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Genetic variability among coat protein of *Prune dwarf virus* variants from different countries and different *Prunus* species

Elżbieta Kalinowska · Karolina Mroczkowska · Elżbieta Paduch-Cichal · Maria Chodorska

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Abstract *Prune dwarf virus* (PDV) isolates have been investigated for genetic diversity. Full-length nucleotide and amino acid sequences of viral coat protein from 23 isolates collected from different stone fruit trees (sour and sweet cherry trees, wild cherry tree, plum tree, almond tree, peach tree) and different countries (Poland, Italy, Germany, USA, Israel) were analysed and compared to 57 others available in GenBank. Comparison of all sequenced virus isolates revealed diversity of 86–100 % at nucleotide level and 79–100 % at amino acid level. The ratio of non-synonymous to synonymous polymorphic sites indicated that purifying selection dominated in case of PDV. However, six codons showed to be under strong positive selection, including the codon located inside the structure involved in RNA-binding activity.

Keywords Prune dwarf virus · PDV · Coat protein · Genetic diversity

Stone fruit trees are infected by several viruses, which cause significant economic losses. One of the notable pathogens is *Prune dwarf virus* (PDV). PDV is distributed worldwide and infects many *Prunus* species, including almond, sour- and sweet cherry, peach, apricot, and plum trees (Németh, 1986). Disease symptoms caused by PDV vary from no obvious to serious damages resulting in significant yield and vigour reduction. Disease symptoms can be visible as chlorosis, chlorotic rings and spots on the leaves (sour cherry), stunting, and leaf malformations as well as fruit size reduction (peaches, plums, and prunes) and fruit rain cracking (sweet cherries) (Uyemoto and Scott, 1992; Brunt et al., 1996). Symptoms are correlated with virus isolate, host, and climate (Németh, 1986). PDV is transmitted by grafting as well as through pollen and seeds (Kelley and Cameron, 1986). The virus is also carried inside almond pollen grain (Silva et al., 2003).

PDV belongs to the genus *Ilarvirus*, family *Bromoviridae* (King et al., 2012). The virus genome is organized into three plus-stranded RNAs. RNAs 1 and 2 encode conservative proteins involved in viral replication. RNA 3 encodes movement protein (MP) and coat protein (CP), which is expressed via sub-genomic RNA 4 (Bachman et al., 1994).

Despite of differences in serological and biological properties of PDV isolates a molecular characterization of viral coat protein sequences did not reveal a correlation between amino acid composition and host species and/or origin of the viral isolate (Vaškova et al., 2000). Subsequent studies of PDV CP nucleotide sequences revealed higher divergence in almond isolates of PDV than in isolates obtained from other hosts (Fonseca et al., 2005) and indicated presence of four phylogroups: 1.

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E. Kalinowska (✉) · K. Mroczkowska · E. Paduch-Cichal · M. Chodorska
Warsaw University of Life Sciences, Department of Plant Pathology, Nowoursynowska 159, 02-776 Warsaw, Poland
e-mail: elzbieta_kalinowska@sggw.pl

