Detection and Partial Molecular Characterization of Two *Plum pox virus* Isolates from Plum and Wild Apricot in Southeast Kazakhstan

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ABSTRACT

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Plum pox virus (PPV) was detected in wild apricot and cultivated plum maintained in a germ plasm collection in Kazakhstan. Both isolates were typed as D strain, with no evidence of recombination. The virus was detected by triple-antibody sandwich enzyme-linked immunosorbent assay (ELISA) utilizing the universal PPV-specific monoclonal antibody (MAb) 5B as the secondary antibody, and by reverse-transcription polymerase chain reaction (RT-PCR) assay using primers that amplified a 243-bp fragment in the C-terminus of the coat protein (CP) coding region. Immunocapture (IC) RT-PCR was used to detect PPV in nine wild apricot accessions, including eight ELISA-negative and one ELISA-positive. The plum and apricot isolates reacted positively in Western blot assay with the universal MAb 5B, and negatively with the strain-M-specific MAb-AL. Restriction fragment length polymorphism analysis applied to the amplified 243-bp fragment showed that restriction sites for *AluI* and *RsaI* were present in the plum and apricot samples. An amplified 836-bp cDNA fragment derived from the P3-6K₁ coding region of both isolates had restriction profiles typical for strain D. Nucleotide identities of 99 to 100% were observed for the 243-bp fragments of the Kazakhstan isolates when compared with the corresponding regions of strain D, and 94 to 95% identity with strain M. Nucleotide sequence analysis of the entire CP coding region of the plum and apricot isolates resulted in the identification of a unique deletion of six nucleotides (two deduced proline amino acid residues) in the N-terminal region in the plum isolate. This is the first deletion of this nature observed among PPV isolates. The DAG motif was present in both isolates. Several nucleotide substitutions in the CP coding region were common to the plum and apricot isolates and appear to be unique to the Kazakhstan isolates. This suggests a close relationship between the isolates.

Additional keywords: nucleotide deletions, strain typing

Information regarding the distribution of any pathogen is invaluable for effective control and management of the associated disease. This is especially significant when the pathogen is known to be insect transmitted, and where new and specific locations are identified. *Plum pox virus* (PPV), causing plum pox or Sharka disease, is an aphid-transmitted virus (6,13) associated with a devastating disease of stone fruit such as plum, peach, and apricot (18).

PPV has spread over the years from the Balkan region to other Eastern European countries, and later to Western Europe (19,22). The known geographic distribution of PPV seems to be increasing with recent reports of the pathogen in Chile, South America (1), and North America, including the United States (15) and Canada (29). Understanding the geographic distribution of this pathogen facilitates control or eradication from the affected country, and allows the identification of safe or unsafe areas for budwood collection, preventing inadvertent introductions to other countries.

Apricot (*Prunus armeniaca*) and plum (*P. domestica*) are valuable fruit tree crops, consumed fresh, dried, or processed. In some regions in Asia, apricot seed and seed oil also are used and may have a higher cash value than fruit. Central Asia is considered the region of origin for apricot. Wild populations of apricot grow on the slopes of the Tien-Shan mountain range in Kazakhstan; eastern Tibet, and China. This germ plasm material, although reduced in size due to cattle grazing and cutting, offers a valuable source of plant material for

breeding resistance to PPV, as well as other desirable horticultural traits. Among the four ecogeographical groups of *P. armeniaca*, the Central Asian group is the oldest and richest in its biodiversity (11).

PPV has been sequenced completely (28) and characterized as consisting of four recognized strains or serotypes, differing in pathogenicity, host range, aphid transmission efficiency, and geographical distribution (2,17,27). Recently, an unusual isolate of PPV, thought to originate in Eastern Europe, was described but could not be classified as a member of any of the recognized strains (10). Detection and strain typing of other Eastern European isolates may assist in confirming the putative origin of this isolate. PPV is considered a quarantine pathogen and is included in the European Plant Protection Organization's A2 list of quarantine pests (22). Therefore, reliable detection, control, and eradication of this virus are of prime importance for the fruit tree-growing industry as well as for germ plasm collections. Strain determination of any new PPV isolate is essential in developing management or eradication strategies and understanding the epidemiology of the virus. In this study, information is presented on the detection of PPV in apricots from the wild and in cultivated plum in Kazakhstan. Preliminary molecular characterization and strain typing of two PPV isolates are described.

