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Brief Report

Complete nucleotide sequence of a Japanese isolate of *Chrysanthemum virus B* (genus *Carlavirus*)

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Summary

The complete nucleotide sequence of a *Chrysanthemum virus B* isolate from Japan (CVB-S) has been determined. The genomic RNA of CVB-S is 8,990 nucleotides long, excluding the poly(A) tail and, like that of other carlaviruses, contains six open reading frames (ORFs). Multiple alignment and phylogenetic analyses indicated that the phylogenetic relationship among members of the genus *Carlavirus* is very diverse, with phlox virus S being the closest relative of CVB. In aphid transmission tests, CVB-S was transmitted at a very low rate by *Aphis gossypii*, a new vector of the virus.

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Chrysanthemum virus B (CVB) is a species of the genus *Carlavirus*, the family *Flexiviridae* [1, 2], and viruses of this species are distributed in chrysanthemum-growing areas around the world [12]. Chrysanthemum (*Chrysanthemum morifolium*) plants infected with CVB show various effects, usually from mild leaf mottling or vein clearing to severe mosaic or flower malformations, but they sometimes show no symptoms [12, 13, 26]. CVB has been reported to be transmitted by sap inoculation and aphids (*Myzus persicae*, *Macrosiphum euphorbiae*, *Aulacorthum solani*, *Coloradoa rufomaculata*, and *Macrosiphoniella sanborni*) in a non-persistent manner [12] however, aphid transmission has a rather low efficiency [12]. The virus particles are slightly flexuous, measuring ca. 685 nm in length and 12 nm in diameter [13].

The carlavirus genome is a positive, single-strand RNA with a 5′-cap and 3′-poly(A) structure and encompasses six open reading frames (ORFs) [1]. Recently, complete nucleotide sequences of members of several carlavirus species have been reported: aconitum latent virus (AcLV, AB051848 [9]), blueberry scorch virus (BlScV, L25658 [5]), daphne virus S (DVS, AJ620300 [16]), hop latent virus (HpLV, AB032469 [11]), lily symptomless virus (LSV, AJ516059 [8]), narcissus common latent virus (NCLV, AM158439 [28]), narcissus symptomless virus (NSV, AM182569 [7]), nerine latent virus

The nucleotide sequence of the virus described in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB245142.

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(NeLV, DQ098905), passiflora latent virus (PLV, DQ455582 [24]), phlox virus S (EF492068), poplar mosaic virus (PopMV, AY505475 [23]), potato virus M (PVM, D14449 [27]), potato virus S (PVS, AJ863509 [19]), shallot latent virus (SLV, AJ292226 [6]), and sweet potato chlorotic fleck virus (SPCFV, AY46142 [3]). Here, we report the complete sequence of a Japanese isolate of CVB (accession number, AB245142) and evaluate the phylogenetic relationships among carlaviruses. As far as we can ascertain, there is no report on transmission of CVB by *Aphis gossypii* despite this aphid species being known to live on chrysanthemum plants [4]. We examined whether CVB is transmitted by *A. gossypii* and compared its transmission efficiency with *M. sanborni*.

The virus was isolated in Showa Village, Japan (CVB-S), and propagated on chrysanthemum cv. Seikoshinnen provided by Seikoen Co., Hiroshima, Japan. CVB-S showed no symptoms during the propagation. After confirmation of the presence of CVB by immunoblotting analysis, total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen). Complementary (c) DNA was synthesized from total RNA using a First-Strand cDNA Synthesis Kit (Amersham) according to the manufacturer's protocol. The reaction was performed using an oligo-dT/*Not* I primer or viral-gene-specific primers. The cDNAs were amplified by PCR with KOD-plus or KOD-Dash polymerase (TOYOBO) using degenerate and/or sequence-specific primer sets. The 5′-end sequence was obtained by 5′ RACE System ver. 2.0 (Invitrogen), modified as follows: the terminal deoxynucleotidyl transferase reactions were performed using dATP or dCTP, and then PCR was carried out with a gene-specific primer and oligo-dT/*Not* I or 5′ RACE Abridged Anchor polyG Primer. To sequence cDNA clones, PCR products were cloned into pUC19 or pGEM-T Easy Vector (Promega).