Table 1 The author's PDV isolates (bold) and PDV sequences avaible in GenBank

Isolate	Original host	Country of origin	GenBank GI No.	Phylogroup/Subgroup	
RS-40B/1	Cherry	Hungary	45385845	I	
PDV-SW7	**Cherry**	**Poland**	**270036708**		
Skierniewice	Cherry	Poland	905394		
PD10	Cherry	Turkey	148612159		
PD3	Cherry	Turkey	148612145		
PD4	Cherry	Turkey	148612147		
B.11.15	Almond	Portugal	56111823	II	A
cp4.3	Almond	Portugal	56111821		
3.19.A1.2	Almond	Portugal	56111830		
1.8.A3	Almond	Portugal	56111828		
cp1.2	Almond	Portugal	56111819		
-[1]	Almond	Portugal	6606517		
3.12.N.14	Almond	Portugal	56111832		
3.20.1	Almond	Portugal	56111838		
3.17.A1	Almond	Portugal	56111834		
3.20.2	Almond	Portugal	56111836		
-	Peach	Argentina	11545665	II	
4B	Almond	Portugal	56111826		
N.1.2	Almond	Portugal	56111817		
RS-45/1	Cherry	Moldova	45385851		
PDV-PL13	**Plum**	**Poland**	**270036710**		
PDV-SWI-35	**Cherry**	**Poland**	**262092717**		
vanC	Cherry	Czech Republic	11494040		
ch137	Cherry	USA	452499		
PDV-SW6-1	**Cherry**	**Italy**	**262092723**		
2/16	Cherry	Czech Republic	11494028		
Branisovska	Plum	Czech Republic	11494049		
PD5	Cherry	Turkey	148612149		
PDV-Plum5	**Plum**	**Italy**	**270036712**		
PDV-PL1-19	**Plum**	**Italy**	**262092725**		
PDV-SW-Regina	**Cherry**	**Poland**	**262092719**		
PDV-SOF15P1	**Cherry**	**Italy**	**407908777**		
PS7/9a	Cherry	Czech Republic	11494034		
PDV-SO14	**Cherry**	**Poland**	**407908771**		
6/54	Plum	Czech Republic	11494055		
7/12	Plum	Czech Republic	11494058		
sss	Plum	Czech Republic	11494052		
Valticka	Peach	Czech Republic	11494043		
Nem	Peach	Germany	11494046		
RS-50A/1	Wild cherry	Hungary	45385847		
RS-88/1	Cherry	Hungary	45385849		
10 W3	Cherry	Poland	157931543		
K1	Cherry	Czech Republic	11494031		
PDV-PA78	**Wild cherry**	**Poland**	**407908775**	II	
PDV-PE15-28	**Peach**	**Germany**	**262092733**		

Table 1 (continued)

Isolate	Original host	Country of origin	GenBank GI No.	Phylogroup/Subgroup
3 W1	Cherry	Poland	157931537	
PDV-PA63	**Wild cherry**	**Poland**	**407908779**	
21/1	Cherry	Czech Republic	11494037	
PDVSO40E50	**Cherry**	**Israel**	**262092729**	
PDV-SW9-1	**Cherry**	**USA**	**262092721**	
PD6	Cherry	Turkey	148612151	
PDV-AL2	**Almond**	**Italy**	**262092727**	
PDV-SW145W	**Wild cherry**	**Poland**	**262092715**	
4 W1	Cherry	Poland	157931539	
2C3	Cherry	Poland	157931547	
PD2	Cherry	Turkey	148612143	
PD9	Cherry	Turkey	148612157	
PDV-SWM1	**Cherry**	**Poland**	**407908781**	
PD8	Cherry	Turkey	148612155	
RS-38/1	Cherry	Hungary	45385843	
MC1	Peach	Brazil	209944108	
1S2	Plum	Poland	157931531	
5C3	Cherry	Poland	157931549	
4 M2	Apricot	Poland	157931551	
PDV-SOF15P11	**Cherry**	**Italy**	**407908777**	
PD7	Cherry	Turkey	148612153	
PD1	Apricot	Turkey	148612141	
RS-10/4	Cherry	Hungary	45331567	
PDV-Almond2	**Almond**	**Italy**	**270036704**	
PDV-SOF17P17	**Cherry**	**Italy**	**407908773**	
PDV-PE247	**Peach**	**Poland**	**407908783**	
DJ1.2	Wild cherry	China	343129302	
PDV-SO20SZ1	**Cherry**	**Poland**	**262092711**	
PDV-SO20SZ3	**Cherry**	**Poland**	**262092713**	
9 M1	Apricot	Poland	157931555	
5 M4	Apricot	Poland	157931553	
1C3	Cherry	Poland	157931545	
8 W2	Cherry	Poland	157931541	
2S1	Plum	Poland	157931533	
3B9	Peach	Poland	157931535	

[1] Dashes indicate lack of data

mixed 2. cherry I 3. cherry II, and 4. almond (Ulubaş et al., 2009). However, when phylogenetic analysis was based on amino acid sequences, the results indicated the presence of only two groups (Pallas et al., 2012).

