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Short Notes

Identification and characterization of the first complete genome sequence of prune dwarf virus in China

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Summary. *Prune dwarf virus* (PDV; *Ilarvirus*, *Bromoviridae*), is a common virus infecting stone fruit trees (*Prunus* spp.). The complete genome sequence of a sweet cherry isolate of PDV (PDV-DL) from Dalian, Liaoning Province, China, was determined. The RNA1, RNA2 and RNA3 of PDV-DL are, respectively, 3,376 nt, 2,594 nt and 2,129 nt in size, and have the same genome organization as those previously reported. When compared to the available sequences of PDV, isolate PDV-DL shares pairwise identities between 91.1 to 97.4% for the RNA1, 87.2 to 99.0% for the RNA2, and 88.1 to 96.9% for the RNA3. Phylogenetic analyses based on near full-length RNA3 sequences clustered 14 PDV isolates into three groups, and PDV-DL showed the closest relationship with a peach isolate PCH4 from Australia and an experimental isolate CH137 from the United States of America. Nine recombination events were predicted in genomic RNA1-3 among all of the PDV isolates. This is the first report of the complete genome sequence of PDV from China, which provides the basis for further studies on the molecular evolution of PDV, and will assist help molecular diagnostics and management of the diseases caused by PDV.

Keywords. *Prunus*, stone fruit.

INTRODUCTION

Prune dwarf virus (PDV) is a species of *Ilarvirus* in the *Bromoviridae*, which has a tripartite positive-sense single-stranded RNA genome (Pallas *et al*., 2012). RNA1 of PDV encodes the replicase protein P1, and RNA2 encodes the replicase protein P2 (Koonin, 1991; Rozanov *et al*., 1992), whereas RNA3 encodes the movement protein (MP) in 5'-proximal half and the coat protein (CP) in 3'-proximal half (Codoñer and Elena, 2008; Pallas *et al*., 2012). PDV is an economically important virus of stone fruits (*Prunus* spp.), causing losses especially in cherry, almond and peach (Çağlayan *et al*., 2011;

Kinoti *et al.*, 2018; Kamenova *et al.*, 2019). The virus is distributed widely in the world, and can be transmitted by vegetative propagation materials and through pollen and seed (Mink, 1993).

In China, PDV was first detected on sweet cherry in 1996 (Zhou *et al.*, 1996), and subsequently reported on cherry or peach from the main stone fruit planting districts of China, including Shaanxi, Liaoning, Beijing and Shandong (Ruan *et al.*, 1998; Hou *et al.*, 2005; Zhao *et al.*, 2009; Zong *et al.*, 2015). The virus has been regarded as a serious threat to the commercial production of cherry in China (Zong *et al.*, 2015). No complete genomic sequence of Chinese PDV isolate was available. The present study determined the first complete genomic sequence of PDV in China, and compared this with other available PDV genomic sequences. This will provide the basis for further research on the molecular evolution of PDV, and assist molecular diagnostics and management of the diseases caused by PDV.

MATERIALS AND METHODS

Virus source

In July 2019, sweet cherry (*Prunus avium* L.) 'Meizao' trees with symptoms of leaf mosaic and crinkle, indicative of virus infection, were observed in an orchard in Dalian, Liaoning Province, China. Leaf samples were collected from a symptomatic tree, frozen in liquid nitrogen, and then transferred to -80°C for further research.

RNA extraction, small RNA sequencing and analysis

High-throughput sequencing (HTS) of small RNAs was used to identify viruses in the samples. Total RNA was extracted from leaf samples using TRIzol reagent (Invitrogen, 15596) according to the manufacturer's instructions. A library of small RNAs was constructed using a TruSeq Small RNA Sample Preparation Kit (IIIumina, RS-200-0024), and was sequenced using IIIumina HiSeq2000 platform at Biomarker Technologies (Beijing, China). The obtained raw reads were cleaned by trimming adapter sequences and eliminating reads less than 18 nt or more than 35 nt, using an in-house Perl script from Biomarker Technologies. The clean reads were assembled *de novo* into contigs using Velvet Software (Zerbino and Birney, 2008). The contigs were used for Blast analysis against the GenBank Virus RefSeq Nucleotide and Virus RefSeq Protein databases, with e-values of 10^{-5} (Wu *et al.*, 2010).

RT-PCR validation of candidate viruses

To validate the occurrence of HTS-detected viruses in the samples, RT-PCR was carried out using specific primers designed based on the obtained viral contigs and conserved regions of the published genome from GenBank (Table S1). Single-stranded cDNA was synthesized from the total RNA using M-MLV Reverse Transcriptase (Promega, M1701) with random hexamer primer following the manufacturer's instructions. PCR was performed in a volume of 25 μL containing 1.0 μL cDNA, 12.5 μL Premix *LA Taq* DNA polymerase Mix (TaKaRa, RR900A), 1.0 μL 10 μM corresponding upstream and downstream primers, with 35 cycles of 94°C for 30 s, 54°C for 30 s and 72°C for 1 min. The PCR products were examined by 1% agarose gel electrophoresis and ethidium bromide staining.