The CVB-S sequence was compared with those of the other carlaviruses listed above using GENETYX ver. 7.0 (GENETYX Co., Tokyo, Japan). To obtain the multiple alignments and phylogenetic relationships in each ORF and 5′- or 3′-untranslated regions (UTRs), the ClustalW program (DDBJ) was executed. Phylogenetic trees were drawn using the TreeView program [21]. Partial sequences of a large number of CVB isolates also have been reported, however, we compared the CVB-S sequence only with those of Germany (CVB-Ger, S60150 [17]) and India (CVB-Cal, AJ831425, AJ831426, AJ629842, AJ619744, AJ629841 and CVB-Pun, AM493895).

The genomic RNA of CVB-S is 8,990 nucleotides long excluding the poly(A) tail and, like other carlaviruses, contains six ORFs. ORF1 to ORF6 have 6285, 696, 324, 195, 948, and 324 nucleotides, respectively. ORF1 encodes a large protein involved in viral replication, ORF2 to ORF4 encode triple gene block (TGB) proteins (TGBp1, 2 and 3, respectively), ORF5 encodes a coat protein (CP), and ORF6 encodes a hypothetical nucleotide-binding protein. The 5′- and 3′-UTRs were 64 and 84 nucleotides (nts) in length, respectively. The 5′-terminal sequence of the CVB-S RNA is 5′-GGAUAAAC-3′, being almost indistinguishable from those of HpLV, NCLV, NSV, phlox virus S and PopMV, but different from those of other fully sequenced carlaviruses.

The CVB-S ORF1 encodes a polypeptide of 2094 amino acids (aa) with molecular mass (M_r) of 237 kDa, and shares amino acid sequence identities of 40.9–58.9% with other carlaviruses (Table 1). Analysis of the amino acid sequence of CVB-S ORF1 has shown domains conserved in the corresponding protein of other carlaviruses [8, 15, 23]; methyl-transferase (MTR, aa 1–371), papain-like proteinase (P-PRO, aa 993–1183), NTP-binding helicase (HEL, aa 1268–1598), and RNA-dependent RNA-polymerase (POL, aa 1734–2094). The HEL and POL domains have typical motifs of $G_{1276}X_{1277}G_{1278}K_{1279}S_{1280}$ and $G_{1966}D_{1967}D_{1968}$, respectively. Comparison of sequences between CVB-S and other carlaviruses indicated that the POL domain is the most conserved among carlaviruses (Table1).

There is an intergenic region (IGR) 32 nts in length between ORF1 and the TGB ORFs of CVB-S. The TGB ORF2 to ORF4 encode proteins with a M_r of 25.6 kDa (231 aa), 11.5 kDa (107 aa) and 6.9 kDa (64 aa), respectively. The identities of the TGB proteins of CVB-S to those of other carlaviruses are 20.9–56.2%, and to those of other CVB isolates are 78.1–93.4% (Table 1). ORF4 shows the lowest degree of identity of all the ORFs.

Table 1. Comparison of the percentage identities of nucleotide and amino acid sequences between CVB-S and other carlaviruses

