Archives of Virology (2019) 164:1931–1935
https://doi.org/10.1007/s00705-019-04261-6

ANNOTATED SEQUENCE RECORD

Complete nucleotide sequence of a new potexvirus, 'Cnidium virus X', isolated from *Cnidium officinale* in Japan

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Received: 1 February 2019 / Accepted: 27 March 2019 / Published online: 22 April 2019

Abstract

A flexuous virus was detected in a *Cnidium officinale* plant in Japan showing mosaic symptoms. The virus was assigned to the genus *Potexvirus* based on analysis of its complete nucleotide sequence. The genomic RNA of the virus was 5,964 nucleotides in length, excluding the 3′-terminal poly(A) tail. It contained five open reading frames (ORFs), consistent with other members of *Potexvirus*. The ORF sequences differ from those of previously reported potexviruses. Phylogenetic analysis indicated that the polymerase of the virus is closely related to that of strawberry mild yellow edge virus; and the CP, to those of both yam virus X and vanilla virus X. We propose that this virus be designated as "cnidium virus X" (CnVX).

Cnidium officinale is a medicinal herb that is used in Asia, mainly China, Korea, and Japan, to treat diseases such as anemia, suppurative skin disease, and gynecological disorders [12]. It has been reported that medicinal plants are infected with many viruses [15, 16], and cnidium vein yellowing virus (CnVYV: family *Secoviridae*) was first detected in *C. officinale* in Korea [23]. Additionally, this virus was also found in *Rehmannia glutinosa* in Japan [21]. In 2017, we collected *C. officinale* samples showing mosaic symptoms from the Hokkaido and Iwate prefectures in northern Japan. Total RNA was extracted from these samples using an RNeasy Plant Mini Kit (QIAGEN, Venlo, The Netherlands) and a reverse transcription (RT)-PCR assay was performed by using genus- universal primer sets [11]. RT-PCR products of the expected sizes were obtained in both the potexvirus and cucumovirus assays. Since this was the first time that a potexvirus had been detected in *C. officinale*, we determined the full-length sequence of this virus. Here, we report the complete nucleotide sequence of a potexvirus infecting *C. officinale*, and based on molecular criteria, designate it as a member of a new potexvirus species.

To determine the complete nucleotide sequence of this virus, a cDNA fragment containing the polymerase motif within ORF 1 was amplified by the above RT-PCR assay using potexvirus universal primers, 'potex1' and 'potex2' (Supplementary Table 1) [8]. The fragment obtained (fragment I) was purified using a GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, England) and cloned using a TOPO TA Cloning Kit for Sequencing (Thermo Fisher Scientific, MA, USA). DNA sequence analysis was performed by the Biotechnology Center at Akita Prefectural University, and the sequences were analyzed using GENETYX-Mac 16.0.7 software (Software Development Co., Tokyo, Japan).

Based on this nucleotide sequence, two specific primers, 'CnV-1R' and 'CnV-1S', were designed to amplify the fragments of the 5′ upstream and 3′ downstream regions, respectively. The 5′ upstream region fragment was amplified using the sense primer 'potex5′-2', which contained a *Not*I site and was designed based on the 5′-terminal conserved sequence of previously published potexviruses [7], and the antisense primer 'CnV-1R'. The 3′ downstream region fragment was also amplified using the sense primer 'CnV-1S' and primer 'M4dT' [3]. RT-PCR was performed according to the manufacturer's protocol (PrimeScript™ II High Fidelity One Step RT-PCR Kit; Takara, Kyoto, Japan) with the following conditions: one cycle of 45 °C for 15 min and 94 °C for 2 min, followed by 35 cycles of 98 °C for 10 s,

Handling Editor: Ioannis E. Tzanetakis.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00705-019-04261-6) contains supplementary material, which is available to authorized users.

