PPV-C specific fragment of 193 bp (15). Lane M, 100-bp DNA ladder; lane 1, healthy GF305; lane 2, 20-4-1 (D); lanes 3 and 4, PPV-C (sweet cherry isolate); lane 5, 20-4-1 (D); lanes 6–8, isolate 3174-01. The arrow indicates the position of the 193 bp fragment.

4 and 5). The M-specific primer detected isolate Q1902-01 (Fig. 2, lane 9), but did not detect isolate Q2185-02 or any samples of 3174-01 (Fig. 2, lanes 8, 10, and 11, respectively). All negative controls were negative (Fig. 2, lanes 1 and 7).

RFLP analysis. *CP coding region.* The amplified 243-bp fragment was treated with the enzymes *AluI* and *RsaI*. When treated with the restriction endonuclease *AluI*, the PPV-associated 243-bp products of Q2185-02 (D) and Q1902-01 (M) were digested into fragments of approximately 95 and 148 bp (Fig. 1, lanes 1 and 2, respectively). The 3174-01-associated 243-bp fragments (Fig. 1, lanes 3 to 6) were unaffected by the enzyme *AluI*. Treatment with the endonuclease *RsaI* resulted in the digestion of the PCR product of isolate Q2185-02 (D strain; Fig. 1, lane 8). Isolate Q1902-01 (M strain) and all samples of 3174-01 were unaffected by the enzyme *RsaI* (Fig. 1, lanes 9 and 10 to 13, respectively).

Nlb region. The 1,040-bp fragment required for digestion with the enzymes *DdeI*, *RsaI*, and *TaqI* was not amplified

when samples of 3174-01 were treated with the recommended primers. Isolates of strain D and M included as controls gave the expected fragment and RFLP profile (*data not shown*).

P3-6K₁ region. The 836-bp fragment required for digestion with the enzymes *AsnI*, *DdeI*, and *EcoRI* was amplified when samples of 3174-01 were treated with the recommended primers. Isolates of strain D and M included as controls gave the expected fragment and RFLP profile (Figs. 3 and 4). The 3174-01-derived fragment lacked an *AsnI* restriction site and contained a single *EcoRI* restriction site, which was similar to the pattern observed for Q2185-02 (D). The results after treatment with *AsnI* are shown in Figure 3. Treatment of the 3174-01-derived fragment with the enzyme *DdeI* produced a restriction profile different from that of Q2185-02 (D) and Q1902-01 (M) (Fig. 4). Four fragments were observed after *DdeI* treatment of the 3174-01 product (Fig. 4, lanes 3 and 4), indicating the presence of at least three *DdeI* restriction sites. Q2185-02 (D) and Q1902-01 (M) had one

and no *DdeI* sites, respectively (Fig. 4, lanes 1 and 2).

Strain-specific RT-PCR. The only sample for which amplification was observed when RT-PCR was carried out with the HsoC-1/CsoC-1 primer pair was the sample of PPV-C (Fig. 5, lane 5). A fragment of approximately 300 bp in size was observed. No amplification was observed for any of the other samples included. When the HsoC-2/CsoC-2 primer pair was used in RT-PCR, amplification of a 193-bp fragment was observed only for the two samples of PPV-C included in the study (Fig. 6, lanes 3 and 4). No amplification was observed for any of the other samples, including 3174-01.

Analysis using the integrated RT-PCR/nested PCR technique produced the expected results with all the positive controls included in this study (Figs. 7 and 8). No amplification was observed for any of the 3174-01 samples with any of the strain-specific primers, including the C-specific primers M10-5' and M11-3'. These primers amplified the expected 224-bp fragment with the PPV-C control (Fig. 8, lane 5). The EA-specific primers (M8-5' and M9-3') amplified a 167-bp fragment with the EA-positive control, but were negative for all other samples, including samples of 3174-01 (*data not shown*).

Western blot analysis. PPV isolate 3174-01 and all positive controls were detected with the universal PPV MAb 5B (Table 2). The rate of migration of the coat protein subunit of 3174-01 was similar to that of isolate Q2185-02 (D), with an approximate molecular mass of 32 kDa. The D-, M-, EA-, and C-specific MAbs did not react with 3174-01 in Western blot analysis (Table 2). The specific MAbs reacted with their appropriate positive controls and no cross-reactions were observed (Table 2).

