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Molecular variability of the capsid protein of the prune dwarf virus

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Abstract

Sequences of the capsid protein gene and the preceding intergenic region of eleven isolates of prune dwarf virus from central Europe were determined. The isolates were obtained from plum, cherry and peach trees. Comparison of all sequenced isolates (including two sequences published previously) revealed high (88%) conservation of the capsid protein gene. The highest degree of identity was observed in the C-terminal half, where only 13 amino acid substitutions could be observed in contrast to the N-terminal half with 22 substitutions. No reasonable correlation between amino acid substitutions and host species and/or geographic origin of the isolates was observed. Alignment with capsid protein genes of other ilarviruses revealed apple mosaic virus, elm mottle virus, lilac ring mottle virus and prunus necrotic ringspot virus as the most related to prune dwarf virus. Unlike the isolates of related prunus necrotic ringspot virus all the isolates of prune dwarf virus shared extensive conservation of the intergenic region. Portions of RNA3 were selected for design of universal primers for PCR detection.

Introduction

Prune dwarf virus (PDV) (genus Ilarvirus, family *Bromoviridae*) is one of the most economically important viruses of stone fruit trees – especially peach, sweet and sour cherry, plum and almond (Neméth, 1986; Uyemoto and Scott, 1992). It frequently occurs in mixed infections with related viruses – prunus necrotic ringspot virus (PNRSV) and apple mosaic virus (ApMV). All three viruses share similar biological properties that contribute to their worldwide distribution: they are transmitted by graft, pollen and seed. Symptoms of PDV vary widely, depending on climate, virus isolate, host species and its cultivar. These range from symptomless infections to serious fruit yield reduction and stunting in peach, yellows in sour cherry, stunting and leaf malformations in plums (Uyemoto and Scott, 1992) and chlorotic spots, rings and fruit rain cracking in sweet cherry (Proebsting et al., 1995).

The variability of symptoms corresponds to the present concept of virus species as a population of variants (Van Regenmortel et al., 1991). Undoubtedly, many variants are of minor phytopathogenic importance and/or are without ascertainable influence on virus fitness. On the other hand, there are numerous examples of virus isolates which overcome natural resistance, change host range, and influence vector transmission, spread in host, and serological and other features.

Natural variability of viruses can also substantially influence their reliable detection by molecular methods which are based on hybridization of oligonucleotides and *in vitro* amplification of parts of virus genomes (polymerase and ligase chain reaction methods, PCR, LCR, respectively). Even the presence of a few nucleotide substitutions in sites adjacent to the 3′-terminus of primers, largely responsible for directing the amplification (Caetano-Anollés, 1993), can result

in false negative results. This fact may further increase the risk that numerous virus variants could escape detection, in particular in woody hosts, where the isolation of pure viral nucleic acid free of PCR inhibitors is difficult and distribution of the virus in organs and tissues of the plant is uneven (Rampitsch et al., 1995; Spiegel et al., 1996).

The knowledge of sequence variability is of the highest importance when designing PCR primers capable of detecting all known virus variants. Only two comparable PDV sequences have been published to date – GenBank accesion numbers U31310 and L28145 (Rezler et al., 1995; Bachman et al., 1994).

The aim of this work was to study the variability of PDV isolates and compare it with variability of related viruses – PNRSV and ApMV. We present capsid protein (CP) sequence data of 11 new isolates of PDV found in central Europe. In addition, we examined whether any correlations exist between amino acid substitutions and host species or the geographic origin of the isolates.

Materials and methods

Virus isolates

The source and sequence accession numbers of the isolates used are listed in Table 1. Most of the samples were collected during winter and spring months from cherry and plum trees. The two exceptions were peach isolates 'Nem' maintained on *Prunus tomentosa* and 'Valtická' maintained on a peach seedling in a greenhouse.

