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Complete nucleotide sequence and affinities of the genomic RNA of Narcissus common latent virus (genus *Carlavirus*)

Brief Report

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Summary. The complete sequence of an isolate of Narcissus common latent virus (NCLV) from Zhangzhou city, Fujian, China was determined from amplified fragments of purified viral RNA. Excluding the poly(A) tail, the genomic RNA of NCLV was 8539 nucleotides (nt) long and had the typical organization for a member of the genus *Carlavirus*. The most closely related species were *Potato virus M*, *Hop latent virus* and *Aconitum latent virus*, which had 58–59% nt identity to NCLV in their entire genomes. These relationships were confirmed by a phylogenetic analysis using a composite nucleotide alignment of all the open reading frames.

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Various filamentous viruses associated with yellow stripe or mosaic symptoms have been reported from *Narcissus* spp., often in complex mixtures. These include four members of the genus *Potyvirus*, Narcissus yellow stripe virus, Narcissus late season yellows virus, Narcissus degeneration virus (only in *N. tazetta*) and Ornithogalum mosaic virus [4, 6, 7, 9, 15]. Narcissus mosaic virus, a member of the genus *Potexvirus*, and Narcissus latent virus, originally described as a carlavirus [5, 7] but which has now been shown to be a member of the genus *Macluravirus*, family *Potyviridae* [3, 14], also occur. Chinese narcissus (*Narcissus tazetta var. chinensis*) is an economically important ornamental plant in China, and virus disease causes loss of yield and poor quality. Recently, we used degenerate primers to detect and then to sequence the 3′-terminal regions of carla- and potyvirus isolates from Chinese narcissus plants with yellow stripe symptoms growing in

Fujian province and Shanghai city, China. The plants contained a complex mixture of viruses and strains, including a possible new member of the genus *Carlavirus*, which was tentatively named Narcissus common latent virus (NCLV) [9]. We now report the complete sequence and genomic organization of NCLV and discuss its classification.

Viral particles were purified from Chinese narcissus from Zhangzhou city, Fujian province, using a published method [17], and stored at −80 °C before use. RNA was isolated from the virions using the RNeasy Plant Mini Kit (QIAGEN), and first-strand cDNA was then synthesized using M-MLV-reverse transcriptase (Life Technologies Ltd) according to the manufacturers' instructions and with M4T (5′-GTT TTC CCA GTC ACG ACA C $(T)_{16}$-3′) as the initial primer. The use of primer M4T, which is complementary to the 3′-terminal polyA tail, ensured that the complete 3′-non-coding region (NCR) was amplified. Genome fragments were then amplified by PCR using degenerate primers designed from published carlavirus sequences (Car-TGB2+, 5′-ATG CCW CTW AXX CCX CC-3′; Car-Pol+, 5′-ATX TGY TTY GCX GGX GAY GAY ATG TG-3′; Car-HELV+, 5′-ACX TTY GGX GAR WSX ACX GG-3′; Car-MTRI+, 5′-CAY WSX CAY CCX GYX TGY AAR AC-3′; X = A, T, C or G; Y = T or C; R = A or G; W = A or T; S = G or C) in combination with downstream primers designed from the NCLV sequences already determined. 5′-RACE was done to ensure amplification of the complete 5′-end sequence as previously described [8]. The LA Taq DNA polymerase system (TaKaRa) was used according to the manufacturer's protocols and the lower Tm of the primer pairs was selected as the annealing temperature during PCR amplification. Successful amplification of fragments of the expected size was confirmed by electrophoresis through 1% (w/v) agarose gels. PCR fragments were purified using the Gel Extraction Kit (QIAGEN) and cloned into the pGEM-T vector (Promega) following the manufacturers' protocols. Additional primers were designed to internal sequences for subcloning and sequencing. At least three independent clones were auto-sequenced in both directions by the ABI PRISM^{TM} 3770 DNA Sequencer. Sequence analysis used programs from the Wisconsin (GCG) package [2] and, in particular, pairwise comparisons were done with GAP (using a gap creation penalty of 50 and a gap extension penalty of 3 for nucleotides and values of 8 and 2, respectively, for amino acids). Phylogenetic analysis was done by Neighbor-Joining (Tamura-Nei distances) using MEGA 3.0 [12].

