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SHORT COMMUNICATION

Genetic diversity of viruses infecting cnidium plants (*Cnidium officinale*) in Japan

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Abstract

Cnidium vein yellowing virus (CnVYV), cnidium virus X (CnVX), cucumber mosaic virus (CMV) and cnidium virus 1 (CnV1) were detected at extremely high levels in *Cnidium officinale* plants showing viral symptoms collected from Iwate and Hokkaido Prefectures, Japan. The complete nucleotide sequence of the newly detected CnVYV and CnV1, and genetic diversity of the cnidium-infecting viruses (CnVYV, CnVX, and CnV1) indicated that South Korean and Japanese cnidium plants had close relationship with each other. All three viruses can infect vegetatively propagated perennials and are vertically transmitted once infection occurs.

Keywords Phylogenetic tree · Cnidium vein yellowing virus · Cnidium virus X · Cucumber mosaic virus · Cnidium virus 1 · DNA sequencing

Cnidium officinale is a perennial herbaceous plant thought to have originated in China. Japanese botanists hypothesize that *C. officinale* was introduced to Japan from China, but *C. officinale* is not included in current lists of Chinese flora [8]. Cnidium plants were introduced to Japan in the mid-1600s, first cultivated in Hokkaido in the middle of the 1800s, and are mainly grown in Hokkaido and Iwate Prefectures at present. *C. officinale* is vegetatively propagated and cultivated from developed rhizomes. Rhizomes have medicinal uses in treating anemia, pyogenic skin diseases, and gynecological disorders in South Korea and Japan, and other Asian countries [3, 13]. Many types of viral infections, such as cnidium vein yellowing virus (CnVYV: a tentative member of the family *Secoviridae*), cnidium virus X (CnVX, genus *Potexvirus*, family *Alphaflexiviridae*), cucumber mosaic virus (CMV: genus *Cucumovirus*, family *Bromoviridae*) and cnidium virus 1 (CnV1: genus *Betanucleorhabdovirus* in the family *Rhabdoviridae*) infections, have been described in plant species growing in South Korea and Japan [2, 5, 6, 11, 14]. In this study, we report the bio-geographical diversity of CnVYV, CnVX, CMV and CnV1 in Japan based on the genetic diversity of these viruses harvested from cnidium

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plants growing in Hokkaido and Iwate Prefectures. The complete nucleotide sequences of CnVYV and CnV1, two newly detected viral species in Japan, are also reported.

In Japan, cnidium leaf samples showing mosaic, yellowing and vein clearing were randomly collected from Yubari, Hokkaido Prefecture (one field, 2022) and Obihiro, Hokkaido Prefecture (seven fields, 2020; three fields, 2022) and Iwate, Iwate Prefecture (three fields, 2021) and Hachimantai, Iwate Prefecture (two fields, 2021), respectively. RNA extraction and the subsequent RT-PCR assay from collected plants or a purified virus preparation were performed using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and a Primescript II High Fidelity One Step RT-PCR Kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol. The complete nucleotide sequence of CnVYV and CnV1 and the coat protein (CP) region of four virus species were amplified using primer sets listed in Table S1 under the following conditions: for the RT-PCR reaction, 1 cycle of 45 °C 15 min and 94 °C 2 min, followed by 35 cycles of 98 °C 10 s, 50 °C 15 s, 68 °C 10 s and finally 68 °C 10 min. After agarose gel electrophoresis, PCR products of the expected sizes were purified using a GFX PCR DNA and gel band purification kit (Cytiva, Chicago, Illinois, USA). To determine the complete nucleotide sequence of CnVYV and CnV1, 5′/3′ RACE was also performed using the 5′/3′ RACE Kit, 2nd Generation (Roche, Basel, Switzerland) or the 5′-Full RACE Core Set (Takara Bio, Shiga, Japan) according to the manufacturer's protocol (Tables S2 and S3). The 3′ RACE of CnV1 was performed after the addition of poly (A) tails with Poly (A) polymerase (Takara Bio, Shiga, Japan; Table S3). These amplified products were sequenced directly or after cloning into the pCR4 TOPO vector (Thermo Fisher Scientific, Carlsbad, CA, USA). The pairwise comparison of amino acid identities between the determined sequences and those of the cognate or related species was calculated using the GENETIX 20.0 program (GENETIX, Tokyo, Japan). Amino acid sequences were aligned using Clustal X version 2.0 [9], and the phylogenetic tree was constructed using the neighbor-joining method in MEGA11 [12]. The reliability of the obtained phylogenetic trees was analyzed by bootstrap analysis with 1000 replications.