MATERIALS AND METHODS

Plant material and virus isolates. The tested trees included wild apricot and cultivated plum growing under field conditions in the Pomological Garden in Talgar (east of Almaty) in Kazakhstan. The apricot trees originated from wild seedling populations growing in their natural habitat in Zailiyski Alatau at the northern edge of the Tien-Shan mountains. The plum trees included hybrids from free pollination of cvs. Stanley, Monfor, Tamga, and local selections, maintained in the plum selection orchard of the Pomological Garden since the mid-1980s. Virus symptoms were observed on leaves of the plum trees and on fruit from one apricot tree.

A minimum of five fully expanded young leaves from several branches on each tree were collected during the active growing season (June 2003) and stored over CaCl₂ at 4°C for about 20 days, until

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completely dry. These dried samples were shipped in sealed bags and analyzed as described below. The transfer of this plant material was done following the export regulations of Kazakhstan and the import regulations of Canada (import permit #P-2003-02993). Isolates of the virus used as positive controls in this study included PPV-D (Q2185-02) from W. Jelkmann (Germany), and PPV-M (Q1902-01) from F. Dosba (France).

Preparation of clarified tissue extracts. Approximately 25 mg of dried leaf tissue (equivalent to about 100 mg in fresh weight) per sample was transferred to a 2-ml tube containing 0.75 g of glass beads and 1 ml of grinding buffer consisting of 0.01 M potassium phosphate buffer (pH 7.0), 0.02 M diethyldithiocarbamic acid (sodium salt), 0.04 M sodium thioglycolate, and 0.5% nicotine. Samples were triturated using the Hybaid RiboLyser Cell Disrupter (InterScience Inc., Markham, Ontario, Canada) and centrifuged (5 min at 5,000 × g) to remove plant debris. The clarified extracts were used for serological assays and immunocapture (IC) reverse-transcription polymerase chain reaction (RT-PCR).

Serological analysis. Triple-antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) was carried out as described by Cambra et al. (2) using the polyclonal antibody A1PPV, and the universal monoclonal antibody (MAb) 5B as a secondary antibody (REAL Durviz TAS-ELISA kit). The supplier's instructions were followed except for (i) use of the sample grinding buffer described above and (ii) inclusion of a blocking step using phosphate-buffered saline (PBS)-Tween with 0.2% skim milk powder for 30 min at room temperature. Clarified extracts (100 µl/well) were added to microtiter plates precoated with the polyclonal antibody A1PPV and stored overnight at 4°C. The MAb and conjugate were used at a dilution of 1:1000 and incubated at 4°C for 16 h and 37°C for 3 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 mean of the absorbance of the healthy controls were considered positive.

Western blot analysis was carried out as described previously (10) using clarified tissue extracts (described above) from healthy, infected, and positive control plants. The protein subunits were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14). The samples were screened with MAb-5B (universal) and MAb-AL (M-specific).

Total RNA extraction. Total RNA was extracted from dried leaf samples using either the modified LiCl method (26) or the Qiagen RNeasy Total RNA kit (Qiagen, Chatsworth, CA). The latter procedure was used essentially as recommended by the

supplier, except for minor modifications described by James et al. (10).

