



# Genomic detection and molecular characterization of two distinct isolates of cycas necrotic stunt virus from *Paeonia suffruticosa* and *Daphne odora*

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## Abstract

Complete genome sequences of two cycas necrotic stunt virus (CNSV) isolates from *Paeonia suffruticosa* and *Daphne odora* were determined. Phylogenetic trees and pairwise comparisons using complete RNA1- and RNA2-encoded polyproteins showed that the two CNSV isolates are divergent (83.19%–89.42% in polyprotein 1 and 73.61%–85.78% in polyprotein 2). A comparative analysis based on taxonomic criteria for the species demarcation of nepoviruses confirmed that they are not new species but distinct variants. This is the first report of the complete genome sequences of CNSV detected in *P. suffruticosa* and *D. odora*, and the first report of CNSV infecting *P. suffruticosa*.

**Keywords** Cycas necrotic stunt virus · *Nepovirus* · *Paeonia suffruticosa* · *Daphne odora* · HTS

Nepoviruses (subfamily *Comovirinae*, family *Secoviridae*, order *Picornavirales*) are divided into three subgroups with cycas necrotic stunt virus (CNSV) belonging to subgroup B [5]. CNSV was first identified in *Cycas revoluta* in Japan [5, 7]. Several other CNSV isolates have been detected in various plant species, including *Daphne odora*, *Gladiolus* spp., *Lilium longiflorum*, *Paeonia lactiflora*, and *Primula sieboldii*

[4, 6, 8, 11, 12, 15]. However, only the genome sequence of a CNSV isolate from *C. revoluta* has been reported to date [5], and the genomic RNA2 sequence of a CNSV isolate from *L. longiflorum* is available [15]. Like other nepoviruses, CNSV has a linear, positive-sense, single-stranded, bipartite RNA genome [5, 13].

In this study, complete genome sequences of two isolates of CNSV from *Paeonia suffruticosa* collected in Uiseong (South Korea; in May 2014) and *D. odora* collected in Daejeon (South Korea; in May 2016) were determined using high-throughput RNA sequencing (HTS). To reduce the cost of HTS, each plant sample was mixed with over 60 different plant samples. Total RNAs extracted from the two pooled samples in two separate experiments using the WizPrep Plant RNA Mini Kit (wizbiosolutions, Seongnam, Korea) were then treated with the Ribo-Zero™ rRNA Removal Kit (Plant Leaf) (Epicentre, Madison, WI, USA). cDNA libraries constructed with the TruSeq RNA sample Prep Kit (Illumina, San Diego, CA, USA) were evaluated with BluePippin™ 2% Agarose Gel Cassettes (Sage Science, Beverly, MA, USA) and the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Raw read data were produced with the Illumina HiSeq 2500 System (San Diego, CA, USA) at the Theragen Etx Bio Institute (Suwon, Korea). De novo assembly and sequence analysis

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were performed by SeqGenesis (Daejeon, Korea), as previously described [9, 10].