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50 °C for 15 s, and 68 °C for 10 s per 1 kb. The amplified fragments, 968 bp (fragment II) and 2,601 bp (fragment III) (Supplementary Fig. S1), were cloned and sequenced as described above. Based on the obtained nucleotide sequence of fragment II, the specific reverse primer 'CnV-2R' was re-designed to amplify the fragment of the 5′ upstream region,

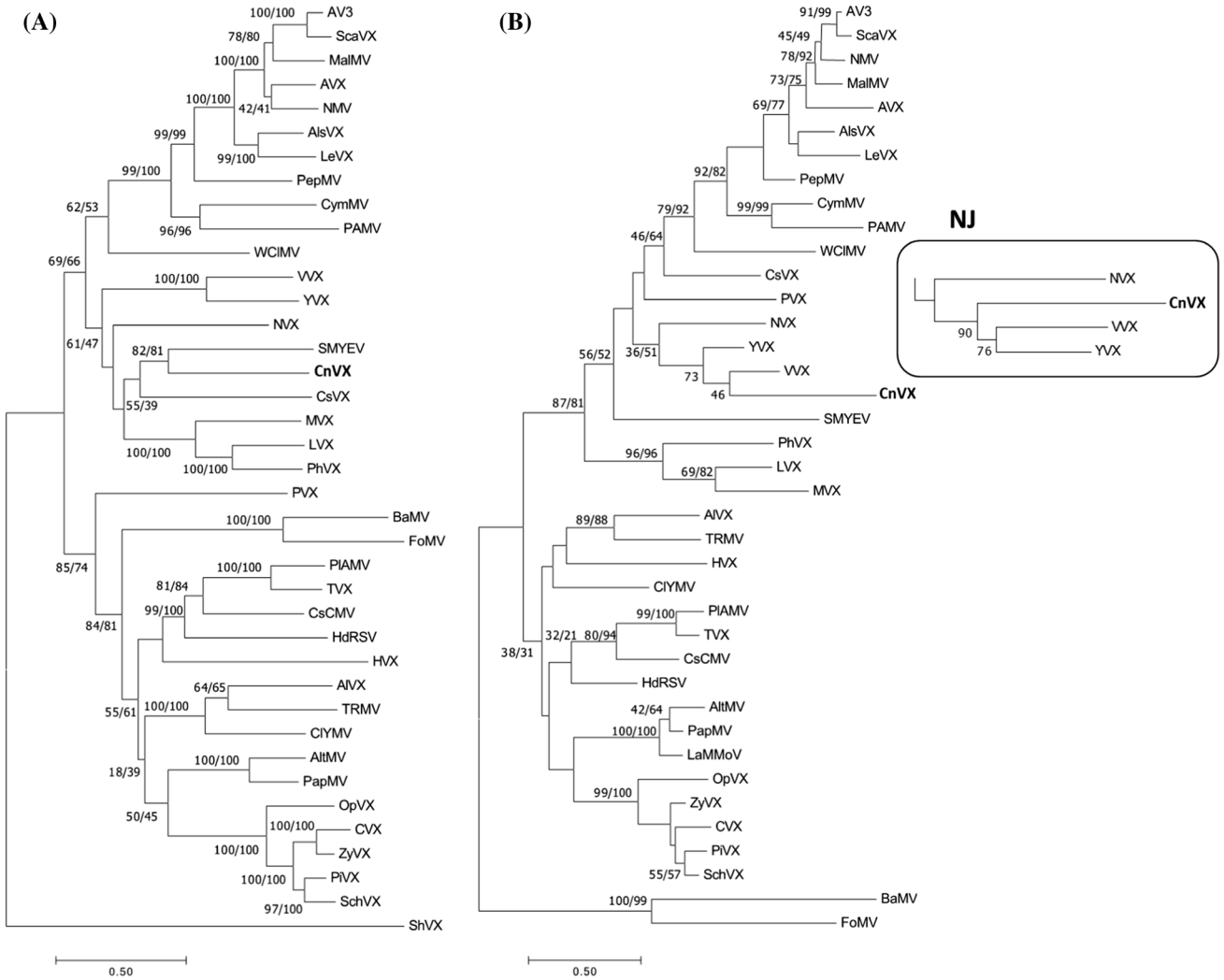

Fig. 1 Phylogenetic analysis of the proposed cnidium virus X and other potexviruses based on published polymerase (A) and coat protein (CP) (B) nucleotide sequences. The maximum likelihood (ML) method [10] was used to construct the phylogenetic trees. The bootstrap values (ML/NJ: 1,000 replications [5]) of identical branches in each tree constructed by the two methods are shown above and below the branches. Branching of the corresponding sequence of shallot virus X (ShVX) was used as the out-group of the ML tree in the polymerase analysis. In the CP analysis, the partially branched tree constructed by the neighbor-joining method (NJ) is shown in an inset. In the ML analysis, the initial tree for the heuristic search was generated automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model [10] and then selecting the topology with a superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.2315)). In the NJ analysis [18], the evolutionary distances were