virus	5′ UTR	ORF1				ORF2	ORF3	ORF4	ORF5	ORF6	3′ UTR
		Total	MTR	HEL	POL						
	nt	aa	aa	aa	aa	aa	aa	aa	aa	aa	nt
AcLV	57.1	45.0	52.3	43.5	73.7	48.9	50.0	29.9	44.1	44.0	72.5
BlScV	67.7	46.5	50.9	47.3	76.5	53.5	55.1	25.8	44.1	38.0	73.9
DVS	60.9	47.9	57.1	47.1	77.6	47.2	50.8	33.8	55.8	59.5	80.8
HpLV	61.7	45.6	51.8	45.0	72.9	50.4	50.0	27.7	44.3	49.1	72.4
LSV	58.0	43.8	50.4	41.4	74.0	51.5	52.3	28.8	41.9	36.6	64.3
NCLV	61.7	53.1	53.9	45.6	73.7	50.9	49.1	32.7	49.8	52.6	68.8
NSV	67.3	44.2	49.5	41.6	67.3	35.4	39.1	30.8	35.9	43.5	61.9
NeLV	57.9	44.6	56.3	43.7	74.8	41.1	46.4	26.6	43.4	50.5	74.6
PLV	49.0	49.2	50.9	41.8	73.7	50.0	49.5	26.5	47.3	58.1	76.0
Phlox virus S	71.0	58.9	62.9	55.3	81.5	51.3	56.2	38.5	54.6	57.9	76.0
PopMV	60.7	46.4	54.5	49.2	72.9	36.7	50.5	29.2	28.0	37.9	68.0
PVM	64.9	43.5	52.2	45.3	74.7	51.5	47.7	27.5	43.4	44.6	69.4
PVS	64.7	44.1	50.9	43.5	77.0	46.6	48.6	30.3	42.4	43.5	65.0
SLV	61.5	41.4	49.3	40.1	70.4	40.3	46.4	31.3	37.7	38.5	71.8
SPCFV	76.9	40.9	48.5	44.2	65.8	40.9	37.0	20.9	32.0	18.5	62.5
CVB-Ger	–	–	–	–	–	87.5	91.6	81.3	91.8	96.3	95.3
CVB-Cal	–	–	–	–	–	93.1	91.6	78.1	90.2	96.3	–
CVB-Pun	–	58.7	77.4	72.0	94.5	90.9	93.4	82.8	91.4	96.3	86.0

MTR, HEL and POL are the functional domains in ORF1.
– indicates no available sequences.

CVB-S ORF2 also has a HEL domain with the conserved motif $G_{31}X_{32}G_{33}K_{34}S_{35}$. Carlaviral TGB proteins belong to the potex-like group [20]. TGB protein activities have been well characterized for potato virus X: TGBp1 is involved in viral movement and suppression of gene silencing [20, 25]; TGBp2 and TGBp3 are transmembrane proteins which are involved in intracellular movement [14, 22].

Following the 55-nt-long IGR from the TGB, CVB-S ORF5 encodes a CP with a M_r 34.6 kDa (315 aa). ORF5 has only 28.0–55.8% identity with other carlaviruses and 90.2–91.8% identity with other CVB isolates. CVB-S CP detected by immunoblotting showed the expected size (data not shown).

Between ORF5 and ORF6, CVB-S has an IGR of 16 nts. CVB-S ORF6 encodes a polypeptide with a M_r 12.5 kDa (107 aa); this polypeptide shows 18.5–59.5% identity to other carlaviruses and 96.3% identity to other CVB isolates. ORF6 of most carlaviruses encodes about 100 amino acids, but the predicted ORF6 products of BlScV, LSV, NSV, PopMV, and SPCFV are 20–40 aa longer. The ORF6 protein of PVM shows nucleic-acid-binding activities in vitro; it has been speculated that it may facilitate aphid transmission or be involved in host gene transcription, gene silencing and/or viral replication [1, 10]. Viruses of some other genera of the family *Flexiviridae* also encode a putative nucleic-acid-binding protein (NBP) [1]: garlic virus E (GVE, AJ292230), garlic virus X (GVX, U89243) and shallot virus X (ShVX, M97264), belonging to the genus *Allexivirus*, Indian citrus ringspot virus (ICRSV, AF406744), belonging to the genus *Mandarivirus*, and grapevine virus B (GVB, X75448), grapevine virus D (GVD, Y07764) and heracleum latent virus (HLV, X79270), belonging to the genus *Vitivirus*. The alignments of NBP amino acid sequences of the carlaviruses, allexiviruses and mandarivirus listed above show conserved motifs in the central region of their proteins, G-X-S-X_2-A-X_2-R-R-A-X_5-C-X_2-C (Fig. 1); in addition, vitiviruses have a similar motif in the N-terminal

