# Cryphonectria nitschkei chrysovirus 1 with unique molecular features and a very narrow host range

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Three supplementary figures and nine supplementary tables are available with the online version of this article.

## Highlights

- ►The alphachrysovirus CnCV1 was thoroughly characterized.
- ►Each genomic segment possesses a mini-ORF preceding the major ORF.
- ►CnCV1 was horizontally transmitted by virion transfection, protoplast fusion, and anastomosis.
- ►Some *Cryphonectria* spp. supported CnCV1 replication but others did not.
- ►CnCV1 induced asymptomatic infections in newly established host fungi.


## Summary

Cryphonectria nitschkei chrysovirus 1 (CnCV1), was described earlier from an ascomycetous fungus, *Cryphonectria nitschkei* strain OB5/11, collected in Japan; its partial sequence was reported a decade ago. Complete sequencing of the four genomic dsRNA segments revealed molecular features similar to but distinct from previously reported members of the family *Chrysoviridae*. Unique features include the presence of a mini-cistron preceding the major large open reading frame in each genomic segment. Common features include the presence of CAA repeats in the 5′-untranslated regions and conserved terminal sequences. CnCV1-OB5/11 could be laterally transferred to *C. nitschkei* and its relatives *C. radicalis* and *C. naterciae* via coculturing, virion transfection and protoplast fusion, but not to fungal species other than the three species mentioned above, even within the genus *Cryphonectria,* suggesting a very narrow host range. Phenotypic comparison of a few sets of CnCV1-infected and -free isogenic strains showed symptomless infection in new hosts.


## 1. Introduction

The family *Chrysoviridae* is a relatively newly established group comprising only a single genus, *Chrysovirus*, and seven fungal virus members, as of 2018 (Ghabrial et al., 2018). Upon the recent discovery of many chrysoviruses from diverse hosts including fungi, plants and possibly insects (Darissa et al., 2011; Li et al., 2013; Nerva et al., 2016; Urayama et al., 2012; Zhai et al., 2018), the renaming of genus *Chrysovirus* as *Alphachrysovirus* and addition of the genus *Betachrysovirus* have been approved by the International Committee of Taxonomy of Viruses (Kotta-Loizou et al., 2020). The first one comprises fungal and plant viruses with either tri- or tetra-segmented dsRNA genomes (alphachrysoviruses), while the second one includes largely fungal viruses with 4 to 7 dsRNA genomic segments (betachrysoviruses). Typical alphachrysoviruses have a tetra-segmented dsRNA genome, terminal genome sequences conserved strictly among the four segments, form spherical particles 40 nm in diameter (Caston et al., 2013; Luque et al., 2014), and lead to asymptomatic infections in most cases in fungal hosts (Ghabrial, 2008). The genomic dsRNA segments designated as dsRNA1 to dsRNA4 are generally monosistronic and encode RNA-directed RNA polymerase (RdRP), capsid protein (CP), plus p3 and p4 with unknown function. Among these

viral proteins, RdRP is the best conserved in all chrysoviruses, while no or little significant sequence similarity is found in other proteins across the genera or often within single genera. Some betachrysoviruses infecting ascomycetes induce macro- and microscopic alterations in their fungal host phenotypes, as exemplified by Magnaporthe oryzae chrysovirus 1 (MoCV1) (Higashiura et al., 2019; Urayama et al., 2010; Urayama et al., 2016; Urayama et al., 2014b), Fusarium graminearum mycovirus-China 9 (FgV-ch9) (Bormann et al., 2018), Colletotrichum fructicola chrysovirus 1 (Zhai et al., 2018) and Alternaria alternata chrysovirus 1 (Okada et al., 2018). Botryosphaeria dothidea chrysovirus 1 and the coinfecting partitivirus (a dsRNA virus) are associated with hypovirulence (Wang et al., 2014).

Recent interesting findings about chrysoviruses include the identification of dsRNA4 of a betachrysovirus, MoCV1, as affecting phenotype of a broad range of fungi from their natural filamentous host fungi (*Magnaporthe oryzae*) to a model organism, i.e. the ascomycetous yeast *Saccharomyces cerevisiae* (Higashiura et al., 2019; Urayama et al., 2012), and the pathogenic basidiomycete fungus *Cryptococcus neoformans* (Urayama et al., 2014a). A protein with a similar symptom determining role was identified in another betachrysovirus, FgV-ch9, in which the viral structural protein coded on dsRNA segment 3 (p3) transcriptionally downregulates a host gene, *vr1* (virus response 1), and leads to the induction of virus-like symptoms (Bormann et al., 2018). Caston and colleagues described the near-atomic 3D structure of chrysovirus particles with approximately 40 nm in diameter for representative alphachrysoviruses, Penicillium chrysogenum virus (PcV) and Cryphonectria nitschkei chrysovirus 1 (CnCV1), that entails a *T*=1 symmetrical lattice (Gomez-Blanco et al., 2012; Luque et al., 2014). The capsid is composed of 60 molecules of CP with a duplicated alpha-helix-rich two domain rather than 60 homo- or hetero-dimers of CP, as observed for other dsRNA fungal viruses. In this regard, betachrysoviruses are differentiated from alphachrysoviruses because betachrysoviruses appear to have two distinct CP components (Higashiura et al., 2019; Urayama et al., 2012; Urayama et al., 2014b), as in the case for other fungal dsRNA virus, Rosellinia necatrix quadrivirus (a quadripartite virus) (Lin et al., 2012; Lin et al., 2013; Luque et al., 2016; Mata et al., 2017) and botybirnaviruses (a bipartite virus) (Liu et al., 2015; Shamsi et al., 2019; Wu et al., 2012).