Sample preparation

Three to five twigs were taken from each tree to overcome the uneven distribution of the virus within its woody host. Blossoms and leaves from forced buds were used immediately for nucleic acid isolation. Fifty to 200 mg of fresh tissue was homogenized with 10 volumes of extraction buffer (500 mM Tris–HCl, pH 8.3, 150 mM NaCl, 0.05% Tween 20, 2% PVP, 1% PEG 6000, 3 mM NaN_3). Nucleic acids were isolated by the phenol–chloroform method and isopropanol precipitation (Sambrook et al., 1989). After washing with ethanol and drying, the pellet was resuspended in 500 μl of sterile water. Two μl were then used as a template in the 20 μl reverse transcription (RT)-PCR reaction.

Primers

Primers used in RT-PCR anneal to conserved regions of RNA3 of PDV and amplify a region between nucleotides (nt) 1088 and 1950 (numbered according to the sequence L28145). The sequence of the upstream primer was: 5′-gtg tag AAA GAA GAG AAG TCC GAC AAG-3′ and that of the downstream one was: 5′-atc tag AAG CAG CAT TTC CAA CTA CGA-3′ (non-viral nucleotides of attached restriction sites are

Table 1. Prune dwarf isolates used in this study

Accession number	Isolate	Original host	Variety (where known)	Locality in the Czech Republic	Origin of the tree variety (where known)
AF208737	2/16	Cherry	Kasinova	North	Germany
AF208738	K1	Cherry	—	South	—
AF208739	PS 7/9a	Cherry	Nanni	North	—
AF208740	21/1	Cherry	Hudson	North	USA
AF208741	vanC	Cherry	Stella Compact	North	Canada
AF208742	Valtická	Peach	—	South-east	—
AF208743	Nem	Peach	—	—	Germany
AF208744	Branisovska	Plum	—	South	—
AF208745	sss	Plum	—	South	—
AF208746	6/54	Plum	Anna Spath	North	Germany
AF208747	7/12	Plum	Lesidrjanskaja	North	Russia
L28145*	ch137	?	?	—	?
U31310	Skierniewice	Cherry	?	—	Poland?

*Bachman et al., 1994.

shown in lower-case letters). The amplified product is 874 bp long and spans the last 50 nt of the movement protein open reading frame (ORF), the intergenic region and the whole CP ORF. The primers were used at final concentration of 10 nmol each.

RT-PCR

The Access RT-PCR System kit (Promega, Madison, WI, USA) containing the AMV reverse transcriptase and *Tfl* polymerase was used for amplification of the target. The thermal cycling scheme was as following: 45 min at 48 °C, 2 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 50 °C and 90 s at 68 °C and a final incubation at 68 °C for 10 min. The RT-PCR was performed in a MiniCycler™ (MJ Research, Watertown, MA, USA).

Sequencing

Amplified products were purified either via the QIAquick PCR Purification Kit (QIAGEN) or the GenElute™ PCR DNA Purification Kit (Sigma) according to instructions of the manufacturers. Sequencing was done with the RT-PCR primers by the Thermo Sequenase dye terminator cycle sequencing pre-mix kit, v 2.0 (Amersham LIFE SCIENCE) on an automated DNA ABI PRISM 310 sequencer (Perkin Elmer Applied Biosystems, Lincoln, USA).

Software

Sequence data were analysed using CHROMAS (Technelysium Pty Ltd, Australia). Relative molecular mass was calculated by DNASTAR (Lasergene, USA). Accession numbers of ApMV isolates used for this calculation were: U15608 (Shiel et al., 1995), S78319 (Guo et al., 1995) and L03726 (Alrefai et al., 1994), PNRSV isolates used were: AF013285–AF013287 (Scott et al., 1998), AF034989–AF034995 (Hammond and Crosslin, 1998), AF170156–AF170171 (Vašková et al., 2000), AJ133199–AJ133213 (Aparicio et al., 1999), L38823 (Hammond and Crosslin, 1995), S78312 (Guo et al., 1995), U03857 (Sanchez-Navarro and Pallas, 1994), U57046 (Scott et al., 1996) and Y07568 (Sanchez-Navarro and Pallas, 1997). Multiple sequence alignments and sequence identities were determined using the www service CLUSTALW, http://www2.ebi.ac.uk/clustalw/ (Thompson et al., 1994). The published alignment was fitted by aid of programme BoxShade (http://www.ch.embnet.org/software/BOX_form.html). RNA folding of subgenomic promotor sequences was done by www service mfold accessible on http://mfold2.wustl.edu/~mfold/rna/form1.cgi (Mathews et al., 1999; Zuker et al., 1999).