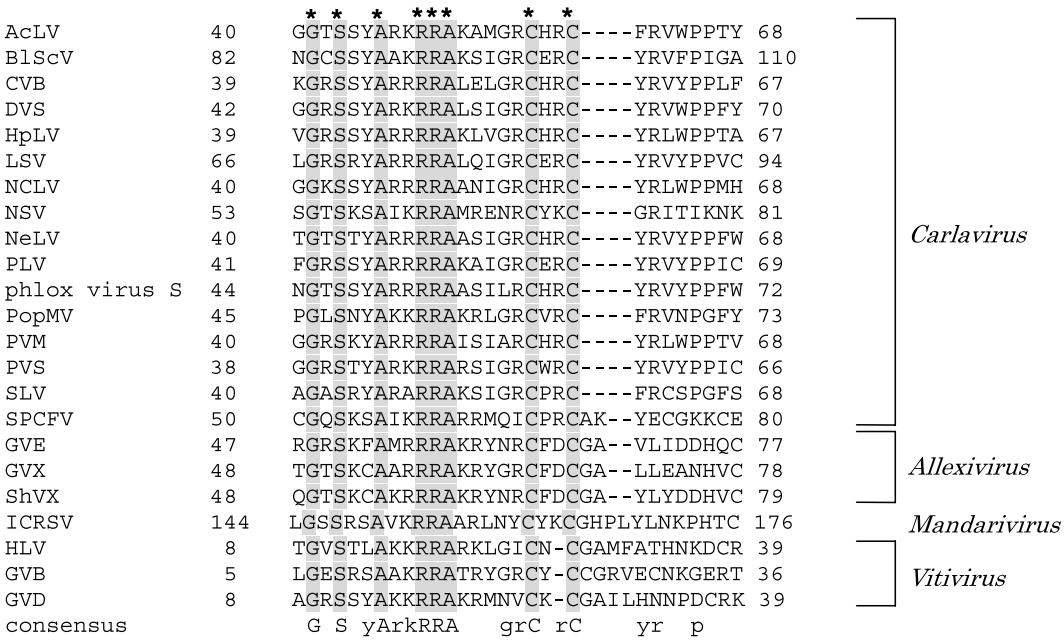

Fig. 1. Multiple alignment of the amino acid sequences of the conserved region within the ORF5 and ORF6 proteins of carlaviruses, allexiviruses, mandarivirus and vitiviruses. The right and left numbers indicate the position of amino acid residues. Invariant residues are indicated by an asterisk. In the consensus sequence, invariant residues are in capital letters, lower-case letters indicate amino acids conserved in at least 12 of the 23 sequences

region. SPCFV is phylogenetically distant from CVB-S; however, this motif is still conserved in SPCFV ORF6, suggesting that it might be important for the function of the ORF6 protein. The role of this protein is not well known, but recently it has been reported that this CVB protein serves as a virus pathogenicity determinant without suppressing RNA silencing [18].

The identity of the amino acid sequences of ORF1 to ORF6 show relatively low identities, whereas nucleotide sequences of the 5′- and 3′-UTR show comparatively high identities of 49.0–76.9% and 61.9–80.8%, respectively, with other carlaviruses (Table 1). These data suggest that phlox virus S is the closest relative of CVB-S in the genus *Carlavirus*.

In an additional experiment, aphid transmission tests were performed with *A. gossypii* and *M. sanborni* using a similar procedure [13] as follows; after starving aphids for 3 h on filter paper, they were fed on infected plants for 5 min, and then these aphids were taken to CVB-free chrysanthemum plants. All tests were performed using 10 aphids/plant. CVB infection was assessed by RT-PCR and immunoblotting. To confirm virus infection, plant sap was used to inoculate seedlings of *Chrysanthemum coronarium* L., a susceptible host for CVB-S. These tests revealed a transmission efficiency of 2/47 (number of CVB-infected chrysanthemums/number of inoculated chrysanthemums) for *M. sanborni* and of 2/24 for *A. gossypi.* These observations corroborate a previous report [12] that aphid transmission of CVB-S is inefficient and provide evidence that *A. gossypii* is able to transmit CVB from chrysanthemum to chrysanthemum.