Sequence analysis. Two clones containing the 243-bp fragment of isolate 3174-01, amplified by using the P1 and P2 primers as described above, were sequenced and the nucleotide sequence analyzed. These clones were derived from samples amplified separately. There were 13 nucleotide changes (5.3%) between the two sequences. The enzymes *RsaI* and *AluI* recognize the restriction sites GTAC and AGCT, respectively. In isolate 3174-01, these sequences are replaced by GTAA/GTGA and GGCA, respectively (Fig. 9), which explains the failure of any digestion with these enzymes. When aligned and compared with sequences of PPV-C (X97398), PPV-M (M92280), PPV-EA (X56258), PPV-D (X16415), and PPV-Fa (a Canadian isolate of PPV strain D), identities of 95 to 99, 92 to 94, 91, 94, and 94%, respectively, were observed.

DISCUSSION

Isolate 3174-01 was detected with the PPV-specific MAb 5B in both TAS-ELISA and Western blot assays. Also, the virus

Fig. 7. Strain typing *Plum pox virus* (PPV) isolate 3174-01 using integrated reverse-transcription-polymerase chain reaction (PCR)/nested PCR (20). The D-specific primers M1-5' and M5-3' (lanes 1–8) and the M-specific primers M6-5' and M7-3' (lanes 9–16) were used. Lanes M, and N, 100- and 50-bp DNA ladder, respectively; lanes 1 and 9, healthy GF305; lanes 2 and 10, 20-13-1 (D); lanes 3 and 11, Q2185-02 (D); lanes 4 and 12, Q1902-01 (M); lanes 5 and 13, PPV-C (sweet cherry isolate); lanes 6–8 and 14–16, isolate 3174-01. The 159-bp D-specific fragment is present in lanes 2 and 3. The 207-bp M-specific fragment is present in lane 12.

Fig. 8. Strain typing *Plum pox virus* (PPV) isolate 3174-01 by integrated reverse-transcription polymerase chain reaction (PCR)/nested PCR using the C-specific primers M10-5' and M11-3' (20). Lane M, 50-bp DNA ladder; lane 1, healthy GF305; lane 2, 20-13-1 (D); lane 3, Q2185-02 (D); lane 4, Q1902-01 (M); lane 5, PPV-C (sweet cherry isolate); and lanes 6–8, isolate 3174-01. The arrow indicates the position of the 224-bp C-specific fragment.

was detected by RT-PCR using the PPV-specific primers P1 and P2 (23), indicating that it is, indeed, an isolate of PPV.

Isolate 3174-01 was not detected with oligonucleotide primers known to be specific for the D or M strain of PPV (18). The 243-bp fragment, amplified using the P1 and P2 primers, can be utilized in RFLP analysis for reliably distinguishing different strains of PPV (23). Two restriction sites (*RsaI* and *AluI*) are associated with this fragment. Members of strain D possess both restriction sites, members of M and EA possess the *AluI* site but lack the *RsaI* site, and members of strain C lack both restriction sites. The 3174-01-associated 243-bp fragment lacks both restriction sites. This suggested the possibility that 3174-01 was a member of PPV strain C. The primer pairs HsoC-1/CsoC-1 and HsoC-2/CsoC-2 (15) have been used for

reliable detection of sour cherry and sweet cherry isolates of PPV strain C. Neither of these primer pairs could detect isolate 3174-01. The primer pair HsoC-1/CsoC-1 amplifies a 259-bp product with sour cherry isolates of PPV-C (15). In this study, a fragment of approximately 300 bp was amplified with a sweet cherry isolate of PPV-C; therefore, it is possible that this primer pair may be useful in distinguishing sweet and sour cherry isolates of PPV-C.

RFLP analysis in the P3-6K₁ region of the PPV genome has been described as a reliable region for confirming the identity of D and M strains (7). Restriction analysis of the 3174-01-associated 836-bp fragment indicated the presence of a single *EcoRI* restriction site and the absence of any *AsnI* site, which is similar to strain D. Analysis with the enzyme *DdeI* revealed the presence of at least three *DdeI* sites,

compared with one and none for strains D and M, respectively. This region has not been used to identify strain C or EA (7).