The complete nucleotide sequence of CnVYV was determined using a purified virus preparation as described by Fuji et al. [4] with some modifications. Symptomatic leaves exhibiting mosaic and vein yellowing symptoms were collected in Iwate in 2018. Virus-infected leaves were homogenized in 200 ml of 0.5 M phosphate buffer (pH 7.6) with 3% (w/v) urea, 0.1% (v/v) 2-mercaptoethanol and 200 ml of chloroform: carbon tetrachloride (1:1), and the mixture was centrifuged at 5000 × g for 10 min. Twelve g PEG6000 (6% (w/v) and 4 g NaCl were added to the supernatant liquid and stirred on ice for 1 h, followed by centrifugation at 10,000 × g for 15 min. The pellets were suspended in 100 ml of 0.1 M borate buffer (pH 7.8) and 0.5% (v/v) Triton X 100, followed by centrifugation at 37,407 × g on a 20% (w/v) sucrose cushion. The resulting pellets were resuspended in 10 mM borate buffer (pH 7.8), and layered on a 10–40% sucrose gradient centrifuged at 100,251 × g for 2 h. The purified virus particles were resuspended in sterile distilled water and incubated in Proteinase K (1 mg/ml, Takara Bio) for 20 min at 37 °C to extract viral RNA.

The infection rate of CMV in leaves ranged from 9.0 to 54.5%, from 35.7 to 73.6% for CnVX in Hokkaido Prefecture in 2020, from 28.5 to 91.8% for CMV and from 10 to 55.5% for CnVX in Iwate Prefecture in 2021 (Table S4). The infection rate of CnVYV and CnV1 in plant leaves was 100% in the Iwate Prefecture fields investigated in 2021 (Table S4). In 2022, in Hokkaido (Yubari and Obihiro), leaves had CMV infection rates ranging from 7.7 to 53.8%, from 75.0 to 94.7% for CnVX, and 92.3–100% for CnVYV and CnV1 (Table S4).

In cnidium plant samples collected from Hokkaido Prefecture (Obihiro) in 2020 and from Iwate Prefecture (Iwate and Hachimantai) in 2021, the nucleotide and amino acid sequence identities of CMV detected in the plants ranged from 98.1–100% and 98.6–100%, respectively, whereas other CMV isolates were 75.7–98.4% and 82.4–99.0%, respectively. The phylogenetic tree also indicated that these cnidium CMV belong to subgroup IA (Fig. 1). The Japanese CMV population is divided into a major clade and a minor clade composed of viruses detected in Iwate Prefecture. Although CMV has also been detected in cnidium plants in South Korea, the South Korean CMV belongs to subgroup II [10].

In cnidium plant samples collected from Hokkaido Prefecture (Obihiro) in 2020 and from Iwate Prefecture (Iwate and Hachimantai) in 2021, the nucleotide and amino acid sequence identities among CnVX detected in the plants were 93.7–99.5% and 99.5–100%, respectively. A phylogenetic tree was constructed based on the nucleotide sequence of the CP. The phylogenetic tree showed that CnVX could be divided into two clades: clade I, composed of Japanese and South Korean CnVX, and clade II, consisting only of Japanese CnVX (Fig. 2). No significant differences were