This is the first report of the complete nucleotide sequence of CVB, and of aphid transmission of CVB by *A. gossypii*. Similar to other carlaviruses [5, 8, 9, 11, 23], the similarities among homologous proteins of carlaviruses were not high (Table 1). Table 1 indicates that genetic diversity is pronounced in TGB regions, particularly in ORF4. Nevertheless, important and functional motifs such

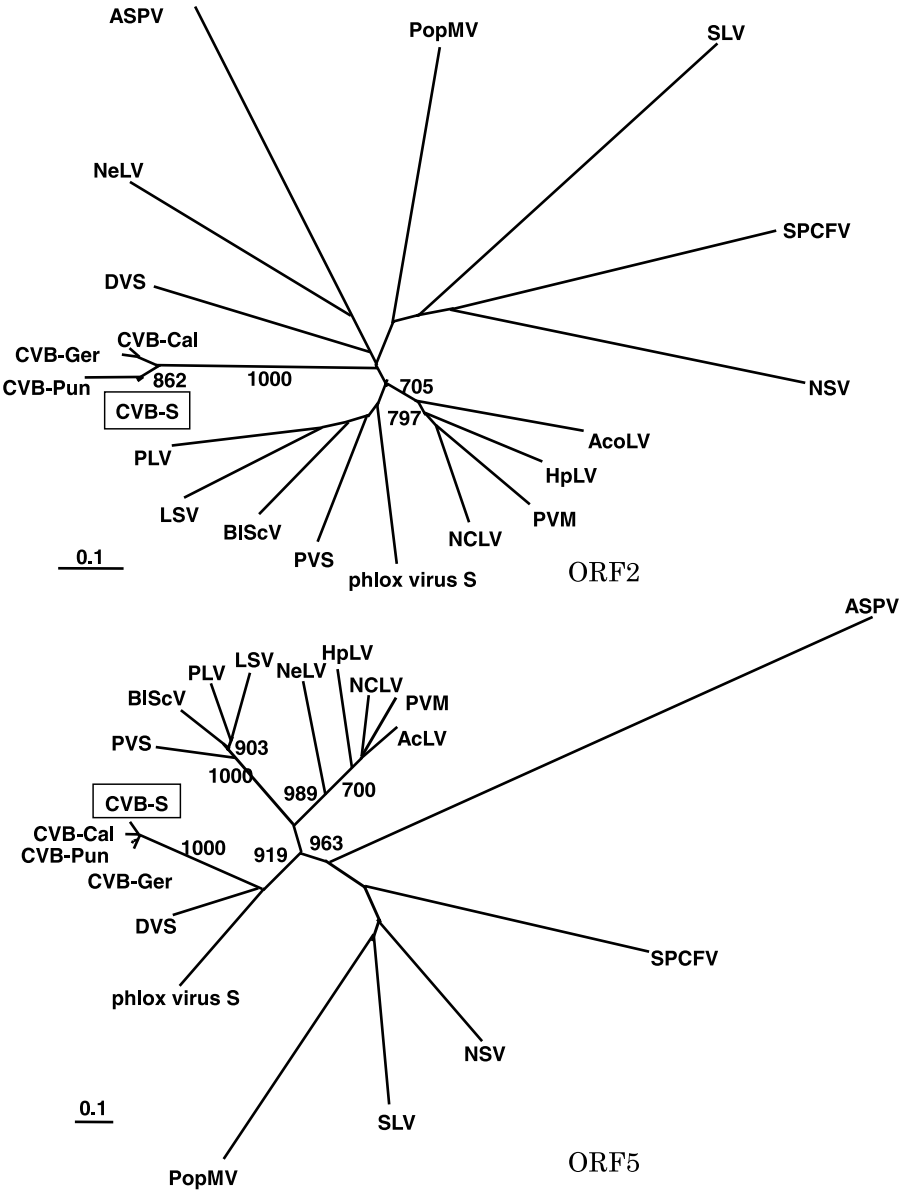

Fig. 2. Phylogenetic relationship of CVB-S and members of the genus *Carlavirus* based on the amino acid sequences of ORF2 and ORF5. Apple stem pitting virus (ASPV), genus *Foveavirus*, was used as an outgroup. Multiple sequence alignments and construction of the phylogenetic trees were generated by ClustalW by the neighbor-joining method. Bars show the ranges of genetic distance, and numbers at nodes indicate the bootstrap values. These data sets were subjected to 1000 replicates

as the HEL and POL domains, the hydropathic profiles of TGBp2 and TGBp3, and the conserved motif of ORF6 exhibit high degrees of sequence conservation among some genera of the family *Flexiviridae*. In CVB isolates, the amino acid identities of TGBp3 (ORF4) are about 80%, while those of CP are about 90%. While this paper was under revision, the sequence of CVB-Pun (AM493895) was recorded. The comparison of ORF1 amino acid sequences of CVB-S and CVB-Pun shows low identity of 58.7% (Table 1). Considering that CVB is widely distributed in the world, the genome of CVB isolates might have a wide diversity.