In contrast to the growing database of chrysovirus genome sequences (Kotta-Loizou et al., 2020) and advances in chrysovirus structural research (Caston et al., 2006; Gomez-Blanco et al., 2012; Luque et al., 2014), little is still known about the biology of chrysoviruses in general. One of the main reasons is the lack of established experimental virus introduction protocols. Transformation with infectious cDNAs (Choi and Nuss, 1992; Sasaki et al., 2002; Zhang et al., 2016), transfection with virions (Chiba et al., 2013b, 2016; Chiba and Suzuki, 2015; Kanematsu et al., 2010; Kondo et

al., 2013; Salaipeth et al., 2014; Xie et al., 2016) or infectious transcripts (Chen et al., 1996; Wang et al., 2020), protoplast fusion (Lee et al., 2011; Shahi et al., 2019c) has allowed for the expansion of host ranges of some fungal viruses other than chrysoviruses. For example, relatively wide host ranges have been recognized for partitiviruses and totiviruses (dsRNA viruses) (Chiba et al., 2013b, 2016; Xie et al., 2016), while a relatively narrow host range was documented for a mitochondrially replicating mitovirus (a fungal ssRNA virus) (Shahi et al., 2019b). Natural interspecies horizontal transfer of an unassigned dsRNA virus, a hypovirus (a fungal ssRNA virus), and mitoviruses was reported between closely related fungal species (Liu et al., 2003; Vainio et al., 2011) and also between distantly related fungal species (Deng et al., 2003; Wu et al., 2007). In contrast, a gemycirculavirus (a single-stranded DNA virus), Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1 (SsHADV-1), was shown to be unable to infect *Botrytis cinerea*, a fungus closely related to its natural host (Yu et al., 2010). However, no information on chrysovirus host ranges is available. Moreover, a cause-and-effect relationship has seldom been established between chrysoviruses and their hosts.

*Cryphonectria parsitica* and *C. nitschkei* (Diaporthales, Ascomycota) are sympatric on chestnut trees (genus *Castanea*) and closely related species (Gryzenhout et al., 2006; Liu et al., 2007). *C. nitschkei* has not been extensively or intensively studied relative to *C. parasitica*. *C. parasitica* is destructive to susceptible chestnut trees (such as the American and European chestnuts, *Castanea dentata* and *Ca. sativa*, respectively), while *C. nitschkei* (also known as *C. japonica*) is not (Dennert et al., 2020). Extensive surveys (screened for virus dsRNA) of these fungi, particularly *C. parasitica*, have been conducted and showed certain percentages, i.e. 1% to 28% for *C. parasitica* (Liu et al., 2007; Park et al., 2008; Peever et al., 1997; Peever et al., 1998) and 27% for *C. nitschkei*, of field isolates carrying viruses. In contrast to many of well-characterized viruses of *C. parasitica* (Hillman and Suzuki, 2004), only a few viruses were reported from *C. nitschkei*: Cryphonectria nitschkei chrysovirus 1 (CnCV1), and two other unidentified viruses (Liu et al., 2007). For CnCV1, two strains (CnCV1/BS122 and CnCV1/OB5-11) were isolated from *C. nitschkei* in geographically different locations, i.e. Japan (OB5-11) and South Korea (BS122) (Kim et al., 2009; Kim et al., 2010; Liu et al., 2007). Some of their properties were reported earlier, which include partial genome sequences, biological effects of virus infection on the host (Kim et al., 2009; Kim et al., 2010; Liu et al., 2007), and particle morphology (Luque et al., 2014). Here, we report the complete genomic sequence of CnCV1/OB5-11 with unusual molecular features. This virus induces asymptomatic infection in *C. nitschkei*, and two closely related *Cryphonectria* species, *C. naterciae* and *C. radicalis* (Braganca et al., 2011; Hoegger et al., 2002), but is non-infective to *C. parasitica* or another species, *Valsa ceratosperma* (Diaporthales, Ascomycota, the pathogenic fungus of apple canker), outside the genus *Cryphonectria*.

## 2. Materials and Methods

### *2-1 Fungal strains*

*C. nitschkei* strains OB5-11 (MYA-4105), originally isolated by Milgroom and colleagues from Jisse and Obuse, Kumamoto Prefecture, Japan (Liu et al., 2007), LFP-E24 (MAFF 410076) and E16 (MAFF 410155) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and the Ministry of Agriculture, Forestry, and Fisheries, Japan (MAFF). *C. parasitica* strains GH2-6, 3K/87, EP155, EP155 RNA silencing deficient mutant Δ*dcl2* (Crouch et al., 2020; Segers et al., 2007; Smart et al., 1999) and *V. ceratosperma* strain AVC53(Sasaki et al., 2002) were generously provided by Drs. Bradley I Hillman (Department of Plant Biology and Pathology, Rutgers University), Donald L. Nuss (Institute for Bioscience and Biotechnology Research, University of Maryland) and Satoko Kanematsu (Institute of Fruit Tree Science, National Agriculture and Food Research Organization). *C. radicalis* strains ph1113 (WSL M2269) and DR1 (WSL 4733) (Hoegger et al., 2002) and one strain of *C. naterciae* (C0754) (C. Cornejo and D. Rigling et al., unpublished results) belong to the collection of Dr. Rigling Laboratory at WSL Swiss Federal Research Institute. These fungal strains were identified by phylogenetic analysis with the internal transcribed spacer sequences (Supplementary Fig. 1) (see below).

Fungal strains were cultured on Difco potato dextrose agar (PDA, Becton, Dickinson and Co.) or PDA containing 40 μg/ml hygromycin (PDA-Hyg). All fungal strains used in this study are listed in Table 1.