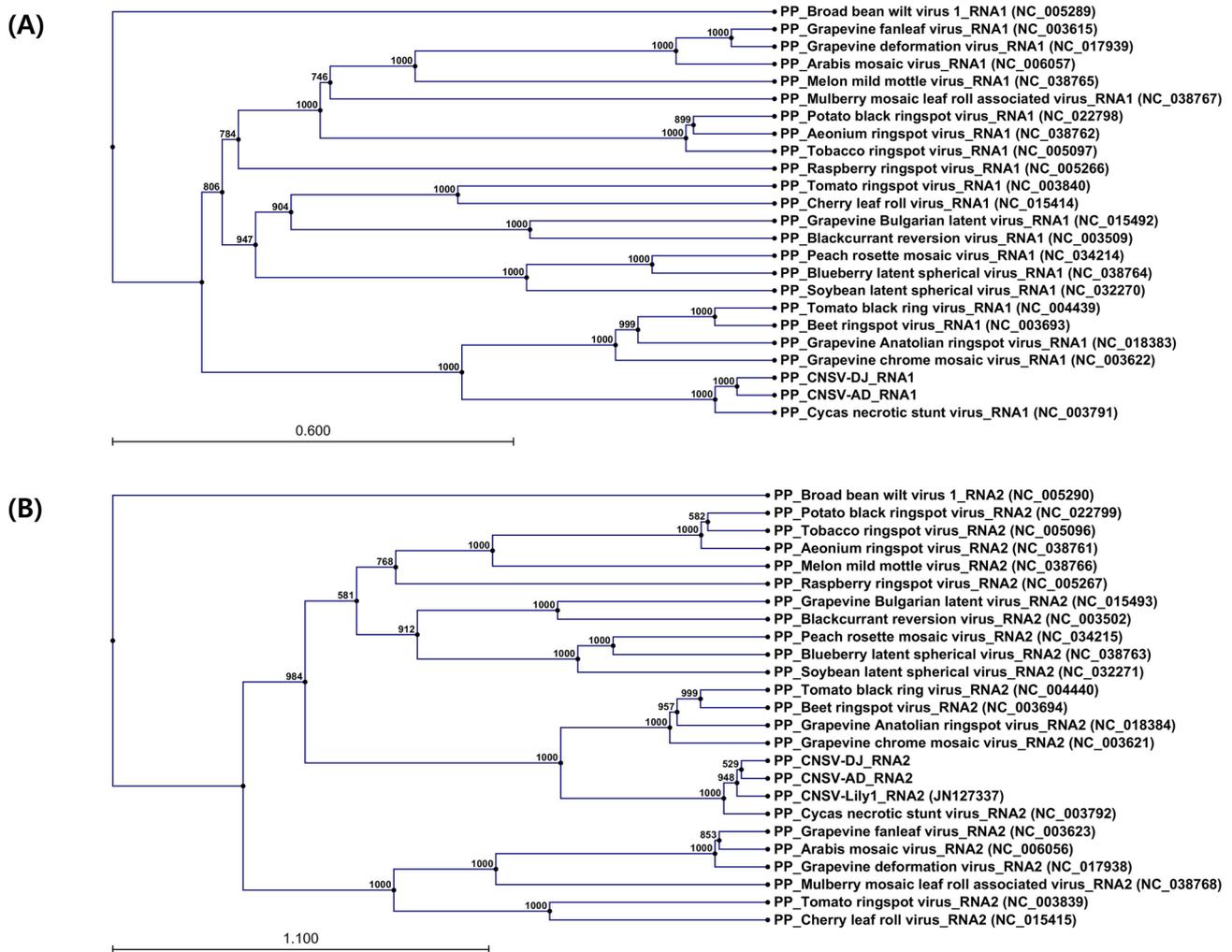
Four contigs corresponding to the bipartite genome of each isolate of CNSV infecting *P. suffruticosa* or *D. odora* were obtained from the two separate experiments. The contigs contained almost-complete segmented genomic sequences: 7492-nucleotide (nt) and 4651-nt contigs from *P. suffruticosa* and 7343-nt and 4616-nt contigs from *D. odora*. To determine the complete genome sequences of the two CNSV isolates, primers were designed based on the contig sequences (Supplementary Table S1). Total RNAs were extracted from each plant sample with the WizPrep Plant RNA Mini Kit and subjected to reverse transcription-polymerase chain reaction (RT-PCR) using SuPrimeScript RT-PCR Premix (GeNet Bio, Daejeon, Korea), with the specific primer sets and a random N<sub>25</sub> primer. Both ends of all genomic RNAs were determined using rapid amplification of cDNA ends (RACE) performed with the 5'/3' RACE System (Invitrogen, Carlsbad, CA, USA). Each PCR fragment was cloned into the RBC T&A Cloning Vector (RBC Bioscience, Taipei, Taiwan), and more than three clones per PCR were sequenced at Macrogen Inc. (Daejeon, Korea). The sequences were assembled with the DNAMAN 5.0 program (Lynnon Biosoft, Quebec, Canada), generating the complete genome sequences and genome organizations of the two CNSV isolates from *P. suffruticosa* and *D. odora* (Supplementary Fig. S1).

The genomic RNAs of the CNSV isolate AD from *P. suffruticosa* consisted of 7503 (RNA1) and 4746 (RNA2) nt (GenBank accession nos. MK512741 and MK512742, respectively), and those of the CNSV isolate DJ from *D. odora* consisted of 7355 (RNA1) and 4627 (RNA2) nt (GenBank accession nos. MK521837 and MK521838, respectively). The BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) searches showed that the complete genomic RNAs of CNSV AD shared nucleotide sequence identities (82.61% identity and 89% query coverage for RNA1; 80.91% identity and 98% query coverage for RNA2) with the *C. revoluta* isolate and that the complete genomic RNAs of CNSV DJ shared nucleotide sequence identities (87.93% identity and 90% query coverage for RNA1; 85.61% identity and 99% query coverage for RNA2) with the *C. revoluta* isolate. The nucleotide sequence similarities between the segmented genomic RNAs of the two CNSV isolates were 83.41% (97% query coverage; RNA1) and 80.39% (98% query coverage; RNA2).