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References

1. Adams MJ, Accotto GP, Agranovsky AA, Bar-Joseph M, Boscia D, Brunt AA, Candresse T, Coutts RHA, Dolja VV, Falk BW, Foster GD, Gonsalves D, Jelkmann W, Karasev A, Martelli GP, Mawassi M, Milne RG, Minafra A, Namba S, Rowhani A, Vetten HJ, Vishnichenko VK, Wisler GC, Yoshikawa N, Zavriev SK (2005) Family *Flexiviridae*. In: Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA (eds) Virus taxonomy: classification and nomenclature of viruses; Eighth Report of the International Committee on Taxonomy of Viruses, Academic Press, San Diego, pp 1089–1124
2. Adams MJ, Antoniw JF, Bar-Joseph M, Brunt AA, Candresse T, Foster GD, Martelli GP, Milne RG, Zavriev SK, Fauquet CM (2004) The new plant virus family *Flexiviridae* and assessment of molecular criteria for species demarcation. Arch Virol 149: 1045–1060
3. Aritua V, Barg E, Adipala E, Vetten HJ (2007) Sequence analysis of the entire RNA genome of a sweet potato chlorotic fleck virus isolate reveals that it belongs to a distinct carlavirus species. Arch Virol 152: 813–818
4. Blackman RL, Eastop VF (1984) Aphids on the world's crops: An identification guide, 226 pp
5. Cavileer TD, Halpern BT, Lawrence DM, Podleckis EV, Martin RR, Hillman BL (1994) Nucleotide sequence of the carlavirus associated with blueberry scorch and similar diseases. J Gen Virol 75: 711–720
6. Chen J, Chen J, Adams MJ (2001) Molecular characterisation of a complex mixture of viruses in garlic with mosaic symptoms in China. Arch Virol 146: 1841–1853
7. Chen J, Shi YH, Lu YW, Adams MJ, Chen JP (2006) Narcissus symptomless virus: a new carlavirus of daffodils. Arch Virol 151: 2261–2267
8. Choi SA, Ryu KH (2003) The complete nucleotide sequence of the genome RNA of *Lily symptomless virus* and its comparison with that of other carlaviruses. Arch Virol 148: 1943–1955
9. Fuji S, Yamamoto H, Inoue M, Yamashita K, Fukui Y, Furuya H, Naito H (2002) Complete nucleotide sequence of the genomic RNA of Aconitum latent virus (genus Carlavirus) isolated from Delphinium sp. Arch Virol 147: 865–870
10. Gramstat A, Courtpozanis A, Rohde W (1990) The 12 kDa protein of potato virus M displays properties of a nucleic acid-binding regulatory protein. FEBS Lett 276: 34–38
11. Hataya T, Uchino K, Arimoto R, Suda N, Sano T, Shikata E, Uyeda I (2000) Molecular characterization of Hop latent virus and phylogenetic relationships among viruses closely related to carlaviruses. Arch Virol 145: 2503–2524
12. Hollings M (1957) Investigation of chrysanthemum viruses II. Virus B (mild mosaic) and chrysanthemum latent virus. Ann Appl Biol 45: 589–602