### *2-2 Sequence determination of CnCV1*

A CnCV1 strain isolated from *C. nitschkei* OB5-11 (ATCC MYA-4105) was partially characterized by Liu et al. (Liu et al., 2007). Partial sequences of the four genomic dsRNA segments (DQ865185-DQ865189), spanning 22%, 86%, 26%, and 18% of the entire length, respectively, were determined. Based on these sequences, we designed primer sets for 3′-RNA ligase mediated rapid amplification of cDNA ends (3′-RLM-RACE). A purified dsRNA fraction of *C. nitschkei* strain OB5-11 was subjected to RACE analysis. Amplified DNA fragments were purified by gel excision and directly sequenced, then all 5′- and 3′-terminal sequences were determined by Sanger sequencing. We further performed RT-PCR to determine internal sequences between the RACE products. The four complete dsRNA segments were designated dsRNA1 to dsRNA4 in the order of length from long to short. The

nucleotide sequences of dsRNA1-4 are deposited in the GenBank/DDBJ/EMBL database as accession numbers LC062729-LC062732, respectively. The detailed methods for dsRNA purification, 3′-RLM-RACE, RT-PCR and sequencing are as described previously (Chiba et al., 2009). Oligonucleotides used in RACE and gap-filling RT-PCR are listed in Supplementary Table S1.

*2-3 Sequence and phylogenetic analyses*

The complete CnCV1 genome sequence was subjected to computational analyses, using sequence processing software. Initiation and termination codon detections and open reading frame (ORF) predictions were performed with GENETX ver. 19 (GENETYX, Tokyo). Sequence similarities were calculated using the BLAST program available from NCBI (nucleotide or protein collection) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide (nt) and amino acid (aa) sequence alignments were obtained with MAFFT ver. 7.471 and used for sequence comparisons or phylogenetic analysis. With the gap-stripped alignment (30% gap tolerance in Gap Strip/Squeeze v2.1.0), a phylogenetic tree was constructed by the maximum-likelihood (ML) method as previously described (Chiba et al., 2013a). Briefly, a cleaned alignment in the Phylip|Phylip4 format was subjected to PhyML 3.0 and analyzed with automated model selection (LG +G+I+F) by SMS, SPR-type tree improvement, and aLRT SH-like fast likelihood-based method (Guindon et al., 2010; Lefort et al., 2017). The phylogenetic tree was drawn with FigTree v.1.4.3 and shown.

The internal transcribed spacer (ITS) region (ITS1, 5.8S rRNA gene, and ITS2) was amplified by PCR using the primer set ITS1-a and ITS1-b (Table S1) and sequenced after cloning into pGEM-T easy (Promega, Madison, WI, USA). The ITS sequences obtained from the fungal strains listed in Table 1 were aligned with MAFFT ver. 7. The conserved sites (451 bases) were used for the tree construction using the neighbor-joining algorithm (Saitou and Nei, 1987) with 1000 bootstrap replicates for statistical support. The tree was refined using FigTree.

*2-4 Protoplast and hyphal fusions*

Protoplasts were prepared from fungal mycelia cultured in potato dextrose agar broth (PDB, Becton, Dickinson and Co.) liquid media according to the method of Eusebio-Cope and Suzuki (Eusebio-Cope and Suzuki, 2015). A similar numbers of protoplasts, approximately $1\times10^7$ were fused in the presence of polyethylene glycol as described by Shahi et al. (Shahi et al., 2019c) in which the recipient strain had been transformed with a hygromycin resistant gene (hygromycin B phosphotransferase) as a selection marker. Cryphonectria hypovirus 1 (CHV1, the prototype of genus *Hypovirus*) was introduced into strain OB5/11 by transformation by plasmid pXH9, which contains the CHV1-EP713

full length cDNA (Choi and Nuss, 1992), and obtained a double infectant (OB5-11/CnCV1/CHV1) after repeated co-culture. After protoplast fusion, subcultures obtained on PDA plates containing hygromycin B (80 μg/ml) were tested for virus infection by a simplified RT-PCR analysis using CnCV1-specific primers (Aulia et al., 2019; Urayama et al., 2015).

Co-culture was also done between different strains in the presence of $ZnCl_2$, which is assumed to impair vegetative incompatibility reactions and enhance horizontal virus transmission, as described Ikeda et al. (Ikeda et al., 2013). The $ZnCl_2$ concentration was adjusted to 0.25, 0.5, 1.0, 1.25, or 1.5 mM.

*2-5 Virion purification and transfection*

Virus particles were prepared by the method of Chiba et al. (Chiba et al., 2016) using differential centrifugation followed by sucrose /cesium chloride equilibrium density gradient centrifugation. Purified particles were stained with an EM stain (EM stainer an alternative for uranyl acetate, Nissin EM Co., Tokyo, Japan) (Nakakoshi et al., 2011). Stained particle samples were observed by electron microscopy (Hitachi model H-7650 transmission electron microscope, Tokyo, Japan). Purified proteins were loaded for 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the gel was stained with Rapid Stain CBB Kit (Nacalai Tesque Inc., Kyoto, Japan).

Virion preparation was also used to transfect protoplasts of virus-free fungal strains of different fungal species listed in Table 1 according to the method of Chiba et al. (Chiba et al., 2016).

## 3. Results

*3-1 Unusual and usual molecular properties of the CnCV1 genome*

The currently available genomic sequences of all the segments of CnCV1/OB5-11 and some segments of CnCV1/BS122 are incomplete (Kim et al., 2010; Liu et al., 2007). This is obvious from comparison of the terminal untranslated regions with previously characterized, established chrysoviruses that have the terminal sequences conserved at both ends of all the genomic segments (Jiang and Ghabrial, 2004; Kotta-Loizou et al., 2020).