Recent studies concerning selective pressure analysis of 36 plant RNA viruses showed that CP amino acid sequences of PDV and other non-vector borne viruses are more divergent than viruses transmitted by vectors (Chare and Holmes, 2004), although it is still unknown which sites of PDV CP amino acid sequence are under positive selection.

Aim of this work was to compare 23 CP gene sequences of the authors' PDV isolates collections with 57 PDV sequences from GenBank database and to perform

a phylogenetic analysis of PDV CP genes in order to find motifs that are characteristic for each group/subgroup. The second goal of the study was to calculate the ratio of non-synonymous and synonymous substitutions among the CP gene to determine how selective pressure shapes the amino acid sequence of the PDV population and determine which sites exactly are under positive selective pressure.

The PDV isolates were collected from various geographic regions and different *Prunus* species (Table 1). Dormant buds from different *Prunus* species infected with PDV were transferred to *Prunus avium* (wild cherry) clone F12/1 seedlings using the chip budding technique. To confirm the presence of the virus, each wild cherry clone F12/1 plant was tested by DAS-ELISA using a PDV commercial polyclonal antiserum (Loewe Biochemica GmbH, Germany) according to the procedure by Clark and Adams (1977). In the following examinations, buds and leaves from wild cherry clone F12/1 collection were used as a source of the virus.

Immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) was conducted according to Malinowski (2005). To amplify the complete sequence of the CP gene, a primer pair designed by Vaškova et al. (2000) (GTGTAGAAAGAAGAGAAG TCCGACAAG and ATCTAGAAGCAGCATTTCCA ACTACGA) was used for one step RT-PCR (One Tube RT-PCR System; Roche, Germany).

Sequences were analysed with the blastn and blastp programs (http://www.ncbi.nlm.nih.gov/BLAST). Alignments, sequence diversity, and phylogenetic tree were made using MEGA6 (Tamura et al., 2013). The number and ratio of nonsynonymous (dN) and synonymous (dS) substitutions for each codon were estimated on the Datamonkey server (Kosakovsky and Frost, 2005), at http://www.datamonkey.org/, using the random effects likelihood (REL) model, single likelihood ancestor counting (SLAC), and fixed effects likelihood (FEL). Only codons with evidence of selection by all three models were mentioned. Since recombination can have effects on phylogenetic analysis, concatenated sequences of the PDV CP genes were examined for recombination using the SPB (Single Breakpoint Recombination) and GARD (Genetic Algorithm Recombination Detection) programs (Kosakovsky et al., 2006). Phylogenetic examinations were repeated using SPB and GARD trees. For SLAC and FEL, *p*-value was 0.1 and the REL Bayes Factor was 50.

The multiple alignment of PDV isolates revealed sequence identity among 23 studies' sequences ranging from 87–100 % at nucleotide level and 83–100 % at amino acid level. Similar ranges were obtained when sequences were compared with corresponding sequences available in GenBank. The percentage of identity among all nucleotide sequences encoding viral coat protein was from 86–100 %. Comparisons of CP amino acid sequences revealed a lower percentage of identity ranging from 79–100 %.

Coat proteins of alfamo- and ilarviruses play an important role in the initiation and propagation of infection (Bol, 1999). One arginine located in the N thermal part of CP is fundamental in RNA-binding activity (Yusibov and Loesch-Fries, 1998). In the case of PDV, the crucial arginine is located at position 14 of the RNA-binding consensus sequence (Q/K/R-P/N-T-X-RS-R/Q-Q/N/S-W/F-A) (Pallas et al., 2013).