computed using the Poisson correction method [24] and are in units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analysis was conducted using MEGA7 [14]. The following viruses were included in the analysis: actinidia virus X (AVX), allium virus X (AlVX), alstroemeria virus X (AlsVX), alternanthera mosaic virus (AltMV), Asparagus virus 3 (AV3), bamboo mosaic virus (BaMV), cactus virus X (CVX), cassava common mosaic virus (CsCMV), cassava virus X (CsVX), clover yellow mosaic virus (ClYMV), cymbidium mosaic virus (CymMV), foxtail mosaic virus (FoMV), hosta virus X (HVX), hydrangea ringspot virus (HdRSV), langenaria mild mosaic virus (LaMMoV), lettuce virus X (LeVX), lily virus X (LVX), malva mosaic virus (MalMV), mint virus X (MVX), narcissus mosaic virus (NMV), nerine virus X (NVX), opuntia virus X (OVX), papaya mosaic virus (PapMV), pepino mosaic virus (PepMV), phaius virus X (PhVX), pitaya virus X (PiVX), plantago asiatica mosaic virus (PlAMV), potato aucuba mosaic virus (PAMV), potato virus X (PVX), scallion virus X (ScaVX), schlumbergera virus X (SchVX), strawberry mild yellow edge virus (SMYEV), tamus red mosaic virus (TRMV), tulip virus X (TVX), vanilla virus X (VVX), white clover mosaic virus (WClMV), yam virus X (YVX), zygocactus virus X (ZVX), and shallot virus X (ShVX)

Table 1 Nucleotide sequence identity/amino acid sequence identity (%) of cnidium virus X to other potexviruses