Genomic dsRNAs extracted from fungal strain OB5-11 were subjected to Sanger sequencing after converted to cDNA and four complete genomic sequences were obtained, designated as dsRNA1 to dsRNA4 in decreasing order of size (Fig. 1A). The largest dsRNA (dsRNA1, 3,411 bp) encodes a putative RdRP, while the smallest dsRNA (dsRNA4, 3086 bp) encodes a putative CP; this is atypical

for chrysoviruses because others generally have the second largest dsRNA as the CP-coding segment (Kotta-Loizou et al., 2020). DsRNA2 (3,159 bp) and dsRNA3 (3,119 bp) encode proteins of unknown function, a putative cysteine protease containing the core of the ovarian tumor gene-like superfamily domain (deubiquitinating enzymes, OTU-Pro) and a replicase-associated protein (a protein sharing low sequence similarity to chrysoviral RdRP at the N-proximal portion, Rep-Assoc) (Liu et al., 2012), respectively. Chrysoviral Rep-Assoc proteins and RdRPs contain the Phytoreovirus S7-like sequence with a putative P-loop nucleotide triphosphate hydrase (NTPase) domain (Pathak et al., 2014). All CnCV1 dsRNA segments but not dsRNA1 possess small ORFs (sORFs), sORF2, sORF3, and sORF4 in the 5′-proximal regions that would encode small proteins of 61 to 227 amino acids (Fig. 1A).

BLASTP analysis was conducted using the deduced amino acid sequence of the major CnCV1 proteins (Supplementary Tables S2 to S5). The search with CnCV1 OTU-Pro, Rep-Assoc and CP as a query detected counterpart proteins encoded by other chrysoviruses (Supplementary Tables S3 to S5). The search with CnCV1 RdRP as a query detected counterpart RdRPs, and in addition with very weak e-values, Rep-Assoc proteins of some chrysoviruses were identified (Supplementary Tables S2). A similar trend was found for CnCV1 Rep-Assoc protein; for example, RdRPs of Anthurium mosaic-associated virus (accession ACU11563, e-value 0.020) and Zea mays chrysovirus 1 (accession AYD75751, e-value 0.026) were detected by a BLASTP search (Supplementary Table S3). These results support the statement of Liu et al. (Liu et al., 2007) that the replicase-associated domain is present in the N-proximal region of those hypothetical proteins homologous to Rep-Assoc.

The 5′- and 3′-terminal ends were well-conserved among the four segments: the strictly conserved stretch in coding strands is 5′-UGAUAAAAA-----UUUACUACU-3′ (Fig. 1B). These terminal sequences are broadly shared by known chrysoviruses, as reported earlier by Cao *et al*. (Cao et al., 2011), and the corresponding regions in the Verticillium dahliae chrysovirus 1 (VdCV1, an alphachrysovirus) genome are identical. The 3′-untranlated regions (UTRs) of the CnCV1 genome arte commonly short (35-60 nt), but the 5′-UTRs vary in length (232-807 nt) (Fig. 1). Interestingly, relatively small ORFs were predicted at the 5′-UTRs of dsRNA2, dsRNA3 and dsRNA4 (sORF2, 3 and 4) that may be expressed (Fig. 1A). In the case of dsRNA1, an internal ribosomal entry site (IRES)-mediated translation initiation appeared to occur at the second AUG codon (positions 233-235) (Chiba et al., 2018). Alternatively, initiation at the first in-frame AUG codon (positions 50-52) by an unknown mechanism would expand the coding capacity from 1047 to 1101 amino acids (Fig. 1). BLASTP analyses of predicted proteins of these mini-ORFs showed that the sORF4 protein shares moderate or very faint sequence similarity with the N-terminal regions of the putative nucleocapsid protein of Plasmopara viticola mymonovirus 1 (a fungal mononegavirus, family *Mymonaviridae*) (e-value <3e-18) and RdRPs of a phytoreovirus (e-value <0.022) and an alphachrysovirus (e-value

<5.6); it may also involve in the S7-like domain similar to other chrysoviral proteins as mentioned above (Supplementary Tables S7). Other sORF proteins revealed no hits or very faint matches (e-values over 0.11) to non-viral proteins such as a bacterial L-rhamnose/proton symporter RhaT (against sORF3 protein) (Supplemental Table S6). These extra-coding capacities enlarged the size of segments dsRNA2 and dsRNA3, resulting in larger sized segments than the CP-coding segment, dsRNA4. Moreover, less-pronounced characteristics of a chrysovirus were found in the CnCV1 5′-UTRs: a conserved box is misaligned and the following CAA rich regions compare poorly to other chrysoviruses (Fig. 1B). The presence of condensed CAA upstream of OTU-Pro and CP ORFs is somewhat suggestive of a translation enhancer, as CAA repeats are considered to be a translational enhancer (Jiang and Ghabrial, 2004). Overall, CnCV1 has unique genomic features when compared with other chrysoviruses.

The molecular features distinguishing CnCV1 from other known chrysoviruses include the presence of relatively small ORFs non-overlapping or partially overlapping with the main large ORFs.

*3-2 Phylogenetic analysis of chrysoviruses*

The evolutionary relationship of CnCV1 with chrysoviruses was reassessed based on the amino acid sequence alignment with chrysoviral RdRps. The analysis confirmed the placement of CnCV1 into the genus *Alphachysovirus* with the closest relationship with VdCV1, and a distant association between CnCV1 and members of the independent genus *Betachysovirus* in *Chrysoviridae* such as MoCV1 and FgV-Ch9 (Fig. 2). This trend was well-supported by sequence similarity levels detected with a BLASTP search with the four major proteins encoded by CnCV1 dsRNA1-dsRNA4 (Supplementary Table S2-S5). The viruses used in this phylogenetic analysis are listed in Supplementary Table S8.