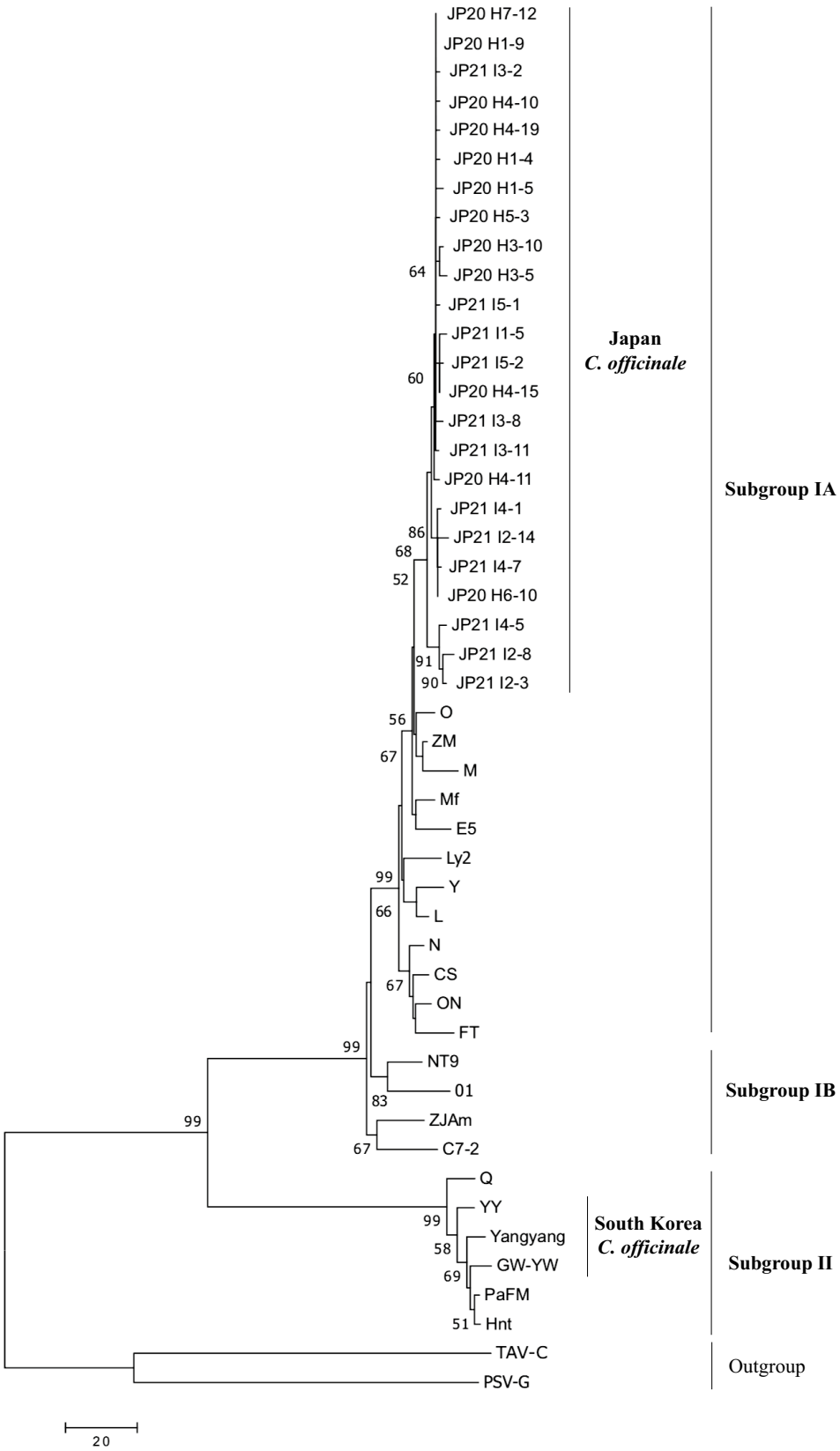


Fig. 1 Phylogenetic analysis of CMV-cnidium accessions associated with previously characterized CMV isolates based on an alignment of CP sequences. The vertical distances are arbitrary, and the horizontal distances are proportional to the calculated mutation distances. The tomato aspermy virus and peanut stunt virus served as the outgroup. Sequences were obtained from GenBank and are identified by the corresponding virus abbreviation