13. Hollings M, Stone OM (1972) Chrysanthemum virus B. Descriptions of Plant Viruses No. 110
14. Ju HJ, Samuels TD, Wang YS, Blancaflor E, Payton M, Mitra R, Krishnamurthy K, Nelson RS, Verchot-Lubicz J (2005) The *Potato virus X* TGBp2 movement protein associates with endoplasmic reticulum-derived vesicles during virus infection. Plant Physiol 138: 1877–1895
15. Lawrence DM, Rozanov MN, Hillman BI (1995) Autocatalytic processing of the 223-kDa protein of blueberry scorch carlavirus by a papain-like proteinase. Virology 207: 127–135
16. Lee BY, Min BE, Ha JH, Lee MY, Paek KH, Ryu KH (2006) Genome structure and complete sequence of genomic RNA of *Daphne virus S*. Arch Virol 151: 193–200
17. Levay K, Zavriev S (1991) Nucleotide sequence and gene organization of the 3′-terminal region of chrysanthemum virus B genomic RNA. J Gen Virol 72: 2333–2337
18. Lukhovitskaya NI, Solovyev AG, Koshkina TE, Zavriev SK, Morozov SY (2005) Interaction of the carlavirus cysteine-rich protein with the plant defense system. Mol Biol 39: 785–791
19. Matousek J, Schubert J, Ptacek J, Kozlova P, Dedic P (2005) Complete nucleotide sequence and molecular probing of potato virus S genome. Acta Virol 49: 195–205
20. Morozov SY, Solovyev AG (2003) Triple gene block: modular design of a multifunctional machine for plant virus movement. J Gen Virol 84: 1351–1366
21. Page RD (1996) TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci 12: 357–358
22. Schepetilnikov MV, Manske U, Solovyev AG, Zamyatnin Jr AA, Schiemann J, Morozov SY (2005) The hydrophobic segment of *Potato virus X* TGBp3 is a major determinant of the protein intracellular trafficking. J Gen Virol 86: 2379–2391
23. Smith CM, Campbell MM (2004) Complete nucleotide sequence of the genomic RNA of *Poplar mosaic virus* (Genus *Carlavirus*). Arch Virol 149: 1831–1841
24. Spiegel S, Zeidan M, Sobolev I, Beckelman Y, Holdengreber V, Tam Y, Joseph MB, Lipsker Z, Gera A (2007) The complete nucleotide sequence of Passiflora latent virus and its phylogenetic relationship to other carlaviruses. Arch Virol 152: 181–189
25. Verchot-Lubicz J (2005) A new cell-to-cell transport model for potexviruses. Mol Plant Microbe Interact 18: 283–290
26. Verma N, Sharma A, Ram R, Hallan V, Zaidi AA, Garg ID (2003) Detection, identification and incidence of Chrysanthemum B carlavirus in chrysanthemum in India. Crop Prot 22: 425–429
27. Zavriev SK, Kanyuka KV, Levay KE (1991) The genome organization of potato virus M RNA. J Gen Virol 72: 9–14
28. Zheng HY, Chen J, Adams MJ, Chen JP (2006) Complete nucleotide sequence and affinities of the genomic RNA of Narcissus common latent virus (genus Carlavirus). Arch Virol 151: 1667–1672