*3-3 Virion morphology and components*

Electron microscopy of purified CnCV1 preparations showed spherical virus particles ~40 nm in diameter (Fig. 3A). A similar agarose gel dsRNA banding pattern was observed between purified dsRNA fractions obtained from virion preparations and directly from infected mycelia (Fig. 3B). SDS-PAGE analysis showed that purified preparations contained a major protein band of 130 kDa along with at least two minor smaller protein bands of 70 kDa and 55 kDa, both of which may be degradation products of the major protein (Fig. 3C). These dsRNAs and proteins were not detectable in CnCV1-free Japanese *C. nitschkei* strain E16 (Fig. 2B and C).

*3-4 A narrow host range of CnCV1*

To answer how widely CnCV1 can infect, we took three approaches to transferring the virus using a total of 10 fungal strains belonging to four species within and outside of the family Cryphonectriaceae (Table 2 and Supplementary Fig. S1). Two lateral transfer methods, i.e. virion transfection (Chiba et al., 2009) and protoplast fusion (Shahi et al., 2019b), involved protoplast preparation, while hyphal anastomosis by coculturing in the presence of zinc compounds (such as $ZnCl_2$) (Ikeda et al., 2013) did not. Firstly, a recently established protoplast fusion technique was employed using the CnCV1-infected *C. nitschkei* strain OB5/11 as a donor and each of the eight fungal strains transformed with the hygromycin resistance gene (HygR) as a recipient (Table 2). Namely, CnCV1 was transmitted to two other *C. nitschkei* strains LFP-E24 and E16, and two closely related *Cryphonectria* species, two strains, ph1113 and DR1, of *C. radicalis*, and one strain, C0754, of *C. naterciae*, but not to either *C. parasitica* EP155 or Δ*dcl2* (an RNA silencing deficient mutant). The other two strains of *C. parasitica*, 3K87 and GH2-6, were also found to not support CnCV1 replication. In order to rule out the possibility of failure in protoplast fusion and to further confirm these results, we next used as a donor *C. nitschkei* strain OB5/11 coinfected with CnCV1 and the prototype hypovirus CHV1 (a positive-sense ssRNA virus). CHV1 was introduced into strain OB5/11 by transformation using the plasmid pXH9 (Choi and Nuss, 1992). The CHV1-infected transformant was anastomosed with the original fungal strain OB5/11 to obtain a hygromycin-susceptible strain co-infected stably with the two viruses (CnCV1 and CHV1). Note that CHV1 was previously shown to have a generally broad host range (Chen et al., 1996; Sasaki et al., 2002). CHV1 was indeed shown to be replicated in all tested fungi, which had previously been reported to be transmitted between two species *C. parasitica* and *C. nitschkei* (Liu et al., 2003). This approach was expected to be helpful to ensure that protoplast fusion occurred successfully if recipients became infected with CHV1. Consequently, all tested fungal strains, which included *V. ceratosperma* (belonging to the family Valsaceae, a different family from that of *Cryphonectria* spp.), received CHV1 from the co-infected strain OB5/11 either in single or co-infections with CnCV1 (Table 2 and data not shown). However, CnCV1 was transferred only to *C. radicalis* (ph1113 and DR1) and *C. nitschkei* (LFP-E24 and E16), whereas no CnCV1 infection was observed in the remaining strains of *C. parasitica* (EP155, Δ*dcl2*, 3K87 and GH2-6) or *V. ceratosperma* (AVC53) (Table 2). The failure of CnCV1 to be replicated in *C. parasitica* EP155, and *V. ceratosperma* was confirmed by RT-PCR (Supplementary Fig. S2).

Secondly, the coculture method with $ZnCl_2$ as a zinc compound was employed for horizontal virus transfer from CnCV1-infected donor strain OB5/11. Zinc compounds are hypothesized (Ikeda

et al., 2013) to slow down programmed cell death during anastomosis between fungal strains belonging to vegetatively incompatible groups and allow virus transmission during coculturing. This method has previously been applied to *Rosellinia necatrix* (white root rot fungus) strains (Ikeda et al., 2013; Zhang et al., 2014) and *C. parasitica* strains (K. Ikeda, personal communication). We tested this method for co-culturing between the CnCV1-carrying *C. nitschkei* OB5/11 and several fungal strains spanning three different fungal species (Table 3). CnCV1 was moved, though not efficiently, to *C. nitschkei* strain LFP-E24 and *C. radicalis* strain DR1. However, no CnCV1 transmission was confirmed in the other tested fungal strains such as *C. parasitica* EP155, *C. nitschkei* E16 or *C. radicalis* ph1113 by this method.

Finally, a conventional virion transfection method was applied for the fungal strains found to be hosts by the above protoplast fusion assay. *C. radicalis* strains ph1113 and DR1, and *C. nitschkei* strains LFP-E24 and E16 could be transfected by CnCV1 virions (Table 4). However, repeated attempts to transfect *C. parasitica* EP155 with CnCV1 were unsuccessful, which is in accordance with the protoplast fusion assay (Table 4). Virus particles could be purified from three new host strains, *C. radicalis* strains ph1113 and DR1, and *C. naterciae* strain C0754. Electron microscopic analyses showed particles indistinguishable in size from those shown in Fig. 3A in each of the three fungal strains (Supplementary Fig. S3).

Taken together, CnCV1 was concluded to be able to replicate in the three *Cryphonectria* species, *C. radicalis*, *C. naterciae* and *C. nitschkei*, but not in the closely related species *C. parasitica* or relatively distantly related fungus *V. certosperma*.