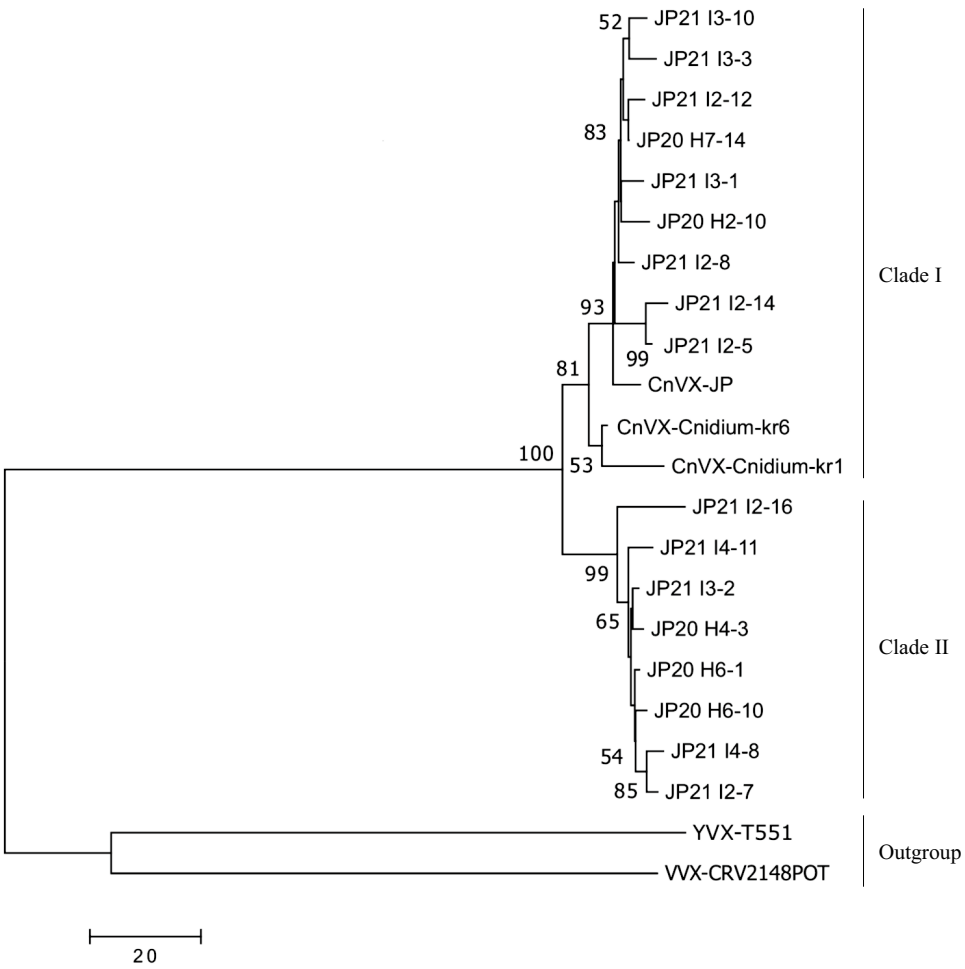


Fig. 2 Phylogenetic analysis of CnVX-JP compared with a previously characterized CnVX from South Korea based on the alignment of CP sequences. The vertical distances are arbitrary, and the horizontal distances are proportional to the calculated mutation distances. Yam virus X and vanilla virus X served as the outgroup. Sequences were obtained from GenBank and are identified by the corresponding virus abbreviation

observed between the Hokkaido and Iwate strains in each clade.

The complete nucleotide sequences of CnVYV RNA-1 and RNA-2 consisted of 7278 and 3737 nucleotides (nt), respectively, excluding the poly A tail (see accession number LC760337 and LC760338). Computer analysis revealed a single long, open reading frame (ORF) in the virus (+) strand. The 3′ and 5′ non-coding regions of CnVYV RNA 1 were 417 nt and 231 nt, respectively. The coding region of CnVYV RNA-1 encoded a 2209 amino acid polypeptide for a 247 kDa protein, identified as a cofactor required for a proteinase (Pro-C, 78 kDa), a putative helicase (HEL, 61 kDa), a genome-linked viral protein (VPg, 3 kDa) a protease (Pro, 29 kDa) and an RNA-dependent RNA polymerase (RdRp, 76 kDa). CnVYV RNA-2 encoded a polypeptide of 993 amino acids for a 109 kDa protein identified as an MP (cell-to-cell movement protein, 39 kDa) and two distinct CPs (LCP [43 kDa] and SCP [26 kDa]). Both polyproteins of CnVYV RNA-1 and RNA-2 are cleaved between serine and glycine residues, presumably by viral proteases, as in CnVYV-South Korea [14]. From plants collected in 2020 from Hokkaido (Obihiro) and in 2021 from Iwate (Iwate and Hachimantai), the nucleotide sequences of a viral large coat protein (LCP) and a small coat protein (SCP) from CnVYV were determined. The nucleotide and amino acid sequences of Japanese isolates of CnVYV were 98.1–100% and 97.4–99.9% identical, respectively. The amino acid identities of LCP and SCP among CnVYV isolates detected from cnidium plants were 97.7–100% and 98.2–100%, respectively. The phylogenetic trees shown in Figs. 3 and 4 indicate that Japanese CnVYV is divided into two clades: a major clade I composed of CnVYV detected from the Hokkaido and Iwate Prefectures and a minor clade consisting of two nucleotide sequence types identified from plants collected in