RT-PCR. Amplification of a 243-bp fragment in the coat protein (CP) coding region was carried out using the primers P1 and P2 (31). Approximately 4 µg of total RNA in 5 µl of double-distilled diethylpyrocarbonate-treated (DEPC) H₂O was combined with 4 µl of antisense P1 primer (5 µM stock) and incubated at 72°C for 5 min. The RT reaction was carried out in a 20-µl volume by adding 11 µl of RT mix consisting of 4 µl of 5× First Strand buffer (Invitrogen, Burlington, Ontario, Canada), 2 µl of 0.1 M DTT, 1 µl of 10 mM dNTP Mix, 0.5 µl of RNase Block (Stratagene, La Jolla, CA), 1 µl of SuperScript II (Invitrogen), and adjusted to a final volume of 11 µl with DEPC H₂O. RT was carried out at 54°C for 30 min. The PCR reaction mix consisted of 0.5 µl of 10 mM dNTP mix, 0.75 µl of 50 mM MgCl₂, 0.25 µl of sense primer (P2, 5 µM stock), 0.25 µl of antisense primer (P1, 5 µM stock), 0.25 µl of Taq DNA polymerase (Invitrogen), 0.25 µl of Taq Extender PCR Additive (Stratagene), 2.5 µl of 10× Taq Extender buffer, adjusted to a final volume of 20 µl with DEPC H₂O. This mix was added to 1 µl of cDNA (generated by RT) adjusted to 5.0 µl with DEPC H₂O, for a final reaction volume of 25 µl. The mixture was denatured at 92°C for 10 min, and amplification carried out at a cycling scheme of 92°C for 1 min, 62°C for 20 s, and 72°C for 1 min, for 35 cycles.

The 836-bp fragment in the P3-6K₁ region of the genome was amplified using the primers PCI and PP3 (7). The RT reaction was done as described above, except that cDNA synthesis was carried out at 42°C for 45 min. Amplification was carried out at 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min for 35 cycles, with a final extension at 72°C for 10 min. Aliquots (10 µl) of the final reactions were assessed by agarose gel electrophoresis.

IC-RT-PCR. Clarified plant extracts (100 µl) were incubated at 4°C for 16 h in microtubes (0.5 ml) precoated with the polyclonal antibody A1PPV (1:100; Durviz). The tubes were washed three times with PBS-Tween, drained thoroughly, and incubated at 65°C for 10 min (16) prior to the addition of the RT Mix (20 µl per tube). The RT mix was as described above except that 2.4 µl of 1.7% Triton-X and 4 µl of P1 antisense primer were included. The tubes were vortexed briefly prior to the RT reaction, for which the initial incubation (5 min at 72°C) was omitted. The PCR reaction was carried out essentially as described above for RT-PCR.

Restriction fragment length polymorphism analysis. Two regions of the PPV genome were targeted for strain typing using restriction fragment length polymorphism (RFLP) analysis of RT-PCR-amplified fragments: (i) the CP coding region (243-bp fragment) treated with the

restriction enzymes *AluI* and *RsaI* (31) and (ii) the P3-6K₁ genomic region (836-bp fragment) treated with *AsnI* and *DdeI* (7).

Cloning of cDNA. A fragment of approximately 1.8 kb, including the PPV CP encoding region, was amplified using the antisense primer 1, located in the 5' terminus of the 3' noncoding region (31), and a sense primer, W3 (5'-GATCAATGCAGT-TCTGAACATTCG-3') that targets a region in the 3' end of the N1b coding region. The RT reaction was as described above for the 836-bp fragment and amplification was carried out at 94°C for 30 s, 55°C for 45 s, and 72°C for 2 min for 35 cycles, with a final extension at 72°C for 10 min. The 1.8-kb fragments were identified by agarose (0.8%) gel electrophoresis with ethidium bromide staining, excised from the gel, and purified using Qiagen's MiniElute Gel Extraction kit (Qiagen). Gel-purified PCR products were ligated into the pCR^(R)2.1 TOPO Vector and cloned using the TOPO TA Cloning Kit, following the supplier's instructions (Invitrogen).

Sequencing and sequence analysis. Sequencing was carried out as described by James et al. (9). Nucleotide and amino acid sequence data were compiled and analyzed using Clone Manager 6 (version 6.00; Scientific and Educational Software, Durham, NC) and Clustal-X (version 1.81) (30). Database searches were performed with BLAST (blastn) programs at the National Centre for Biotechnology Information (NCBI; National Institutes of Health). Phylogenetic analyses were performed using the neighbor-joining method of Saitou and Nei (23), with a bootstrap of 1,000 replicates (10). Trees were produced using NJPLOT (20).

RESULTS

Serological assays. All plum samples (11 trees), one apricot sample (11 samples tested), and the positive controls reacted positively in TAS-ELISA with the PPV-specific universal MAb 5B as the secondary antibody. Negative reactions were observed with all healthy controls.