*3-5 Possible fungal strain-specific positive effect of CHV1 on CnCV1 accumulation*

CnCV1 accumulation in the new fungal host strains and original strain was compared by agarose gel electrophoretic analysis (Fig. 4). When normalized against host rRNA, CnCV1 dsRNA accumulated much less in the two strains of *C. nitschkei* LFP-E24 and E16 than in the original *C. nitschkei* host strain, OB5/11. Particularly in strain E16, CnCV1 was only detectable by RT-PCR and was unstable (Fig. 4A). It should be noted that coinfection by CHV1 led to enhanced CnCV1 accumulation in *C. nitschkei* LFP-E24 (Fig. 4A), likely through suppression of antiviral RNA silencing by coinfecting CHV1 as in the case of *C. parasitica* (Segers et al., 2006). In *C. radicalis* ph1113, CnCV1 accumulated comparably to in OB5/11 and no positive effect of CHV1 on CnCV1 accumulation was detected (Fig. 4B and Supplementary Fig. S4). Similarly, CnCV1 was detectable as much as in the original strain OB5/11 in *C. radicalis* DR1 in the absence or presence of CHV1 and *C. naterciae* C0754 in the absence of CHV1 (Supplementary Fig. S4).

*3-6 Asymptomatic infection of host fungi by CnCV1*

In order to investigate the possible effects of CnCV1 infection on its host, different approaches were taken. First, we tried to obtain virus-cured isogenic strains from CnCV1-infected strain OB5-11 by using single spore isolation. We tested over 300 single-conidial isolates for virus infection, but none of them were virus-free (data not shown). Our attempts to obtain virus-free strains by other methods including hyphal tipping, protoplasting, and mycelial fragmentation (Kim et al., 2013) were also unsuccessful (data not shown). Thus, secondly, taking advantage of the newly extended host of CnCV1, five sets of isogenic CnCV1-free and -infected fungal strains of two species were used: *C. nitschkei* (strains LFP-E24 and E16), *C. radicalis* (ph1113 and DR1), and *C. naterciae* (C0754). For any set, no discernable phenotypic alteration was observed between virus-free and CnCV1-infected strains (Supplementary Fig. S5). This notion was confirmed using multiple CnCV1-infected isolates for each fungal strain that were independently obtained by protoplast fusion and/or virion transfection (data not shown).

## 4. Discussion

This thorough characterization of a chrysovirus from *C. nitschkei* (CnCV1), the partial genome sequence of which was reported earlier (Kim et al., 2010; Liu et al., 2007), revealed intriguing molecular and biological attributes. There are now two genera within the family *Chrysoviridae*, i.e. *Alphachrysovirus* and *Betachrysovirus* (Kotta-Loizou et al., 2020). *Alphachrysovirus* comprises orthodox chrysoviruses that have four genome segments and infect filamentous ascomycetes or probably some insects as well as plant infecting members with three genomic segments. The second genus includes so-called chryso-like viruses that infect filamentous fungi with five to seven genome segments, some of which undergo spontaneous loss of one segment during laboratory maintenance (Higashiura et al., 2019; Urayama et al., 2014a). Kim et al. suggested that the two CnCV1 strains OB5-11 and BS122 belong to a single species (*Cryphonectria nitschkei chrysovirus 1*) based on their partial sequences (Kim et al., 2010). The study confirms this prediction; the two strains of CnCV1 is a member of the genus *Alphachrysovirus* based on the phylogenetic analysis (Fig. 3) and a high level of sequence identity between corresponding genome segments (Supplementary Table S2).

CnCV1 has the conserved terminal sequences in both ends and CAA repeats in the UTR of the genomic segments, like previously characterized alphachrysoviruses. However, unlike other alphachrysoviruses, all of the CnCV1 genomic segments, excepting for dsRNA1, possess single

small-ORFs, preceding the main large ORF (Fig. 1). The dsRNA4-encoded small polypeptide possibly possesses the S7-like domain, which is also conserved in a structural protein (nucleocapsid protein) of the fungal mononegavirus (e-value: ~3e-18), suggesting a further acquisition and wider distribution of the S7 domains in the RNA virosphere (Liu et al., 2012). There were no significant hits with e-values smaller than 0.11 when a BLASTP search was conducted with other small proteins of CnCV1 (Fig. 1). This poses a few interesting questions: 1) are these small cistrons (sORF proteins) expressed and functional in CnCV1-infected cells, and 2) could these mini-ORFs have possible effects on expression of the downstream large ORFs. With regard to 2), CnCV1 dsRNA2 and possibly other segments has IRES activities in its 5′-terminal region containing the mini-cistron (Chiba et al., 2018). Of note is a conserved sequence upstream of the initiation codons of their large ORFs (Fig. 1). Such conserved sequence stretches are detectable in other alphachrysoviruses such as VdCV1 (Cao et al., 2011), Aspergillus fumigatus chrysovirus (Jamal et al., 2010) and PcV (Jiang and Ghabrial, 2004). Investigation of the effects of the conserved sequence stretch, mini-ORF and CAA repeats, presumed translational enhancers, of the CnCV1 genomic segments on IRES activities is under way.

This study also developed a virion transfection method for introducing a chrysovirus (CnCV1) into *C. radicalis* and *C. nitschkei* (Table 4). Virion transfection has thus far been established for diverse icosahedron dsRNA viruses including victoriviruses (Chiba et al., 2013b; Xie et al., 2016), partitiviruses (Chiba et al., 2013a; Sasaki et al., 2006), megabirnaviruses (Salaipeth et al., 2014), botybirnaviruses (Wu et al., 2012), yadonushiviruses (toti-like viruses) (Zhang et al., 2016), and mycoreoviruses (Hillman et al., 2004). However, no successful transfection has been reported for multi-particulate dsRNA viruses with over three dsRNA segments, such as chrysoviruses, quadriviruses and alternaviruses. The efficiency of CnCV1 transfection appeared to be lower than those of other transfectable fungal viruses when examined by a standard protocol (Chiba et al., 2016; Salaipeth et al., 2014). In this study, we used a similar number of protoplasts and roughly double amounts of virus particles. This method may be applicable to betachrysoviruses, many of which show symptomatic infections (Bormann et al., 2018; Urayama et al., 2016; Urayama et al., 2014b).