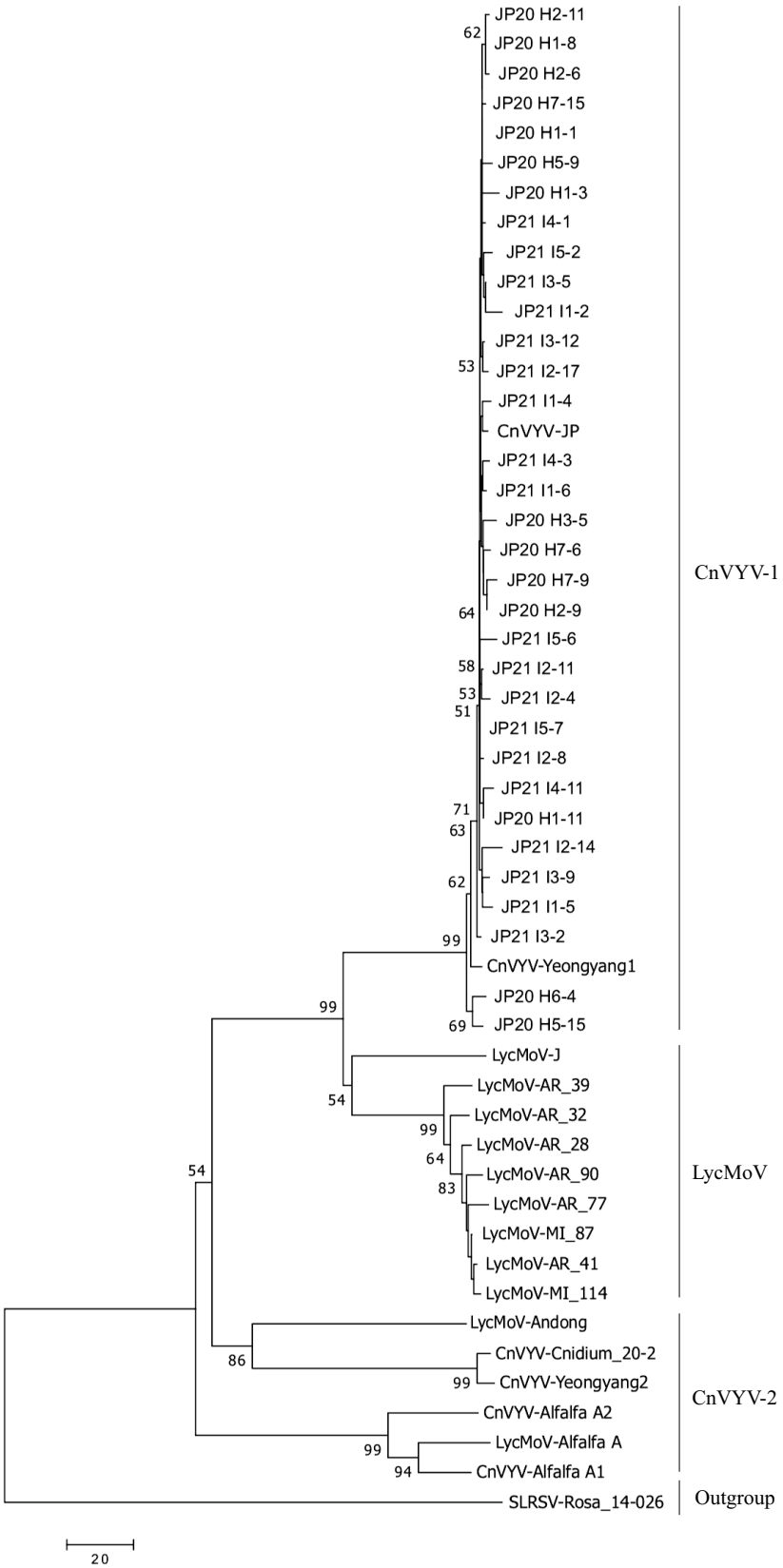


Fig. 3 Phylogenetic analysis of CnVYV-JP compared to previously characterized CnVYV-1, CnVYV-2 and LycMoV based on the alignment of LCP sequences. The vertical distances are arbitrary, and the horizontal distances are proportional to the calculated mutation distances. Strawberry latent ringspot virus served as the outgroup. Sequences were obtained from GenBank and are identified by the corresponding virus abbreviation