Results and discussion

Nucleic acids, isolated directly from the original infected tissue to omit introduction of additional selection pressure during virus transmission to non-related herbaceous hosts, were amplified and sequenced. Sequences of the CP gene and the intergenic region were determined and compared with the previously published data (U31310 and L28145).

Variability of the isolates

The CP gene showed more than 88% identity with all the PDV isolates. This value was computed both at the nucleotide as well as at the protein level. Between the PDV isolates, more than one but less then 25 differences were observed in the primary sequence of the CP. In total, 35 amino acid (aa) positions varied within the 218 aa long protein – 22 in the amino (N) terminal half and 13 in the carboxy (C) terminal half of the protein (Figure 1). A detailed comparison did not reveal any association of these substitutions with host species or with geographic origin of the isolates. Surprisingly, similar results were recently found for PNRSV, where a correlation of variation in the primary amino acid sequence of the CP with extensive serological and biological variability associated with PNRSV infections was expected (Scott et al., 1998).

The greatest difference in computed relative molecular mass of the CPs of PDV isolates was 277 Da. The smallest value was computed for the Polish cherry isolate U31310 (23,928 Da) and the highest one for the Czech plum isolate AF208745 (24,205 Da). CPs proteins of the other two comparable ilarviruses, ApMV and PNRSV, differed much more: ApMV isolates by about 520 Da (24,566 Da of S78319 and 25,086 Da of L03726) and PNRSV by 491 Da (24,852 Da of 'Valticka' isolate AF170171and 25,343 Da of AF034992). We cannot explain, why the range of masses of these related viruses differ so much.