There are many examples in which fungal viruses have been experimentally shown to replicate in fungal species distinct from their original hosts. For example, many dsRNA viruses of *R. necatrix* were found to be replicable in *C. parasitica*, which belongs to a different order than *R. necatrix* (Eusebio-Cope et al., 2015). Similar examples can readily be found in other systems (Sasaki et al., 2002; Xie et al., 2016). Even cross-kingdom virus transmission and/or infection has been reported for some fungal and plant hosts (Andika et al., 2017; Bian et al., 2020). However, there are only a limited number of fungal viruses in which the host range has been investigated systematically. For the mitochondrially replicating mitovirus, Cryphonectria mitovirus 1, host range was determined

using a protoplast fusion-based method for inoculation (Shahi et al., 2019a). Another example is a hypovirus CHV1, for which a cloned viral cDNA or its transcripts are available (Chen et al., 1996; Choi and Nuss, 1992; Sasaki et al., 2002). It was assumed that *Cryphonectria* spp. could support the replication of the chrysovirus (CnCV1) originally isolated from *C. nitschkei*. However, this study clearly showed its very narrow host range; only *C. nitschkei*, *C. naterciae*, and *C. radicalis* supported CnCV1 replication (Tables 2 and 3). The inability of CnCV1 to replicate in other *Cryphonectria* spp. such as *C. parasitica* was also confirmed in a compelling way. First, we used an antiviral RNA silencing deficient *C. parasitica* strain (Δ*dcl2*) with the genetic background of the EP155 standard strain. Repeated attempts to transfect with CnCV1 virions failed (Table 4). Second, we took a protoplast fusion technique in which *C. nitschkei* strain OB5-11 infected CnCV1 alone as well as that co-infected by CnCV1 and CHV1 was used as the donor strain. CnCV1 horizontal transmissibility was not observed, regardless of whether the donor strain OB5-11 was singly or doubly infected. In the latter case, no CnCV1 transfer was detected even under conditions in which the coinfecting virus CHV1 was 100% transferred (Table 2). From these observations and considerations, CnCV1 is concluded to show species-specific infection with a very narrow host range.

Previous large-scale surveys of *Cryphonectria* spp. for viruses have been conducted. Particularly, Milgroom's group collected *C. parasitica* field isolates from the USA, Japan and China (Liu et al., 2007). The fact that CnCV1 has thus far been detected only in *C. nitschkei*, but not in *C. parasitica*, may support our conclusion that CnCV1 is unable to infect *C. parasitica*, which is taxonomically closely related and sympatric to its natural host, *C. nitschkei*. An extensive survey of *Cryphonectria* spp. in Korea is largely congruent with the surveys in USA that both CHV1 and CnCV1 occur in both *C. parasitica* and *C. nitschkei* (Park et al., 2008). However, as a rare case, a few Korean isolates of *C. parasitica* were shown to harbor a chrysovirus (Park et al., 2008). How the *C. parasitica* chrysovirus is similar to CnCV1 is unknown. It will be of great interest to investigate whether other chrysoviruses show narrow or broad host ranges.

There are several factors that restrict the host range of a virus, such as host defense that the virus cannot overcome and a host factor(s) that the virus requires for its replication and systemic spread. Among them is antiviral RNA silencing, which has been shown to be involved in the inability of fungal viruses to be stably maintained (Aulia et al., 2019; 2020). The failure of CnCV1 to replicate in *C. parasitica* appears to be attributable to the lack of host factors necessary for CnCV1 replication. This hypothesis is supported by a few observations. First, a *dcl2* knock-out mutant of *C. parasitica* deficient in the fungal primary antiviral defense could not support the replication of CnCV1 (Table 2). Second, neither of the two other tested strains of *C. parasitica* supported CnCV1 replication (Table 2), even in the presence of CHV1, which encodes an RNA silencing suppressor (Chiba and Suzuki,

2015; Segers et al., 2006; Sun et al., 2009) and often enhances the replication of co-infecting unrelated fungal viruses (Aulia et al., 2019; Sun et al., 2006).

By comparing a set of virus-free and -infected isogenic *C. nitschkei* strains, Kim et al. showed that CnCV1-BS122, another strain belong to the same species as CnCV1-OB5/11, caused reduced mycelial growth (Kim et al., 2013). On the other hand, Liu et al. suggested that CnCV1-OB5/11 may induce no overt phenotypic alterations (Liu et al., 2007). We compared a few sets of virus-free and CnCV1-infected isogenic strains of *C. nitschkei* from Japan and *C. radicalis* from Europe. As a result (Supplementary Fig. S1), CnCV1-OB5/11 showed asymptomatic infection in the two fungal strains. This difference in infection pattern may be attributable to differences in the viral and host genotypes. A symptomless nature has been reported for other alphachrysoviruses such as PcV (Ghabrial, 2008), whereas many betachrysoviruses have been shown to be symptomatic (Kotta-Loizou et al., 2020). The currently established methods for the experimental introduction of CnCV1 will be useful for the biological characterization of other chrysoviruses, as discussed above.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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## Figure legends

### Fig. 1 Genome organization of CnCV1.

(A) Schematic representation of CnCV1 genomic dsRNA segments. The segment length is shown on the right in bp, and positions of start/stop codons for potential ORFs are indicated as well. The major ORF on each segment is shown by a gray or black box, while the small ORF (sORF) preceding the major one is indicated by a light gray box. The semi-conserved sequence stretch upstream of the

major ORF on each segment is indicated by a small open box. (B) Terminal sequence domains of the CnCV1 genomic segments dsRNA1 to dsRNA4. Terminal sequence elements are composed of 5′- and 3′-terminal conserved sequences shaded in gray, putative semi-conserved sequences putatively serving as IRESs and repeated CAA. The latter two elements are located in the 5′-UTR followed by major ORFs.