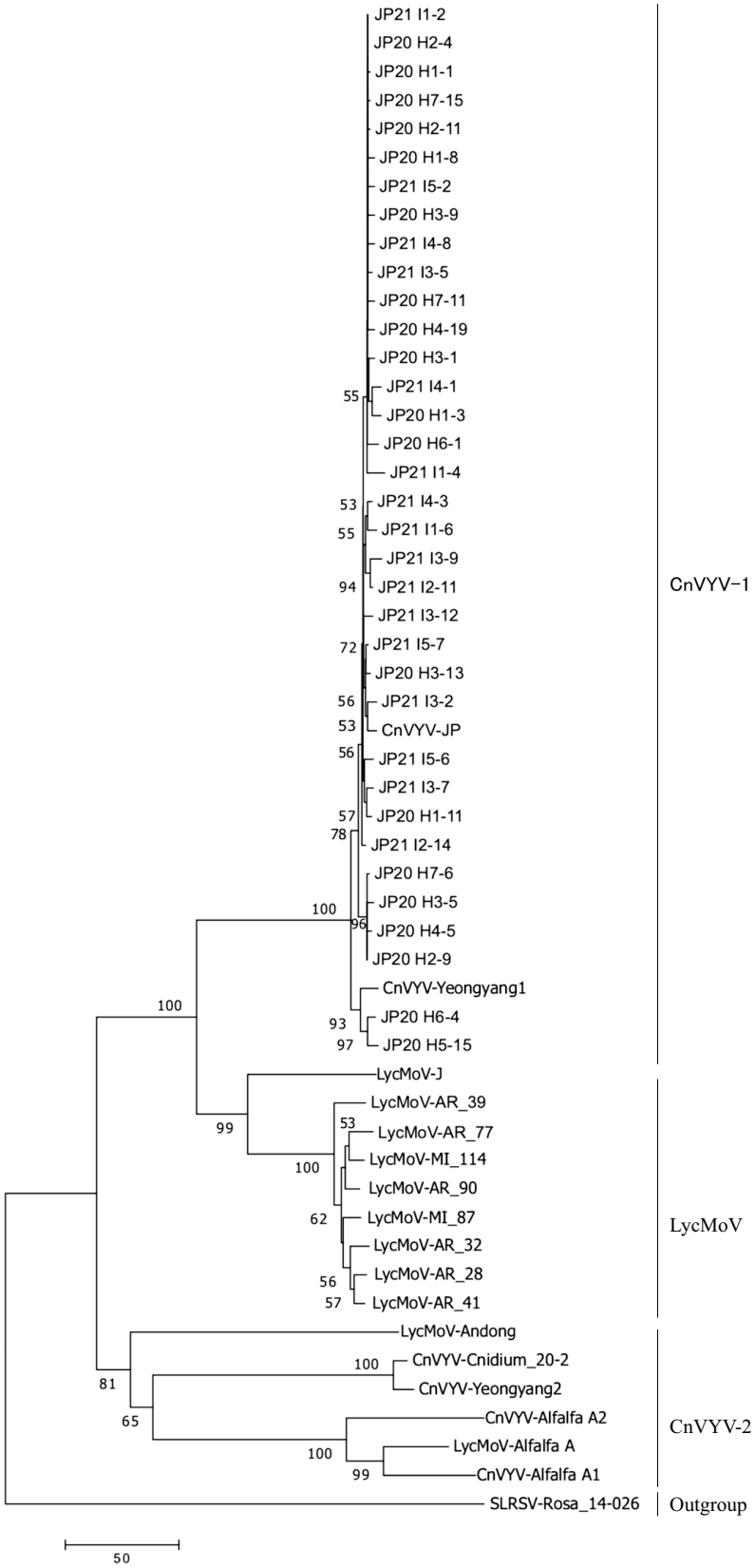


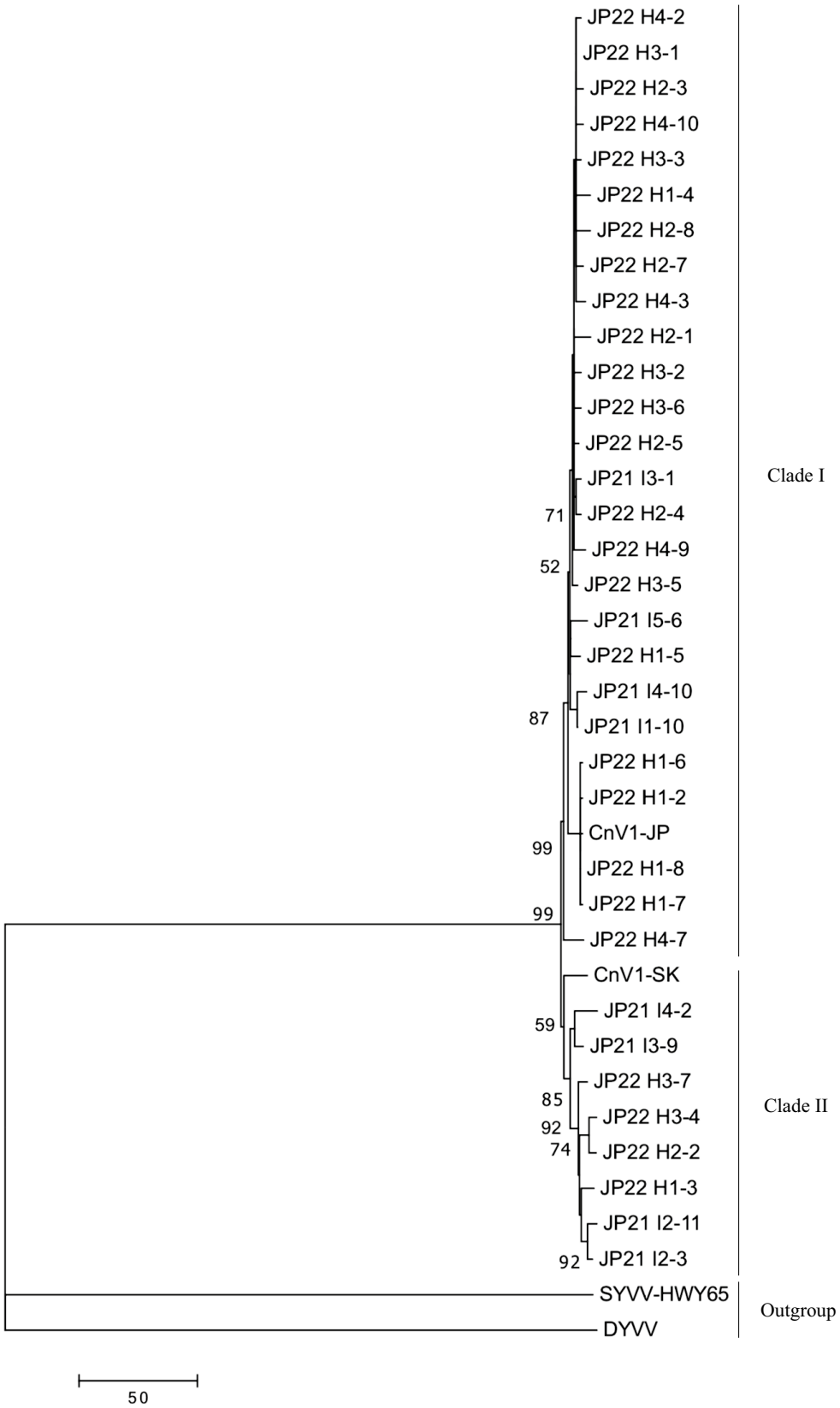
Fig. 4 Phylogenetic analysis of CnVYV-JP with previously characterized CnVYV-1, CnVYV-2 and LycMoV based on the alignment of SCP sequences. The vertical distances are arbitrary, and the horizontal distances are proportional to the calculated mutation distances. Strawberry latent ringspot virus served as the outgroup. Sequences were obtained from GenBank and are identified by the corresponding virus abbreviation

Hokkaido Prefecture. A phylogenetic tree was constructed based on the nucleotide sequence of SCP and LCP, including available sequence information for CnVYV and a closely related species, lychnis mottle virus (LycMoV) from the Genbank database [14, 15]. A phylogenetic tree generated from nucleotide sequences of selected CnVYV and LycMoV isolates from East Asia revealed that the CnVYV Japanese isolates clustered with CnVYV and LycMoV (Figs. 3 and 4). Jiang et al. [7] and Bejerman et al. [1] reported that CnVYV should be classified as two strains (CnVYV-1 and CnVYV-2); thus, CnVYV-2 was tentatively named cnidium vein yellowing-like virus (CnVYLV). If CnVYLV is a distinct species from CnVYV, then co-infection with the two viral species may occur, and CnVYLV may be detectable from Japanese cnidium plants. In this study, however, we did not find CnVYLV associated with these plants. Therefore, we propose that CnVYV, LycMoV and CnVYLV should be classified as identical viral species until the biological characteristics of all three species are reported.