Virus	ORF1 (Polymerase)	ORF2 (TGBp1)	ORF3 (TGBp2)	ORF4 (TGBp3)	ORF5 (CP)	GenBank accession number
AVX	55.1/42.3	55.5/33.6	53.9/30.6	54.1/11.2	52.2/32.9	NC028649
AlVX	55.3/39.8	51.2/31.8	52.8/29.7	42.6/26.5	49.8/27.9	FJ670570
AlsVX	57.6/42.2	50.8/30.8	56.4/26.0	48.1/14.2	55.3/36.5	AB206396
AltMV	57.4/34.6	56.0/33.1	53.0/26.0	43.9/21.6	50.7/28.0	AY863024
AV3	58.8/41.9	52.9/25.3	51.1/28.0	40.9/16.6	51.8/35.1	NC010416
BaMV	53.1/32.4	51.5/30.5	56.4/29.7	50.9/**32.0**	45.3/18.5	D26017
CVX	56.3/34.0	53.2/34.0	53.1/37.0	47.3/12.6	50.3/28.4	AF308158
CsCMV	55.0/34.7	49.2/33.1	49.3/29.7	50.2/20.8	55.4/30.4	U23414
CsVX	58.0/53.2	55.0/28.6	54.4/24.4	N.D.	46.6/35.2	NC034375
ClYMV	56.0/43.5	48.1/33.7	51.4/35.6	55.6/25.8	56.8/34.9	D29630
CymMV	56.6/41.4	53.4/26.4	46.3/22.7	45.5/14.4	49.9/31.6	AF016914
FoMV	53.2/33.5	51.0/28.3	53.0/34.6	48.1/18.0	48.7/20.0	AY121833
HVX	56.3/37.4	48.1/33.7	57.0/31.6	49.7/17.4	51.8/30.5	AY181252
HdRSV	55.3/34.9	50.0/27.9	53.1/39.6	45.9/29.5	51.8/31.0	AY707100
LaMMoV	N.D.	48.4/32.2	52.3/31.6	47.4/14.0	50.5/32.3	AB546335
LeVX	56.8/41.8	50.4/31.2	55.2/33.0	51.9/13.6	50.6/31.6	NC010832
LVX	58.0/51.3	49.4/28.9	52.9/37.6	43.2/12.9	53.6/34.0	AJ633822
MalMV	54.2/40.4	52.6/32.3	55.0/27.7	48.6/16.4	49.4/31.8	NC008251
MVX	56.4/49.1	46.8/31.5	52.7/34.6	60.1/12.3	54.0/30.6	AY789138
NMV	57.6/42.4	49.7/29.1	55.5/33.6	50.8/11.9	51.2/33.3	AY225449
NVX	60.0/44.1	49.3/30.2	51.1/29.7	45.3/15.1	55.3/39.2	NC007679
OVX	57.2/34.6	50.5/31.8	54.2/34.6	49.1/26.2	52.9/30.0	AY366209
PapMV	56.4/39.0	50.6/30.4	53.0/30.0	47.2/22.7	52.7/30.2	D13957
PepMV	57.5/42.2	52.4/27.9	51.3/25.0	48.8/26.8	50.5/35.8	AF484251
PhVX	57.1/52.1	47.9/30.9	51.6/36.6	51.6/19.0	50.0/31.5	NC010295
PiVX	56.8/34.3	52.2/34.7	53.1/37.3	58.1/18.1	52.8/30.1	NC024458
PlAMV	55.9/35.1	54.6/31.8	46.8/29.7	44.8/18.7	55.4/31.0	Z21647
PAMV	56.9/38.7	46.4/29.8	52.1/37.0	50.7/18.3	47.4/27.3	S73580
PVX	56.2/38.6	47.4/30.6	52.4/27.7	43.1/22.0	54.8/33.4	X05198
ScaVX	57.9/42.2	50.0/25.8	50.6/33.0	55.4/12.5	52.8/33.7	AJ316085
SchVX	56.7/34.2	49.0/31.3	53.9/**43.0**	49.7/15.2	53.0/27.8	AY366207
SMYEV	58.2/53.7	53.6/**38.4**	55.2/40.0	49.5/16.2	52.5/38.0	D12517
TRMV	55.0/34.5	52.6/32.7	51.9/31.6	54.9/27.4	48.7/27.2	NC016003
TVX	55.8/38.9	51.3/30.0	50.4/28.7	46.4/24.1	49.0/30.5	AB066288
VVX	57.6/48.0	51.3/37.9	53.5/33.3	43.9/24.1	60.2/**51.1**	NC035205
WClMV	59.3/**54.2**	48.5/36.9	60.7/31.0	48.2/28.7	53.5/36.0	X16636
YVX	56.2/42.0	51.1/35.2	54.3/38.6	50.0/21.8	60.1/48.7	NC025252
ZVX	56.2/35.1	48.6/29.0	53.1/41.0	44.9/14.7	51.9/28.1	AY366208
ShVX	52.0/29.7	51.6/31.1	53.2/29.0	51.2/11.6	46.4/27.3	M97264

Values were obtained using the global homology search of the GENETYX-Mac 16.0.7 program. Bold characters indicate the highest percentage of amino acid identity between cnidium virus X and the other potexviruses. N.D., no data: excluded from the analysis because it was not registered in the database. Virus abbreviations are provided in the legend to Figure 1

and the amplified fragment (2,136 bp, fragment IV) was amplified in combination with the sense primer 'WM-5F' [22]. This RT-PCR product was also cloned and sequenced as described above. Finally, to determine precisely the 5′-terminal nucleotide sequence, 5′ RACE was performed using two specific primers, 'CnV5′-1' and 'CnV5′-2', designed from the nucleotide sequence of fragment IV. 5′ RACE was performed according to the manufacturer's protocol (5′/3′ RACE Kit, 2nd Generation; Sigma-Aldrich, MO, USA), and the purified 5′ RACE products were also cloned and

sequenced as described above. The nucleotide sequence of each fragment was aligned and analyzed using GENETYX-Mac 16.0.7 software (Software development Co., Tokyo, Japan). For phylogenetic analysis, the amino acid sequences of the polymerase and coat protein (CP) regions were aligned with sequences representing members of the genus *Potexvirus*, using the multiple sequence alignment tool MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/). Phylogenetic trees were then constructed from the alignment data using the maximum-likelihood (ML) and neighbor-joining (NJ) methods, using MEGA7 software (Fig. 1) [14].