The amino acid alignment of 80 PDV isolates revealed substitution of R_{16}/Q_{16} amino acids by K_{16} in one sequence (GI45331567). Additional replacements

Table 2 Evidence of positive selection among CP of PDV calculated using SLAC, REL and FEL methodologies (dN-dS values). For SLAC and FEL significance level was at 0.1 (*p*-values), for REL significance level was at 50 (Bayes Factor)

Codon	SLAC		FEL		REL	
	dN-dS	*p*-value	dN-dS	*p*-value	dN-dS	Bayes Factor
6	4.294	0.011	1.960	0.011	0.717	246.525
13	2.730	0.017	1.176	0.001	1.376	9823.450
36	2.129	0.070	0.951	0.028	1.152	208.542
48	3.965	0.004	1.758	0.001	1.336	12805.400
97	3.276	0.019	1.369	0.017	1.100	1791.800
126	1.969	0.090	0.876	0.027	1.091	155.442

were present at $Q_{17}/N_{17}/S_{17}$ positions; in two sequences, these amino acids were changed to R_{17} (GI407908779) and G_{17}, (GI56111838). In two other sequences (GI262092711, GI262092713), the position A_{19} was replaced by S_{19}.

A phylogenetic tree of PDV CP amino acid sequences was made using the maximum likelihood method based on the JTT model (Jones et al., 1992). The tree constructed separated all PDV isolates analysed into two clades (Supplementary material - Fig. 1). The first group (phylogroup I) contained all isolates from 'cherry I group' described by Ulubaş et al. (2009). Sequence similarity within the phylogroup I was between 89–99 % at nucleotide level and 94-99 % at amino acid level. The second group (phylogroup II) included 73 of the 80 analysed isolates and contained one almond subgroup (subgroup A). Sequence similarity within the phylogroup II was between 86–100 % at nucleotide level and 79–100 % at amino acid level. There was no obvious correlation between coat protein sequence and geographic origin of the isolates confirming earlier studies (Vaškova et al., 2000; Fonseca et al., 2005; Ulubaş et al., 2009). Analysis of PDV CP amino acid alignment did not reveal any characteristic motifs in any of the two groups except the presence of V at position 38 and S at position 186 in the first cluster.

The CP genes of 80 PDV isolates were initially analysed for recombination using GARD and SBP methods. Although the GARD algorithm did not find recombination events in the analysed data set, the SBP algorithm revealed a recombination point at the 574 nucleotide in the CP PDV alignment using Akaike Information Criterion (AIC), with 100 % of the model average support. It is known that maximum likelihood methods of the codon substitution model used to estimate sites under positive selection may generate false positive results when recombination is not taken into account. For this reason, SBP inferred trees were used in further analysis. The results of SLAC, REL, and FEL estimations revealed that purifying selective pressure dominates in the case of PDV coat protein. All methods estimated strong signals of negative selection for 56 sites, including codons (bold) inside the Q/K/R-P/N-T-X-RS-R/Q-Q/N/S-W/F-A structure. Our result meets with the hypothesis concerning domination of the purifying selection as one of the ways to remove deleterious mutations (García-Arenal et al., 2001). Despite domination of the purifying selective pressure, our estimations revealed that 2.73 % of codons (6, 13, 36, 48, 97, and 126) were under significant positive selection (Table 2), including codon 13 located at position X (bold) in the Q/K/R-P/N-T-X-RS-R/Q-Q/N/S-W/F-A context. The mean dN/dS value calculated with SLAC method was 0.314 and the mean pairwise diversity was 0.114. Previous results of selective pressure analysis of PDV CP revealed a mean dN/dS of 0.227 and a mean pairwise genetic diversity of 0.032 (Chare and Holmes, 2004). Lower values obtained by Chare and Holmes (2004) may be connected with a smaller dataset available in public databases. Despite differences between estimations, our results meet with the hypothesis that the presence of positive selection in viral populations is connected with the mode of virus transmission and coat proteins of non-vector borne viruses are more tolerant to sequence variability than CP of vector borne viruses (García-Arenal et al., 2001; Chare and Holmes, 2004). The presence of sites with diversifying selective pressure in viral coat protein may also be connected with changes in host-plant interaction, e.g., gene silencing. Viral gene silencing suppressors may be located in the CP genes (e.g., citrus tristeza closterovirus). An analysis of selective pressure among genes from 17 ssRNA plant viruses revealed dN/dS ratios for silencing suppressors, suggesting that diversifying selection may be a viral respones to plant defense (Obbard et al., 2009).

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