Cloning and sequencing of PDV genome

The primer pairs PDV-F1/PDV-R, PDV-F2/PDV-R and PDV-F3/PDV-R (Table S1) were selected to amplify, respectively, the near full-length sequences of RNA1, RNA2 and RNA3. PCR was performed as described above, except that the elongation time was increased from 1 min to 3 min at 72°C. The 5' and 3' terminal sequences were obtained from the total RNA, which had been treated with Poly(A) polymerase (TaKaRa, D2180A), using SMARTer^{TM} RACE cDNA Amplification Kit (Clontech, 634923). All of the amplicons were gel-purified using Gel Extraction Kit (CWBIO, CW2302M), cloned into pMD18-T simple vector (TaKaRa, D103A) and sequenced. Three independent clones were sequenced for each amplicon.

Sequence, phylogenetic and recombination analyses

The genomic sequences were assembled from the near full-length sequences and 5' and 3' terminal sequences using Vector NTI based on overlapping regions of 127-723 nt, and the ORFs were identified using ORFfinder (https://www.ncbi.nlm.nih. gov/orffinder). Blast analysis was performed online (http://blast.ncbi.nlm.nih.gov/ Blast.cgi). Only four full genomes of PDV are available in GenBank (sweet cherry isolate 1046C from Slovakia, sweet cherry isolate Niagara D5 from Canada, and peach isolates PCH4 and NS9 from Australia). In addition, one full-length RNA1 from sweet cherry isolate Salmo BC from Canada, one full-length RNA2 from an unknown isolate (AF277662), and one full-length RNA3 from experimental isolate

CH137 maintained in squash from the United States of America were available. SDT 1.0 software (Muhire *et al.*, 2014) was used to calculate the pairwise identities of nucleotide and amino acid sequences by the ClustalW algorithm. Multiple sequence alignment was performed using the ClustalW algorithm of MEGA 6.0 (Tamura *et al.*, 2013), with gap opening penalty of 15 and gap extension penalty of 6.66. Phylogenetic trees were constructed using the neighbor-joining (NJ) method with 1,000 bootstrap replicates, and genetic distance was calculated by the Tamura three-parameter model which was determined as the best-fitting model of substitution using MEGA 6.0. Recombination events were predicted by seven methods in RDP3 Software (Heath *et al.*, 2006), including RDP, Geneconv, Chimera, BootScan, MaxChi, SiScan and 3Seq, with default settings. Only the recombination events predicted by at least five methods with *P* values <0.01 were accepted.

RESULTS AND DISCUSSION

A total of 16,858,859 raw reads were obtained by HTS of small RNAs. After removing adapter sequences, a total of 15,488,787 clean reads with lengths of 18-35 nt were assembled into 3,742 contigs with N50 of 66 nt using Velvet Software. Blast analysis against the GenBank database showed that 11 out of the 3,742 contigs with lengths of 53-204 nt were mapped to the genome sequence of PDV, seven mapped to cherry virus A (CVA) with lengths of 46-189 nt, five to prunus necrotic ringspot virus (PNRSV) with lengths of 49-230 nt, one of 42 nt to papaya ringspot virus (PRSV) and one of 43 nt mapped to pepper chlorotic spot virus (PCSV). RT-PCR detection with specific primers derived from the contigs and sequencing of the amplicons confirmed the occurrence of PDV, CVA and PNRSV. However, PRSV or PCSV were RT-PCR negative, which might indicate false positive results of HTS considering the limited numbers and lengths of contigs. Previous research showed that virus detection based on HTS of small RNAs could be 10 times more sensitive than RT-PCR (Santala and Valkonen, 2018). Therefore, it cannot be excluded that the low titres of PRSV and PCSV were below the sensitivity of RT-PCR.

The complete genomic sequence of the PDV isolate PDV-DL was determined and submitted to GenBank with accession numbers MT013233-MT013235. It shares the same genome organization as those previously reported. The RNA1 of PDV-DL is 3,376 nt, encoding P1 of 1,055 aa from 39 to 3,206 nt. This contains the methyltransferase domain at N-terminal and the NTPase/helicase-like domain at C-terminal (Rozanov *et al.*, 1992; Gorbalenya and Koonin, 1993). The RNA2 is 2,594 nt, encoding the RNA-dependent RNA polymerase (P2) of 788 aa from 32 to 2,398 nt. The RNA3 is 2,129 nt, encoding MP of 293 aa from 261 to 1,142 nt and CP of 218 aa from 1,215 to 1,871 nt.