The RNA genome of the virus is composed of 5,964 nucleotides (nt), excluding the poly(A) tail. Four of the five ORFs overlap but ORF1 does not overlap with any other ORFs. The overlapping of ORFs 4 and 5 (Supplementary Fig. S1), like that found in the plantago asiatica mosaic virus (PlAMV) [19], mint virus X (MVX) [20], nerine virus X (NVX) [6], phaius virus X (PhVX) [11] and strawberry mild yellow edge virus (SMYEV) [9], is an unusual arrangement. An extensive database search failed to match the virus detected in *C. officinale* to any other potexvirus sequences. ORF1 (nt 72–3,974) presumably encodes a putative polymerase of 1,300 amino acids (aa) with a calculated M_r of 146,323. ORF 2 (triple gene block [TGB] p1: nt 4,111–4,803), ORF 3 (TGBp2: nt 4,766–5,071) and ORF 4 (TGBp3: nt 5,019–5,258) together constitute a TGB whose organization is conserved in members of the genera *Potexvirus*, *Mandarivirus*, *Carlavirus*, and *Foveavirus*. TGBp1, 2 and 3 encode presumptive polypeptides of 230, 101 and 79 aa, with a calculated M_r of 25,164, 11,035 and 8,722, respectively. ORF 5 (nt 5,080–5,787) encodes a putative CP of 234 aa with a calculated M_r of 25,006.

A comparison of the nucleotide and amino acid sequences of the virus detected in *C. officinale* with those of other potexviruses indicated that the polymerase (ORF1) and CP had the highest sequence similarity to those of white clover mosaic virus (WClMV) [2] and vanilla virus X (VVX), respectively. TGBp1, 2 and 3 were highly similar to the corresponding genes of SMYEV, schlumbergera virus X (SchVX) [13], and bamboo mosaic virus (BaMV) [17], respectively (Table 1). A phylogenetic tree constructed based on the amino acid sequences of the polymerase and CP also indicated that the polymerase of the virus is closely related to that of SMYEV; and the CP, to those of both yam virus X and VVX (Fig. 1).

The polymerase and CP amino acid sequences of the virus detected in *C. officinale* were 32.4–54.2% and 18.5–51.1% identical, respectively, to those of a series of potexviruses (Table 1). According to the molecular criteria for species demarcation established by Adams et al. [1], 40.4–73.3% polymerase and 20.5–79.8% CP amino acid sequence identity, indicates a distinct species, while sequence identity exceeding 88.8 and 74.4%, respectively, indicates that the viruses being compared belong to the same species [1]. The Ninth Report of the International Committee on Taxonomy of Viruses indicates that members of distinct species have less than ~ 72% nucleotide sequence identity or less than 80% amino acid sequence identity between their CP or polymerase genes [4]. Since the highest scores for the polymerase and CP genes were 54.2% (WClMV) and 51.1% (VVX), respectively, these data indicate that the *C. officinale* virus is different from members of any other potexvirus species that have been published previously.

The complete nucleotide sequence of this newly identified potexvirus isolated from *C. officinale* indicates that it is closely related to WClMV and VVX but differs from all other known potexviruses and by molecular criteria must be considered a member of a new species. We propose that the newly isolated virus should be classified as a member of a new potexvirus species and designated as "cnidium virus X" (CnVX).

Nucleotide sequence accession number: The complete genome sequence of cnidium virus X (CnVX) with annotation was deposited in the DDBJ nucleotide sequence database under the accession number LC460456.

Acknowledgements The DNA sequencing was performed at the Biotechnology Center of Akita Prefectural University.

Compliance with ethical standards

Conflict of interest The author declares no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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