Three ELISA-positive plum (P5R8, P9R2, and P3R2) and the positive apricot (A8R2) were selected for Western blot analysis. The four isolates from Kazakhstan and the positive controls Q2185-02 (D) and Q1902-01 (M) were detected with the universal PPV MAb 5B. No reaction was observed with the healthy controls. The rate of migration of the CP subunit of the four tested isolates was similar to that of the M isolate (Q1902-01). However, in further Western blot analysis with the M-specific MAb-AL, no reaction was detected with any of the Kazakhstan isolates, whereas a positive reaction was associated with the M isolate (*data not shown*).

RT-PCR. The PPV-specific 243-bp fragment of the CP coding region (Fig. 1) and the 836-bp product in the P3-6K₁ re-

gion (Fig. 2) were amplified from plum and apricot samples using RT-PCR analysis. In several samples, the 243-bp product was observed on the gel as a weak ethidium bromide-stained band (Fig. 1, lanes 3 and 5). Total RNA extraction using the LiCl method resulted in more distinct PCR products for both the CP and P3-6K₁ regions of A8R2 (apricot sample), whereas the Qiagen extraction procedure was more effective for the plum samples.

The use of IC-RT-PCR targeting the PPV CP region allowed the amplification of the 243-bp fragment in nine wild apricot accessions, including eight ELISA-negative samples and the ELISA-positive A8R2. The reaction of several apricot samples, observed in the ethidium bromide-stained gels, was weak (*data not shown*). A similar size fragment of 243 bp was obtained for two plum samples and the positive control (Q2185-02, D isolate). No amplification was observed for the healthy controls included (*data not shown*).

RFLP analysis. The amplified 243-bp fragment, derived from the C-terminal region of the CP coding region, was treated with the endonucleases *AluI* and *RsaI*. The amplified fragments associated with A8R2, P5R8, the D control (Q2185-02), and the M control (Q1902-01) were cleaved by *AluI* into fragments of 95 and 148 bp. Treatment with the endonuclease *RsaI* resulted in digestion of the fragments associated with A8R2, P5R8, and the D control, but not the M control (*data not shown*). Treatment of the 836-bp fragment, derived from the P3-6K₁ region, with the endonuclease *AsnI* (7) did not result in cleavage of A8R2-, P5R8-, and Q2185-02 (D)-associated fragments (Fig. 3, lanes 1, 3, and 4), whereas isolate Q1902-01 (M) was digested showing a single cleavage site (Fig. 3, lane 2). Treatment with *DdeI* resulted in cleavage of A8R2, P5R8, and the D isolate Q2185-02 at a single location (Fig. 3, lanes 6, 8, and 9). Isolate Q1902-01 (M) was not cleaved by *DdeI* (Fig. 3, lane 7).

Sequence analysis. The 243-bp product in the region of the C-terminus of the CP region was sequenced and the *AluI* and *RsaI* restriction sites identified (Fig. 4). The percentage of identity of the 243-bp fragment of the Kazakhstan isolates was 100%, and between the D and M isolates identity was 99 to 100% and 94 to 95%, respectively. The entire CP coding regions of the PPV plum isolate (P5R8) and the apricot isolate (A8R2) from Kazakhstan were cloned and sequenced. The cDNA clone derived from P5R8 was identified as KZPLa1, and the cDNA clone derived from A8R2 was identified as KZAPR. The percentage of identity between the apricot clone and the plum-associated clone was estimated at 97%.

The nucleotide sequence of the entire CP coding region of the plum isolate P5R8 consisted of 984 nucleotides (nt; consistent

Fig. 1. Reverse-transcription polymerase chain reaction showing amplification of the *Plum pox virus*-specific 243-bp fragment (31) from plum and apricot trees from Kazakhstan. Lane M, 100-bp DNA ladder; lane 1, healthy *Nicotiana benthamiana* (herbaceous host); lane 2, healthy apricot cv. Haggith (woody host); lanes 3 and 4, plum trees P7R1 and P5R8, respectively; lanes 5 and 6, apricot accessions A40R1 and A8R2, respectively; lane 7, PPV isolate D (Q2185-02) in *N. benthamiana*.