Compared to available full-length genomic sequences, PDV-DL shares pairwise nucleotide sequence identities of 91.1–97.4% for RNA1, 87.2–99.0% for RNA2, and 88.1–96.9% for RNA3 (Figure 1). The P1 of PDV-DL shares the highest amino acid sequence identity of 97.7% with that of isolate Salmo BC (YP_611154). The P2 shares the highest amino acid sequence identity of 98.9% with that of isolate Niagara D5 (QGA70956). The MP shares the highest amino acid sequence identity of 98.3% with that of isolate CH137 (AAA46818), and the CP of PDV-DL shares the highest amino acid sequence identity of 99.5% with that of a cherry isolate PD6 from Turkey (ABQ96001).

There are only two records of CP genes of Chinese PDV isolates available from sweet cherry in GenBank, HSY4-1 (KC965106) and DJ1-2 (JF333587). The CP gene of PDV-DL shares 93.5% nucleotide and 96.3% amino acid identities with that of HSY4-1, and 92.7% nucleotide and 95.4 amino acid identities with that of DJ1-2.

The phylogenetic NJ trees based on RNA1, RNA2 and RNA3 showed different topological structures. PDV-DL in this study clustered together with isolate Niagara D5 in the RNA2 tree, and together with isolates PCH4 and CH137 in the RNA3, both of which were supported by high bootstrap values, but PDV-DL did not form a distinct clade with any other isolates in the RNA1 tree (Figure 1). The different clustering of RNA1, RNA2 and RNA3 may be related with the potential recombination and segment re-assortment during the evolutionary history of PDV, which are common for the family *Bromoviridae* (Codoñer and Elena, 2008; Song *et al.*, 2019). However, this is difficult to confirm because of the limited number of published PDV genome records. Therefore, the information of genome sequences from more PDV isolates are needed to confirm the evolutionary history of this virus.

Recombination and segment reassortment are two of the important mechanisms for driving the evolution of RNA plant viruses, which mainly occur during mixed infections (Codoñer and Elena, 2008). The present study has shown that a total of nine recombination events were predicted by RDP3, and three events were identified in each of genomic RNA1, RNA2 and RNA3 (Table 1).

Due to limited number of PDV complete genomes and in order to eliminate the potential effects of recombination events on phylogenetic analysis, the near full-

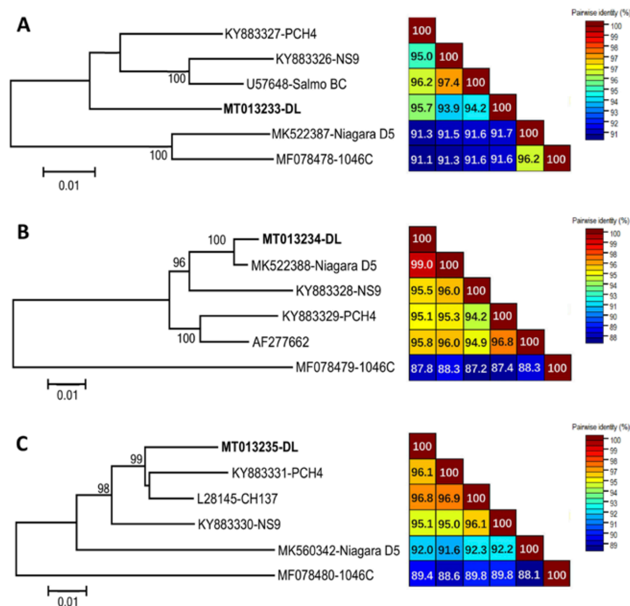

Figure 1. Phylogenetic analyses and matrix of pairwise identities based on prune dwarf virus (PDV) full-length genomic sequences of RNA1 (A), RNA2 (B) and RNA3 (C). The PDV-DL isolate examined in this study is marked in bold. Phylogenetic trees were constructed by the neighbor-joining (NJ) method with 1,000 bootstrap replicates, and genetic distance was calculated by the Tamura three-parameter model using MEGA 6.0. The pairwise identities were calculated by the ClustalW algorithm using SDT 1.0 software.