The RNA1- and RNA2-encoded polyproteins of isolate AD (polyprotein 1: nt 212–7132, 2306 amino acids [aa]; polyprotein 2: nt 284–4357, 1357 aa) and isolate DJ (polyprotein 1: nt 207–7058, 2283 aa; polyprotein 2: nt 241–4311, 1356 aa) contained the conserved domains identified in a Pfam analysis (protein families database: <http://pfam.xfam.org/>; [2]) (Supplementary Fig. S1). Their

cleavage sites were predicted based on a comparative analysis of nepoviral sequences [3, 13, 14], including the reference genome sequence of CNSV (GenBank accession nos. NC\_003791.1 and NC\_003792.2) (Supplementary Fig. S1). The 5'/3'-untranslated regions (UTRs) of CNSV isolate AD were 211/371 nt (RNA1) and 283/389 nt (RNA2) in length, respectively. Their nucleotide sequence similarities to existing CNSV isolates were 78.57% (90% query coverage; 5'-UTR) and 93.77% (69% query coverage; 3'-UTR) for RNA1 and 83.9% (83% query coverage; 5'-UTR) and 93.77% (79% query coverage; 3'-UTR) for RNA2. The 5'/3'-UTRs of CNSV isolate DJ were 206/297 nt (RNA1) and 240/316 nt (RNA2) in length, respectively. Their nucleotide sequence similarities to existing CNSV isolates were 81.77% (94% query coverage; 5'-UTR) and 89.11% (100% query coverage; 3'-UTR) for RNA1 and 82.49% (70% query coverage; 5'-UTR) and 89.44% (100% query coverage; 3'-UTR) for RNA2. The 5'-UTRs of the genomic RNA1s of the two isolates shared the highest nucleotide sequence identities with the corresponding regions of the RNA2s of existing CNSV isolates. This is because the homology of the corresponding UTRs between RNA1 and RNA2 was quite high, and the only available genomic RNA1 sequence of CNSV with a complete coding sequence might have an incomplete 5'-UTR.

To understand the genetic relationships between the two CNSV isolates and other nepoviruses, including CNSV isolates (the reference genome sequences used in this study are publicly available at <https://www.ncbi.nlm.nih.gov/genome/viruses/>; [1]), phylogenetic analyses were conducted, and pairwise comparisons were made using CLC Main Workbench 6.1.1 (CLC Bio, Aarhus, Denmark). Phylogenetic trees and pairwise comparisons based on the complete RNA1-encoded polyproteins or complete RNA2-encoded polyproteins of the CNSV isolates and nepoviruses showed that the two viruses identified in *P. suffruticosa* and *D. odora* are divergent (83.19%–89.42% in polyprotein 1 and 73.61%–85.78% in polyprotein 2), when all the CNSV isolates were compared (Fig. 1 and Supplementary Fig. S2). However, the polyproteins of isolates AD and DJ were more similar to each other than to those of other isolates. Some of the taxonomic criteria for the species demarcation of nepoviruses are conserved Pro (proteinase)-Pol (RNA-dependent RNA polymerase) region aa sequence, which is defined by the sequence between CG and GDD motifs, with less than 80% identity and coat protein (CP) aa sequence with less than 75% identity. Phylogenetic analyses and pairwise comparisons based on the species demarcation criteria confirmed that the two viruses are not new species, but are distinct variants (Supplementary Figs. S3 and S4). Interestingly, the amino acid sequence between motifs CG and GDD of isolate DJ was closer to that of the *C. revoluta* isolate than to that of the AD isolate, and the CP amino acid sequences of isolates



**Fig. 1** Phylogenetic trees constructed based on alignments of RNA1-encoded complete polyprotein (a) or RNA2-encoded complete polyprotein (b) amino acid sequences of two distinct isolates (isolate AD, GenBank accession nos. MK512741 and MK512742; isolate DJ, GenBank accession nos. MK521837 and MK521838) of cycas

necrotic stunt virus (CNSV) and those of known nepoviruses. Phylogenetic trees were made with CLC Main Workbench 6.1.1 using the UPGMA method, and branch lengths and bootstrap values are indicated. Broad bean wilt virus 1 was used as the outgroup

AD and DJ were more distantly related to each other than to those of other isolates.

Here, the complete genome sequences of two distinct isolates of CNSV were determined and molecularly characterized. This is the first report of the complete genome sequences of CNSV isolates identified in *P. suffruticosa* and *D. odora*. To the best of our knowledge, this is also the first report of CNSV infecting *P. suffruticosa*.

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**Author contributions** SL, SHL, and JSM designed this study. SL, JHL, and JSM contributed to the sample collection and virus detection. The

experiments are performed by SL. SL, SYK, HSC, HSK, JMP, SHL, and JSM contributed to analysis of high-throughput sequencing data. SL wrote the initial draft, and SHL and JSM edited the manuscript. All authors read and approved the final manuscript.

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**Data availability** The dataset generated during the current study is available in GenBank (accession nos. MK512741, MK512742, MK521837, and MK521838).

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest to disclose.

**Research involving human and animal participants** This article does not contain any studies with human participants or animals performed by any of the authors.

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