The complete sequence of NCLV was obtained from the sequences of five overlapping fragments. Each of them was derived from sequencing at least three independent clones that showed no variation. The sequence of the extreme 5′-end of NCLV RNA was derived from sequencing 11 independent clones; the sequences of 8 clones were identical and the other 3 clones were several nucleotides shorter. Excluding the poly(A) tail, the genomic RNA of NCLV was 8539 nucleotides (nt) long and the predicted arrangement of the open reading frames (ORFs) was similar to that of other carlaviruses.

The NCLV ORF1 (78–5978 nt) encodes a polypeptide of 1966 amino acids (aa) with a calculated M_r of 222.8 kDa. The various motifs for methyltransferase (MTR), helicase (HEL) and RNA-dependent RNA polymerase (POL), the active

Table 1. The percentage nucleotide and amino acid identity between NCLV and other carlaviruses in each of the ORFs using GCG GAP

	Potato virus M	Hop latent virus	Aconitum latent virus	All other carlaviruses*
(a) Nucleotide identity				
Polymerase	58.0–58.7	58.1	57.2	48.6–52.9
TGBp1	59.9–60.4	58.0	56.7	34.4–54.2
TGBp2	58.2–60.9	52.9	58.4	36.7–62.6
TGBp3	48.4–50.0	49.2	51.9	36.3–49.7
Coat protein	64.9–65.8	62.2	64.0	36.0–61.3
Nucleic acid binding protein	60.6–62.1	69.2	61.3	35.9–63.6
(b) Amino acid identity				
Polymerase	56.4–57.4	57.4	56.1	42.7–49.3
TGBp1	63.0–64.8	63.3	60.0	34.6–52.9
TGBp2	59.6–62.4	57.8	57.8	39.4–59.4
TGBp3	38.7–40.3	38.3	43.5	17.7–44.3
Coat protein	74.1–75.1	65.2	71.9	34.9–63.8
Nucleic acid binding protein	63.1–65.0	60.8	53.4	23.8–57.3

*All available sequences of definite or probable members of the genus were used provided they included one or more complete ORF

site of the papain-like cysteine protease and a probable proteolytic cleavage site similar to those previously reported were identified [16, 19]. Over the whole replicase gene, there was 48.7–58.7% nucleotide identity between NCLV and the other sequenced carlaviruses (Table 1).

Following the 46-nt-long intergenic region, there are the three ORFs of the triple gene block known to be involved in cell-to-cell movement [13]: TGBp1 (6025–6714 nt; 229 aa, 25.3 kDa), TGBp2 (6692–7018 nt; 108 aa, 12.0 kDa) and TGBp3 (7018–7203 nt; 61 aa, 6.6 kDa). TGBp1 contains an NTP-binding motif, GAGKS, at amino acid position 31–35, which is similar to that in the TGB1 of furo-, carla-, potex- and hordeiviruses, suggesting that the protein may function as an NTP-dependent helicase [10, 11, 18]. TGBp2 is predicted to have a helix-coil-helix structure by computer analysis, indicating the presence of two transmembrane (TM) regions with N- and basic C-termini exposed inside (data not shown). TGBp3 is a small peptide rich in hydrophobic residues. The TGBp1 gene of NCLV has 34.4–60.4% nucleotides identical to other carlaviruses, and the corresponding values for TGBp2 and TGBp3 are 36.7–62.6% and 36.3–51.9%, respectively (Table 1).

Following an intergenic region of 29 nt, ORF5 (7231–8136 nt) encodes a putative CP of 301 aa with a calculated M_r of 33.1 kDa. ORF6 (8142–8450 nt) encodes a putative nucleic acid binding protein (NABP) of 102 aa with a calculated M_r of 11.5 kDa. The nucleotide identity of NCLV CP and NABP genes to other carlaviruses is 36.0–65.8% and 35.9–69.2%, respectively (Table 1).

In all parts of the genome, the most closely related sequences were those of potato virus M (PVM), hop latent virus (HpLV) and Aconitum latent virus (AcLV), and this was confirmed by BLAST. Using the entire genomic sequences, the nucleotide identity between NCLV and the most closely related sequences was determined by GCG GAP to be 59.2–59.5% (PVM), 58.8% (HpLV) and 58.1% (AcLV).