An integrated RT-PCR/nested PCR technique has been described (20) that reliably distinguishes all four strains of PPV, including isolates of PPV-C. All recommended combinations were assessed in this study but all were negative for 3174-01, including the C-specific primers. Western blot assays were attempted and these included analyses with D-, M-, EA-, and C-specific MAbs. All were negative for 3174-01. Only the universal monoclonal antibody 5B reacted with 3174-01, and the molecular mass of 3174-01 was estimated to be approximately 32 kDa, similar to that of the member of the D strain included in the study. In all cases where the strain-specific MAbs were used, reactions were observed with the corresponding positive controls but not with isolate 3174-01.

It is interesting that the changes to 3174-01 in the region of the *AluI* and *RsaI* restriction sites in the C-terminus of the CP coding region are similar to that observed in association with members of PPV-C. Analysis of the cDNA of 3174-01 corresponding to the C-terminal region of the coding region revealed either GTAA or GTGA at the position of the *RsaI* restriction site. This, along with other nucleotide differences among cDNA clones, provides evidence of a mixed population, with all members lacking the restriction sites. Mixed populations of PPV in a single tree

Table 2. Western blot analysis using the universal *Plum pox virus* (PPV)-specific monoclonal antibody (MAb) 5B, and D-, M-, EA-, and C-specific MAbs for the detection of PPV isolate 3174-01

Isolate ^b	MAb ^a				
	5B	4D	AL	EA24	AC
3174-01	+	—	—	—	—
Q2185-02 (D)	+	+	—	—	—
Q1902-01 (M)	+	—	+	—	—
20-4-1 (D)	+	+	—	—	—
PPV-C	+	—	—	—	+
PPV-EA	+	—	—	+	—
Healthy <i>Nicotiana benthamiana</i>	—	—	—	—	—

^a Detection: + = positive and — = negative.

^b D = PPV strain D and M = PPV strain M.

Fig. 9. Multiple alignment of the nucleotide sequence of the 243-bp amplified fragment in the C-terminal region of the coat protein coding region of *Plum pox virus* (PPV) showing the *RsaI* and *AluI* endonuclease restriction sites (23). Samples (with accession number where appropriate) include PPV-3174-2b, PPV-3174-3b, PPV-C (X97398), PPV-M (M92280), PPV-EA (X56258), PPV-D (X16415), and PPV-Fa (Canadian D isolate).

are not uncommon (8). The identity in this 243-bp region was highest between 3174-01 and PPV-C (95 to 99%). It is possible that 3174-01 was derived from PPV-C, or is more closely related to PPV-C than to any other PPV strain. PPV-C and 3174-01 lack the *RsaI* and *AluI* restriction sites. This does not mean that they must, therefore, belong to the same strain. PPV-EA and PPV-M are different strains that are similar in terms of the restriction profile in this region. They both lack the restriction site *RsaI*.

It would appear that 3174-01 is not a member of any of the previously described strains of PPV. This conclusion is based on (i) the absence of both restriction sites *AluI* and *RsaI* in the C-terminal region of the coat protein coding region of 3174-01; (ii) failure of amplification with two sets of C-specific primers; (iii) an inability to detect this isolate with any of the strain-specific primer combinations described for integrated RT-PCR/nested PCR detection (20), including C-specific primers; (iv) failure of amplification using primers that target the replicase gene region (8) for RFLP analysis; and (v) a lack of recognition by D-, M-, EA-, and C-specific MAbs. All studies were replicated at least twice; in some cases, four replicates were used. This provides a high level of confidence in the results obtained.

This isolate of PPV was detected in plum (*P. domestica*) from a homeowner's property and appears to represent an isolated find, with no other positive plants detected to date in the immediate vicinity. The homeowner indicated that the plant germ plasm originally was introduced into Canada from Eastern Europe as "seed" and that the trees tested were approximately 10 years old. There are two important issues worth noting: (i) if the original plant material was infected with this virus and was introduced as seed, then there is circumstantial evidence for seed transmission of this isolate of PPV, although this must be confirmed by experimental analysis; and (ii) the activities of homeowners in moving plant germ plasm across borders without proper certification poses a serious threat to agricultural and horticultural industries.

Studies are underway to completely sequence this PPV isolate and assess its host range, including budding or grafting infected tissue onto cherry, to further characterize and obtain data that may facilitate

accurate classification of this unusual isolate of PPV. Vernalization and germination of seed collected from the infected plum trees are underway. The seedlings will be tested for the presence of PPV.

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