Fig. 2. Reverse-transcription polymerase chain reaction showing amplification of the *Plum pox virus*-specific P3-6K₁, 836-bp fragment (7) from plum and apricot trees from Kazakhstan. Lane M, 1-kb DNA ladder; lane 1, healthy *Nicotiana benthamiana*; lane 2, healthy plum cv. Shiro (woody host); lanes 3 and 4, plum trees P7R1 and P5R8, respectively; lanes 5 and 6, apricot trees A40R1 and A8R2, respectively; lane 7, isolate D in *N. benthamiana*. The arrow indicates the position of the 836-bp fragment.

Fig. 3. Reverse-transcription polymerase chain reaction and restriction fragment length polymorphism analyses of the *Plum pox virus*-specific P3-6K₁, 836-bp fragment (7) from plum and apricot samples from Kazakhstan. The P3-6K₁ fragments were treated with the restriction endonucleases *AsnI* (lanes 1–4) and *DdeI* (lanes 6–9). Lane M, 50-bp DNA ladder; lanes 1 and 6, isolate D in *Nicotiana benthamiana*; lanes 2 and 7, isolate M in *N. benthamiana*; lanes 3 and 8, plum P5R8; lanes 4 and 9, apricot A8R2. Lane 5 is blank.

for two independently derived clones), and 990 nt for isolate A8R2. This translated into 328 and 330 deduced amino acid residues, respectively. Nucleotide deletions in P5R8 were detected at position 91 to 96 (CCACCT), resulting in the deletion of two deduced proline residues (Fig. 5). The N-terminal region of the PPV CP is a variable region, but three prolines at amino acid positions 30, 31, and 32 of the CP amino acid sequence appear to be highly conserved across most D isolates. Only in rare cases have substitutions been observed: PPL in accessions X81074 and X81083, or TPP in X81084 (accession numbers from GenBank database). Isolate P5R8 is the only PPV isolate that has been described to date with a single proline residue in this position.

There were six positions (48, 226, 243, 270, 318, and 381) in the CP coding region of D isolates with nucleotides unique to the three Kazakhstan isolates (Fig. 5). The nucleotides at position 318 to 320 are interesting because the sequences of almost all isolates published to date consist of the bases TAC or CGC in this position; the Kazakhstan isolates and accession S57404 (GenBank database) are the only isolates with CAC at this position. The DAG motif, essential for aphid transmission, is present at positions 11 to 13 of the deduced amino acid residues of the CP of isolates A8R2 and P5R8. The nucleotide sequence data reported in this study will appear in GenBank under the accession numbers AY591253 (KZPLa1) and AY591254 (KZAPR).

Phylogenetic analysis. An analysis based on alignments of the deduced amino acid residues in the CP region of the PPV

isolates from Kazakhstan was done. A wide range of PPV strain D isolates, described in the database, was included (Fig. 6). The Kazakhstan isolates from plum and apricot grouped together in the same branch and appear to be closely related (Fig. 6). The next-closest related D isolate was isolate AIN from Poland (accession number AF332871, host plum). Geographical location-associated clustering of some PPV isolates was observed, for example: D-Fan and D-Vul detected in Canada (D. James and A. Varga, *unpublished*); PENN1 (AF401295), PENN2 (AF401296), PEN Pch (AF354268), PEN Plm (AF354269), and PPV-D DCKT1 (AF360580) found in Pennsylvania; and some isolates originating from Chile (21), such as Chile112 (AF440741) and Chile 12 (AF440744).

DISCUSSION

Wild apricot trees (*P. armeniaca*) grow on the slopes of the western, central, and eastern Tien-Shan mountain range in Kazakhstan; in eastern Tibet, and in the Tsiling Shan range up to the mountains north of Beijing. There is considerable genetic diversity among apricot species and ecotypes evident in the wild and also among major collections such as those in the former USSR (11,12). The genetic diversity of the natural, wild apricot populations in the Tien-Shan mountain range in Kazakhstan offers an exceptionally important gene pool for breeding programs aimed at extended harvest periods for fresh fruit, frost tolerance, and resistance to pathogens. Therefore, it is of great importance to maintain this valuable genetic material in a germ plasm collection. Determining the

disease status of plants in a collection and the surrounding region might prevent inadvertent movement of undesirable pests into germ plasm collections and their distribution to new regions both within the source country and internationally (25).