In studies to determine the most appropriate criteria for discriminating between species in the carla-, potex-, allexi-, and related viruses, it appeared that distinct

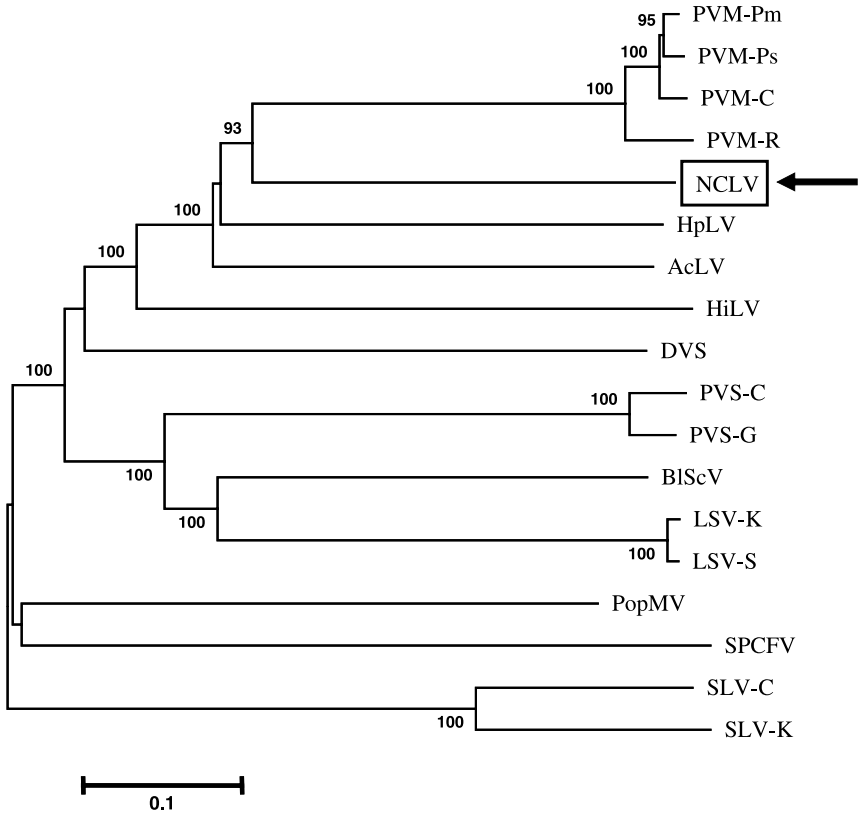

Fig. 1. Phylogenetic tree for the entire coding sequence of Narcissus common latent virus (NCLV, AM158439) and other completely sequenced carlaviruses. Abbreviations and accession numbers are AcLV, Aconitum latent virus (AB051848); BlScV, Blueberry scorch virus (L25658); DVS, Daphne virus S (AJ620300); HiLV, Hippeastrum latent virus (DQ098905); HpLV, Hop latent virus (AB032469); LSV-K, Lily symptomless virus [Korea] (AJ516059); LSV-S, Lily symptomless virus [China] (AJ564638); PopMV, Poplar mosaic virus (X65102); PVM-C, Potato virus M [China] (AJ437481); PVM-Pm, Potato virus M [Poland mild] (AY311395); PVM-Ps, Potato virus M [Poland severe] (AY311394); PVM-R, Potato virus M [Russia] (D14449); PVS-C, Potato virus S [Czech] (AJ863510); PVS-G, Potato virus S [Germany] (AJ863509); SLV-C, Shallot latent virus [China] (AJ292226); SLV-K, Shallot latent virus [Korea] (Z68502); SPCFV, Sweet potato chlorotic fleck virus (AY461421). The values at the forks indicate the percentage of trees in which this grouping occurred after bootstrapping the data with 10000 replicates (shown only when >60%). The scale bar shows the number of substitutions per base

species have less than about 72% identical nucleotides or 80% identical amino acids between their coat protein or polymerase genes [1]. Therefore, analysis of the complete sequence of NCLV supports the earlier suggestion [9] that it should be regarded as a distinct species.

Phylogenetic analysis was done using the entire coding sequence of all fully sequenced members of the genus *Carlavirus*. This was assembled by first preparing separate amino acid alignments of the six ORFs and using these as templates to generate the corresponding nucleotide alignments. These were then combined into a single nucleotide alignment, which reflects virtually the whole genome, since the 5′- and 3′-UTRs are each <100 nt long. The tree confirmed NCLV as a member of the genus and grouped it reliably with PVM, HpLV and AcLV (Fig. 1).

The NCLV sequence reported here was deposited in the EMBL/Genbank/DDBJ databases with the accession number AM158439.

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