Leaf samples used to detect CnV1 components were detected in virus-infected cnidium plants growing in Yubari, Hokkaido Prefecture in 2022. PCR-amplified cDNA fragments from CnV1-infected samples were cloned and sequenced. Nucleotide sequence analysis revealed that the Yubari isolate shared the highest sequence identity with CnV1, previously reported from South Korea [2]. Thus, this putative new CnV1 was named CnV1-Japan (CnV1-JP). The complete genome sequence of the CnV1-JP isolate consists of 14,003 nt (see accession number LC754523). The 3′ and 5′ non-coding regions of CnV1-JP were 228 nt and 178 nt. The coding regions contained six ORFs, including a nucleocapsid protein [N (nucleotides: 229–1626: 465 amino acids), 51.7 kDa], a phosphoprotein [P (nucleotides: 1815–2816: 333 amino acids), 36.6 kDa], a cell-to-cell movement protein [P3 (nucleotides: 3646–4017: 323 amino acids), 36.1 kDa], a matrix protein [M (nucleotides: 4210–5019: 269 amino acids), 30.1 kDa], a glycoprotein [G (nucleotides: 5267–7219: 650 amino acids), 74.3 kDa] and an RdRp [L (nucleotides: 7460–13816: 2118 amino acids), 240.2 kDa]. The genomic structure of CnV1-JP was the same as that of CnV1-South Korea [2]. Based on the full-length nucleotide sequence, CnV1-Japan was found to be the most closely related to the CnV1-South Korea isolate (97.4% identity at nt level). From cnidium plants collected from Iwate Prefecture (Iwate and Hachimantai) in 2021 and Hokkaido Prefecture (Yubari and Obihiro) in 2022, the nucleotide and amino acid sequence identities among CnV1 isolates detected in cnidium plants were 97.5–100% and 97.8–100%, respectively. The phylogenetic tree constructed, based on the nucleotide sequence of the CP, indicated that the virus CP is closely related to that of CnV1 from South Korea (Fig. 5, [2]). This result suggests that CnV1 infection also occurred after the introduction of cnidium plants to Japan.

The phylogenetic analysis indicates that the four viral species, CMV, CnVX, CnVYV and CnV1, infected vegetatively propagated perennials and suggest vertical transmission after the occurrence of the first infection.

The cnidium plants surveyed in this study are mainly cultivated in Hokkaido and Iwate Prefectures in Japan. The *C. officinale* was introduced to Japan in the mid-1600s and first cultivated in Hokkaido prefecture in the mid-1800s, and then its exported from Japan to South Korea [8]. The *Ligusticum chuanxiong* plants in China, which has a different basal plant, is not distributed in Japan. Although our present results could not indicate the infection route of CnVYV, CnVX and CnV1, these genetic diversities indicated that South Korean and Japanese cnidium plants had close relationship with each other. Phylogenetic analysis of CMV, CnVX, CnVYV and CnV1 enabled deciphering minor clades, suggesting a model for geographical evolution [2, 5, 6, 11, 14]. Our results also suggest that minor mutations continually occurred after cnidium plants were introduced to Japan. In this study, we revealed that almost all Japanese cnidium plants are prone to infection by these closely related viruses; however, the characteristics of these viruses and how these infections affect plant quality have not been elucidated. In areas of cnidium production, it is important to monitor the status of mixed virus infections and disease outbreaks and develop appropriate pest control measures according to the degree of damage and the specific pathogenic virus species. In addition, the effects of virus infection on the quality and yield of cnidium should also be studied in detail to identify infectious virus species and to develop cost-effective control measures. Further investigations will be needed to understand these issues and to incorporate strategies based on such knowledge for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JP22 H1-5
JP21 I4-10
87
JP21 I1-10
JP22 H1-6
JP22 H1-2
CnV1-JP
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JP22 H4-7
CnV1-SK
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JP21 I3-9
JP22 H3-7
85
JP22 H3-4
92
74
JP22 H2-2
JP22 H1-3
JP21 I2-11
92
JP21 I2-3
SYVV-HWY65
DYVV
50
Clade I
Clade II
Outgroup

◂**Fig. 5** Phylogenetic analysis of CnV1-JP with previously characterized CnV1 from South Korea based on an alignment of CP sequences. The vertical distances are arbitrary, and the horizontal distances are proportional to the calculated mutation distances. Datura yellow vein virus and sowthistle yellow vein virus served as the outgroup. Sequences were obtained from GenBank and are identified by the corresponding virus abbreviation

management of the subject viruses in cnidium cultivation in Japan.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13337-023-00835-w.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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

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