In this study, PPV was detected by ELISA, Western blot, RT-PCR and IC-RT-PCR assays in wild apricot and plum growing in South East Kazakhstan. The plum displayed severe leaf symptoms, whereas only a single apricot showed obvious symptoms. ELISA is a simple and useful tool for the detection of plant viruses (5). However, IC-RT-PCR was used to detect infected apricot that were negative by ELISA. IC-RT-PCR is a more sensitive detection technique compared with ELISA (3). Strain typing techniques targeting the CP region in the 3' terminus of the genome (31) and the P3-6K₁ region in the 5' terminus of the genome (7) gave consistent results indicating the D strain. This suggests that these isolates are not recombinants because recombination usually occurs in the region of the Nib-CP junction (4,7), resulting in inconsistencies for typing upstream and downstream of this junction. Strain typing of the detected isolates was especially important because an unusual and potential new strain of PPV was detected recently in plum that originated from Eastern Europe (10). Studies that might provide information as to the exact origin of any new isolates, and the specific identities of new isolates including strain type, are valuable in understanding the epidemiology of this virus. This information will assist in developing effective control strategies. It is interesting that, in spite of identification as D strain based on

			<u>RsaI</u>
D-Tey	9337	cagactacagcctcgccagatatgcctttgatttttaacgaaatgacttcaacgacaccggtacgggcacg	
KZPLa1	1		
KZAPR	1		
M-SK	9337	t.....c.....a..g..t..g..t....	
			<u>AluI</u>
D-Tey	9407	tgaagctcatatccagatgaaggcagcagcattgagaaatgttcaaaatcgtttatttggcttgatgga	
KZPLa1	71		
KZAPR	71		
M-SK	9407	a.....	
D-Tey	9477	aacgtcggaaacacaagaagaggacacagagagacacacgcgtggtgatgttaatcgcaacatgcacaacc	
KZPLa1	141		
KZAPR	141		
M-SK	9477	g.....c..g.....	
D-Tey	9547	tcctcggtgtgaggggagtgtagtgggtctcggt	
KZPLa1	211		
KZAPR	211		
M-SK	9547		

Fig. 4. Multiple alignment of the nucleotide sequence of the 243-bp fragment in the C-terminal region of the coat protein coding region of *Plum pox virus* (PPV) showing the restriction sites of the endonucleases *RsaI* and *AluI* (31). Samples include KZPLa1 derived from plum P5R8, KZAPR derived from apricot A8R2, and PPV D-Tey (X16415) and PPV M-SK (M92280). Dots indicate identical nucleotides.

RFLP analyses, the Kazakhstan plum and apricot isolates displayed atypical electrophoretic mobility of the CP subunits, migrating similar to the M isolate. It is possible that electrophoretic mobility may not always be a reliable indicator of strain type.

The DAG motif was detected in both the plum and apricot isolates of PPV from Kazakhstan, indicating that they have the potential for aphid transmission (24). This must be confirmed experimentally because other factors are

known to be involved in aphid transmission (8).

The 6-nt (2 amino acid residues) deletion observed in the plum isolate of PPV described in this study is the first deletion of this nature observed among isolates of