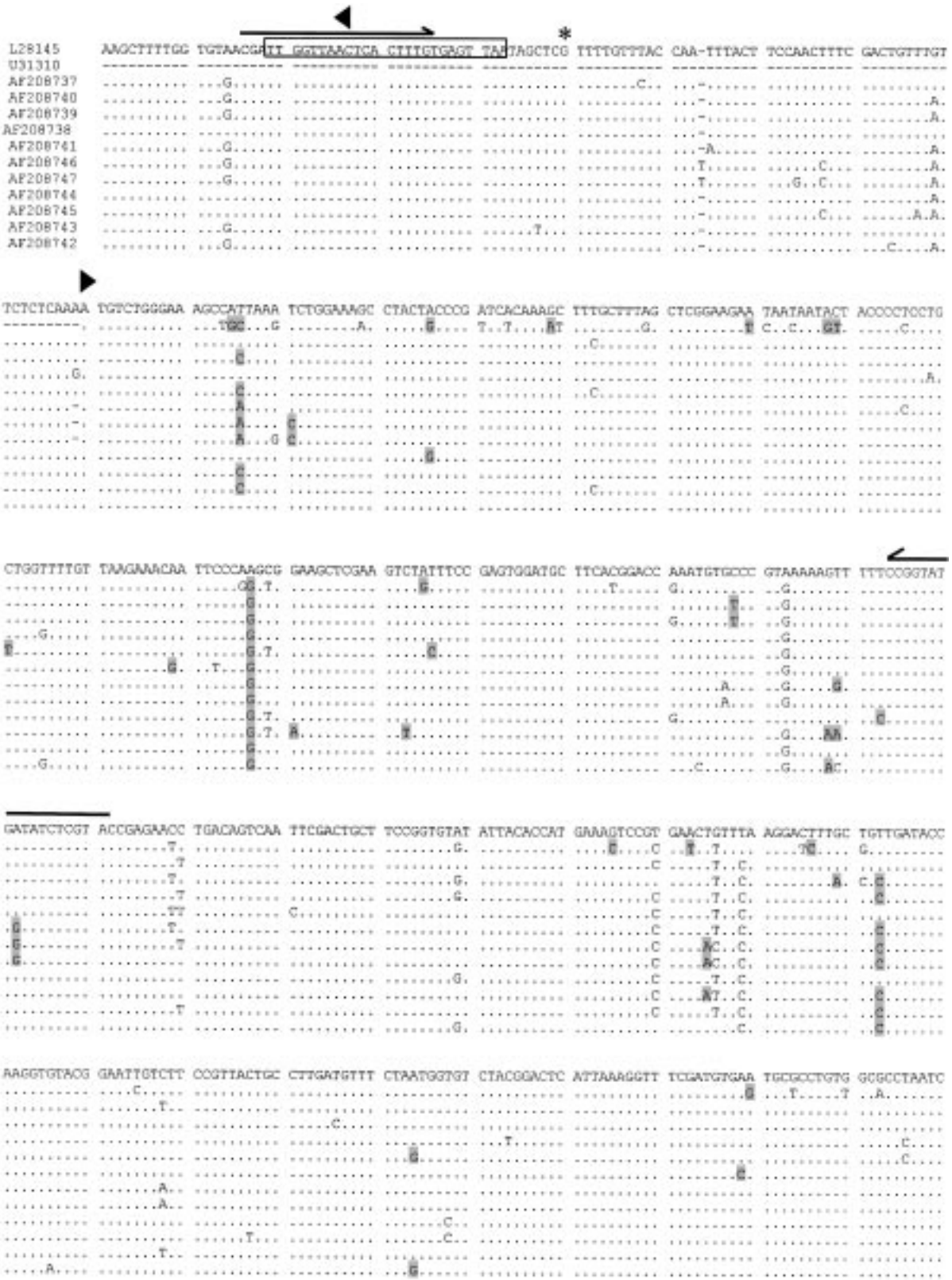

Figure 1.