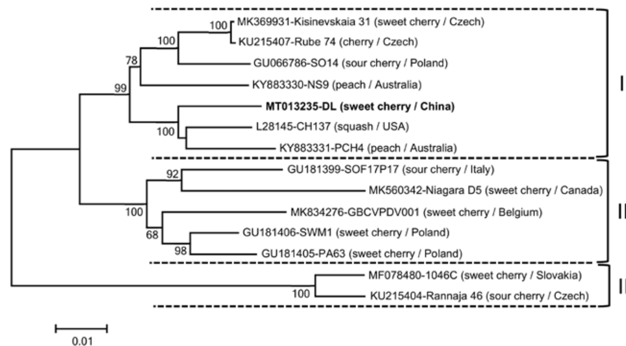

Figure 2. Phylogenetic analysis based on the near full-length sequence of RNA3, covering the complete coding regions of movement protein (MP) and coat protein (CP) genes and the intergenic region (IGR), of the prune dwarf virus (PDV) isolate examined in this study (PDV-DL), and 13 other PDV isolates available in GenBank, without recombination events detected. The accession number, isolate names, hosts and countries for each isolate are indicated, and PDV-DL examined in this study is marked in bold. Phylogenetic tree was constructed by the neighbor-joining (NJ) method with 1,000 bootstrap replicates, and genetic distance was calculated by the Tamura three-parameter model using MEGA 6.0.

length sequence of RNA3 of PDV-DL, covering the complete coding regions of MP and CP genes and the intergenic region (MP-IGR-CP), was used for phylogenetic analysis with 13 PDV isolates from GenBank where no recombination events were detected. The obtained NJ tree based on the MP-IGR-CP regions clustered the 14 PDV isolates into three groups supported by high bootstrap values, and PDV-DL showed the closest evolutionary relationship with isolates PCH4 and CH137 (Figure 2). The phylogenetic grouping showed no clear correlation to host or geographic origin. Individual phylogenetic analysis of the CP and the MP confirmed the same clustering, although this was not supported by high bootstrap values (62 for CP and 69 for MP) (Figure S1).

Previous studies of PDV genetic diversity mostly focused on the CP gene of RNA3, and showed different results. Ulubaş Serçe *et al.* (2009) reported the existence of four distinct phylogenetic groups of PDV isolates based on the CP gene, while Predajňa *et al.* (2017) identified only two groups of PDV isolates based on MP and CP phylogenetic trees. In the present study, three distinct phylogenetic groups were observed in phylogenetic NJ tree based on the near full-length RNA3 sequences,

Table 1. Recombination events in genomic RNA1, RNA2 and RNA3 of prune dwarf virus (PDV) predicted by RDP3.

	Recombinant	Major parent	Minor parent	Region (nt)	RDP	Geneconv	BootScan	MaxChi	Chimaera	SiScan	3Seq
RNA1	PCH4	Salmo BC	DL	1134-1517	5.96E-08	1.06E-05	3.10E-08	1.63E-05	4.33E-06	1.84E-06	3.77E-05
	PCH4	Salmo BC	DL	1757-2145	2.57E-06	1.26E-05	8.92E-07	1.05E-04	9.85E-05	3.31E-10	2.59E-06
	PCH4	Salmo BC	DL	2369-2727	9.47E-09	1.27E-07	4.34E-09	3.03E-08	4.67E-06	1.48E-08	3.28E-05
RNA2	Niagara D5	PCH4	NS9	55-671	7.90E-03	-	6.68E-03	4.64E-03	-	2.01E-03	7.68E-03
	1046C	PCH4	NS9	399-754	7.90E-03	-	6.68E-03	4.64E-03	-	2.01E-03	7.68E-03
	AF277662	NS9	PCH4	436-697	7.90E-03	-	6.68E-03	4.64E-03	-	2.01E-03	7.68E-03
RNA3	PA78	PA63	SWM1	16-1006	-	1.65E-04	2.23E-06	7.26E-08	1.46E-07	3.86E-11	3.77E-13
	SOF15P11	SWM1	SOF17P17	1-933	1.76E-05	1.05E-03	1.78E-05	4.53E-09	2.15E-03	2.47E-12	3.25E-07
	PE247	SOF17P17	Rannaja 46	12-957	-	2.38E-12	9.39E-14	7.74E-17	2.76E-16	2.11E-23	1.20E-36

which was consistent with results from Australia (Kinoti *et al*., 2018) and Bulgaria (Kamenova *et al*., 2019).

PDV is one of the most common viruses infecting stone fruit trees, especially sweet cherry trees (Predajňa *et al*., 2017). Symptom expression induced by PDV is highly variable, ranging from symptomless to leaf yellowing, chlorosis, mosaic, ringspot, necrosis, malformations and fruit reduction, depending on environmental conditions, virus isolate, host species and cultivar (Fonseca *et al*., 2005; Predajňa *et al*., 2017). Stone fruit trees with mixed infections of PDV with other viruses are common in nature and these infections can also affect symptom expression (Gao *et al*., 2016). In the present study, PDV was detected in a sweet cherry tree with symptoms of leaf mosaic and crinkle. However, PNRSV and CVA were also detected in the same tree, so there is no clear evidence supporting the correlation of the observed symptoms to infection by PDV. However, this is the first report of the complete genomic sequence of PDV from China, which provides the basis for further studies on genetic evolution in this virus, and for molecular diagnostics and potential disease management of PDV in this country.

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