D-Tey	1	caagctgacgaaagagaggacgaggaggaagttgatgcaggcaagccgattgtagttactgcaccggcag
KZPLa1	1	a.....a.....
KZAPR	1	a.....a.....
D-PENN2	1	a.....
D-Chile12	1	a.....t.....
D-Vul	1	a.....
D-Fan	1	a.....c.....
M-SK	1	..g....t...gag..a....t..a....g.....a.ga..t.c...g..a.....
D-Tey	71	caactagcccaataacttcaaccacctccagtcatacagcctgcaccccgactacggcgccaatgctcaa
KZPLa1	71	-----.....t....
KZAPR	71	c.....t....
D-PENN2	71	
D-Chile12	71	c.g.....t....
D-Vul	71	t.....t....
D-Fan	71	t....
M-SK	71	gtgg...cgac.....g.....g.....a.....aa..c..a..a.....t....
D-Tey	141	ccccattttcacgccagcaacaactcaaccagcaacaaaaccagtttcacaggtgccaggacctcaactg
KZPLa1	135	t.....
KZAPR	141	t.....t.....
D-PENN2	141	g.....t.....
D-Chile12	141	t.....
D-Vul	141	g.....t.....
D-Fan	141	g...t.....
M-SK	141	t.....g..t..ggt..g.....c..t..caa..tt...gg.ca...c..
D-Tey	211	caaacttttggacatatggtaatgaggatgcacacctagcaactcaaacgcgctagtcaacacaaaaca
KZPLa1	205	c.....c.....t.....
KZAPR	211	c.....c.....t.....
D-PENN2	211	g.....
D-Chile12	211	
D-Vul	211	
D-Fan	211	
M-SK	211	..ggt.....ggt.....a....a..c.....c.....a..tt.g..g..t...gga..
D-Tey	281	gagacagggacgtcgatgcaggatcaattggaacttttacagtgccacgtttgaaggcaatgacttcgaa
KZPLa1	275	c.....
KZAPR	281	...t...a.....c.....
D-PENN2	281	
D-Chile12	281	
D-Vul	281	...tg.....a.....
D-Fan	281	...t.....
M-SK	281	..g..t.....g.....cg.....c..a..aa.....a.....
D-Tey	351	actatctctgccaaggtgaagggaaaggctattatgaacttgaaccatt
KZPLa1	345	c.....
KZAPR	351	g...c.....
D-PENN2	351	
D-Chile12	351	
D-Vul	351	
D-Fan	351	
M-SK	351	gt.....a.g.....a..a.....a..t....

Fig. 5. Multiple alignment of the nucleotide sequence of the N-terminal coat protein coding region (nucleotides 1 to 400) of *Plum pox virus* (PPV) isolates. Samples include KZPLa1 derived from plum P5R8, KZAPR derived from apricot A8R2, and previously described PPV strain D isolates (with GenBank accession number, where appropriate): D-Tey (X16415), D-PENN2 (AF401296), Chile12 (AF440744), M-SK (M92280), and D-Vul and D-Fan (D. James and A. Varga, unpublished). Dots indicate identical nucleotides and dashes denote gaps.

Fig. 6. Phylogenetic analysis of the coat protein regions of two *Plum pox virus* (PPV) isolates from Kazakhstan and previously reported sequences of PPV strain D isolates. Branch lengths indicate phylogenetic distances calculated from distance matrices of deduced amino acid sequences. The following PPV D isolates, with their GenBank accession numbers, where appropriate, were used for the analysis: KZAPR (AY591254), KZPLa1 (AY591253), RB-BF (AF172346), Skierniewice (U27652), AIN (AF332871), NL (X81074), PEN Pch (AF354268), PEN Plm (AF354269), SoC-Der (X81083), AT (X57975), MNAT1 (AF360579), DCKT1 (AF360580), PENN1 (AF401295), PENN2 (AF401296), Almond (AJ000340), Chile116 (AF440743), Chile31 (AF440746), Chile114 (AF440742), Chile20 (AF440745), Chile112 (AF440741), Chile12 (AF440744), Rankovik (M21847), D-Tey (X16415), and D-Fan and D-Vul (D. James and A. Varga, *unpublished*).

PPV. The biological significance of this deletion is unknown at this time. The plum isolate and the apricot isolate appear to be closely related, sharing a number of nucleotides that appear to be unique to the Kazakhstan isolates. These may be useful as markers in epidemiological studies. Based on these results, it is possible that the plum isolate was derived from the apricot isolate because the latter appears to be more typical in terms of the number of nucleotides in the CP coding region of the genome. The close similarity between the plum and apricot isolates also can be observed in their branching pattern in phylogenetic analysis. Some clustering of PPV isolates originating from distinct geographical regions was observed. This could represent patterns of distribution of PPV from a given source in vegetative plant material or aphid vectors, localized genetic variability emerging with isolation over time, or localized selection. However, the available information does not allow any definitive conclusion. Further studies to determine distribution, the host range of the Kazakhstan PPV isolates, assessment of biological effects of the two proline deletions in the plum isolate, and identification of possible aphid vectors should be carried out in order to design a control strategy and minimize further spread of this pathogen.

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