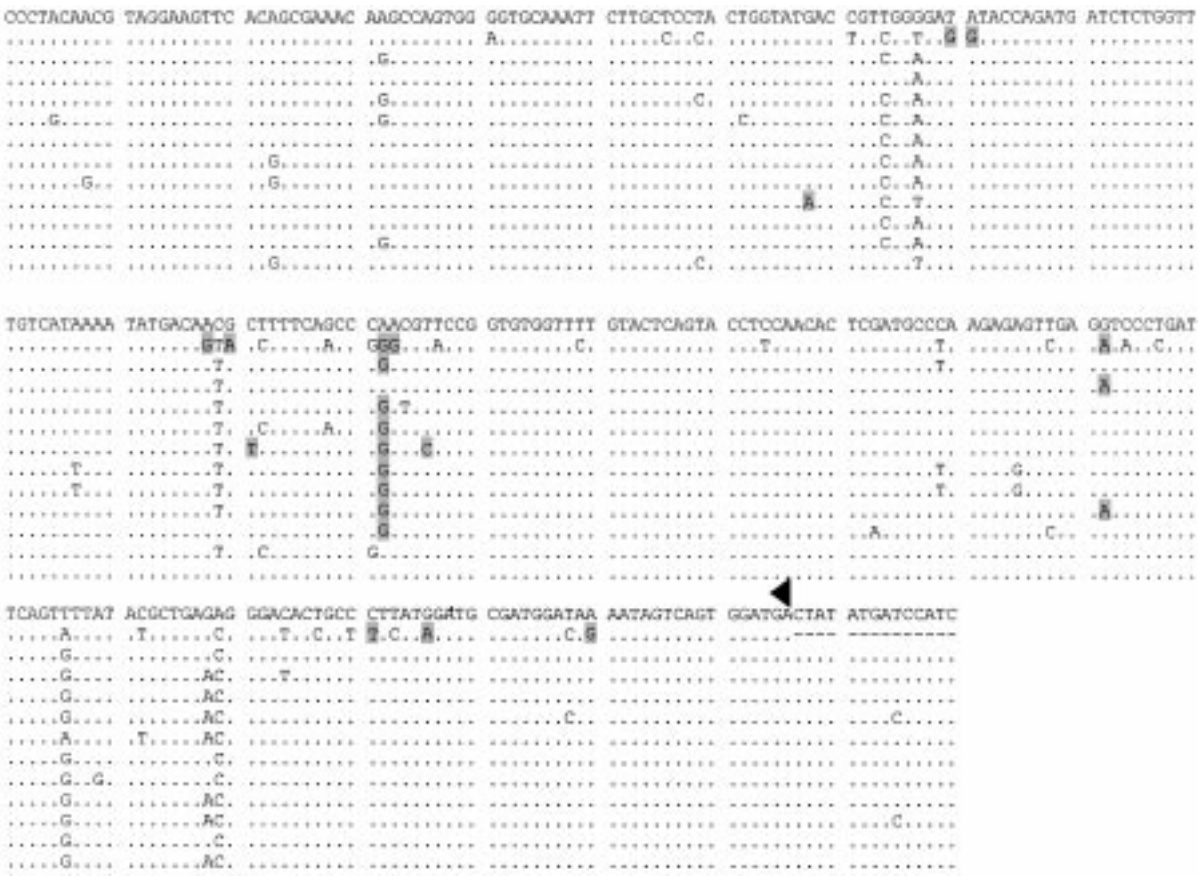

Figure 1. (Continued) Coat protein and putative subgenomic promoter sequence alignment. Alignment of the nucleotide sequence of the coat protein gene and the preceeding intergenic region. Only different nucleotides are shown, unknown or missing nucleotides are marked by dashes. Light shaded boxes indicate nonsynonymous nucleotide substitutions. Initiation codon of the CP is shown by triangle pointing to the right. Stop codon of the CP and the movement protein are shown by triangle pointing to the left. The transcription start of RNA4 is marked by the star. Positions of proposed detection primers are marked by arrows. Hairpin of the putative subgenomic promoter is boxed.

Function of the capsid protein

The CP of ilarviruses is a molecule of key importance. It is not only the structural component, but it has also a major role in initiation and propagation of the infection (reviewed in Bol, 1999). Although little information on the biochemical function of the CP is available for PDV, studies on related ilar- and alfamoviruses indicate, that one of the arginines in the N-terminal part of the CP is crucial for RNA-binding activity (Yusibov and Loesch-Fries, 1998; Swanson et al., 1998). Alignment of the CPs of 12 ilarviruses and alfalfa mosaic virus showed that the amino acid residues surrounding this crucial arginine were well conserved in the majority of viruses of this group (Ansel-McKinney et al., 1996). In PDV, the crucial arginine is located at position 14 of amino acids 10–19 in the KPTT**R**SQSFA context. Variability of PDV observed in this region was limited – two isolates contain an A_{13} instead of prevailing T_{13} and one of them – U31310 – had also N_{17} instead of S_{17}. Amino acids P_{11}, S_{15} and A_{19} which could also be associated with the RNA-binding activity of the CP (Ansel-McKinney et al., 1996) were conserved in all PDV isolates.

The intergenic region

CP is expressed via subgenomic RNA4, which is collinear with the 3′ end of RNA3. The location of the subgenomic promotor has not been determined for PDV. However, studies on alfalfa mosaic virus indicate that the core promoter sufficient for basal level of activity lies directly upstream (−26/+1) of the transcription start site (van der Vossen et al., 1995).

Existence of stable hairpins was predicted for most members of the family *Bromoviridae* at a distance of 3–8 nt from the site of initiation of RNA4 synthesis. Stability of this hairpin was most pronounced in the genera Alfamovirus and Ilarvirus which display genome activation by the CP (Jaspars, 1998).

Calculated free-energy of the hairpins (Figure 2) of all PDV isolates was −8.7 kcal/mol. Conservation of the putative subgenomic region was so extensive, that among 12 isolates (for which sequence data was available) no nucleotide substitution occurs in the hairpin-forming region between nucleotides −7 and −27 (Figure 1).

Unlike PNRSV, where the intergenic region contributes considerably to sequence divergence (Vašková et al., 2000), variation in the intergenic region of PDV isolates was minimal. This phenomenon was most pronounced downstream of the transcription start of RNA4, up to the starting codon of the CP ORF (in Figure 1 defined by a star and a triangle pointing to the right), where the divergence of PNRSV isolates was as high as 75%, while the maximum dissimilarity observed among PDV isolates was only 9%.

Primer design

A detailed inspection of variability of PDV isolates revealed only a few substitutions at the C-half of the CP. Thus it is possible to choose suitable sequences for priming of PCR amplification in that region. Such primers, together with those complementary to the perfectly conserved region of the subgenomic promotor, may be a promising tool for reliable PCR-based detection of PDV.

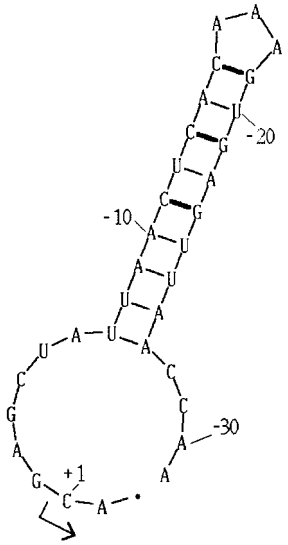

Figure 2. Hairpin structure of putative prune dwarf virus RNA4 subgenomic promotor. The start of synthesis of RNA4 is at nucleotide +1, the direction of transcription is denoted by an arrow.

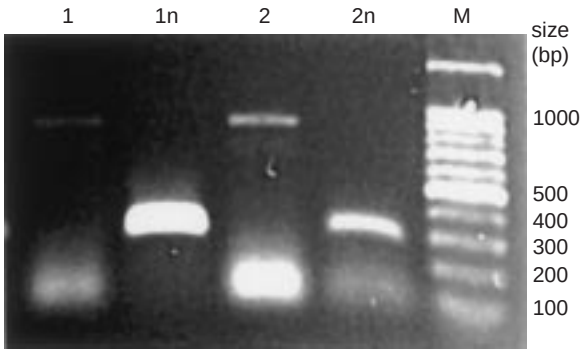

Figure 3. Amplification of low-yield PDV isolates with proposed detection primers. RT-PCR amplification products. Lines 1, 2 – weak bands of 874 bp long products amplified by main primer pair of this study. Lines 1n, 2n – 268 bp product amplified by universal primer pair designed after a detailed inspection of variability of PDV isolates. RT-PCR protocol of both primer pairs was as described in Material and methods. Line M – molecular size marker. Sample 1 – a sweet Cherry isolate, sample 2 – a Plum isolate.

Only a few primer pairs have been published to date (Rampitsch et al., 1995; Rowhani et al., 1995; 1998). Unfortunately, their behaviour on various isolates was only rarely mentioned in the papers describing their use. The primer pair described in this work provided generally good amplification results, however, according to our observations, there exist certain isolates of PDV for which the yields of RT-PCR products were consistently low (not suitable for sequencing). The use of primer pairs designed on the basis of conserved regions detected in this study may further increase the efficiency of PCR amplification. We tested one such pair of primers, (5′-CGATTGGTTAACTCACTTTG and 5′-GTACGAGATATCATACCGG) amplifying the region between nt position 1131 and 1398 (Figure 1). Presence of previously unambiguous PDV infection was clearly discriminated by a high signal of 268 bp product (Figure 3).

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