**Fig. 2. Phylogenetic analysis of CnCV1.**

A phylogenetic tree was generated based on the alignment of RdRP sequences (see Materials and Methods). Chrysoviruses included in the analysis are listed in Supplementary Table 8. Two victoriviruses, Helminthosporium victoriae virus 145S and Rosellinia necatrix victorivirus 1, and one totivirus, Saccharomyces cerevisiae virus L-A, were used as outgroups.

**Fig. 3. Morphology and components of CnCV1 virions.**

(A) Electron micrograph of CnCV1. CnCV1 virions were purified from *C. nitschkei* strain OB5/11 and observed using a Hitachi H-7100 electron microscope after staining with the EM Stainer (Nissin EM Co., Tokyo). The bar represents 100 nm. (B) dsRNA genomic segments of CnCV1. dsRNA fractions were obtained from a set of CnCV1-infected strain OB5/11 and virus-free strain E16 and electrophoresed in 1.0% agarose gel in 1X TAE buffer. RNA was stained with ethidium bromide. The red arrow indicates the CnCV1 dsRNA. M refers to DNA ladders used as size standards (Thermo Fisher Scientific., Inc., Waltham, MA, USA). (C) Protein component of CnCV1 virions. Virus particles purified from *C. nitschkei* OB5/11, after denaturing at 95°C for 5 min in the presence of SDS and beta-mercaptoethanol and electrophoresed on an 8% polyacrylamide gel. Proteins were stained with Coomassie Brilliant Blue R250. A fraction obtained from virus-free *C. nitschkei* strain E16 by the same method as that used for strain OB5/11 were treated in parallel. M refers to the protein size markers (Precision Plus Protein Dual Color Standards, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Fig. 4. CnCV1 accumulation in new fungal hosts.**

CnCV1 dsRNA accumulation in *C. nitschkei* strains LFP-E24 and E16 (A) and *C. radicalis* strains ph1113 (B). Total RNA fractions were purified from respective fungal strains infected singly by CnCV1 or doubly by CnCV1 and CHV1. The names of fungal strains are shown on the top of the gel. The original CnCV1-infected *C. nitschkei* strain OB5/11 and respective virus free (VF) fungal strains were treated in parallel. Total RNAs were electrophoresed on a 1.0% agarose gel as described in the legend of Fig. 3 and stained with ethidium bromide. The blue and red arrows indicate the migration

positions of the CHV1 replicative form dsRNA and CnCV1 genomic dsRNA, respectively. DNA ladders were used as size standards (M).

**Table 1. Fungal and viral strains used in this study.**

| Family/Species | Strain | Original host plant or description | Reference or Source |
|---|---|---|---|
| Cryphonectriaceae | | | |
| *Cryphonectria nitschkei* | OB5/11 | Japanese chestnut (*Castanea crenata*) | ATCC MYA4105 (Liu et al., 2007) |
| | LFP-E24 | Japanese oak (*Quercus crispula*) | MAFF 410076 |
| | E16 | Jolcham oak (*Quercus serrata*) | MAFF 410155 |
| *Cryphonectria parasitca* | EP155 | American chestnut (*Castanea dentata*), Standard funal strain | ATCC38755 (Crouch et al., 2020) |
| | Δ*dcl2* | RNA silencing deficient mutant of *C. parasitica* strain EP155 | (Segers et al., 2007) |
| | GH2-6 | American chestnut (*Castanea dentata*) | (Smart et al., 1999) |
| | 3K/87 | European chestnut (*Castanea sativa*) | Nuss Lab Collection ID: 09G3 |
| *Cryphonectria radicalis* | ph1113 | European chestnut (*Castanea sativa*) | WSL collection M2269 (Hoegger et al., 2002) |
| | DR1 | European chestnut (*Castanea sativa*) | WSL collection M4733 |
| *Cryphonectria naterciae* | C0754 | Cork oak (*Quercus suber*) | (Braganca et al., 2011) |
| Valsaceae | | | |
| *Valsa ceratosperma* | AVC53 | apple tree (*Malus domestica*) | (Sasaki et al., 2002) |

**Table 2. Horizontal transfer of CnCV1 via protoplast fusion**

| Recipient | | Donor | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *C. nitschkei* OB5-11/CnCV1 | | *C. nitschkei* OB5-11/CnCV1+CHV1 | | | | |
| | | CnCv1 transmission | No. of assay* | CnCV1 (single) transmission | CHV1 (single) transmission | CnCV1+CHV1 (double) transmission | No. of assay* | Collective CnCV1 transmission rate |
| *C. nitschkei* | LEP-E24 | 0/40 (0%) | 2 | 0/20 (0%) | 16/20 (80%) | 4/20 (20%) | 1 | 20% |
| | E16 | 1/40(3%) | 2 | 0/20 (0%) | 18/20 (90%) | **2/20 (10%) | 1 | 10% |
| *C. parasitica* | EP155 | 0/60(0%) | 3 | 0/60 (0%) | 54/60 (90%) | 0/60 (0%) | 3 | 0% |
| | Δ*dcl2* | 0/80(0%) | 4 | 0/60 (0%) | 53/60 (88%) | 0/60 (0%) | 3 | 0% |
| | 3K/87 | NT*** | | 0/60 (0%) | 22/60 (37%) | 0/60 (0%) | 3 | 0% |
| | GH2 | NT | | 0/60 (0%) | 59/60 | 0/60 (0%) | 3 | 0% |
| *C. radicalis* | DR1 | 59/60 (98%) | 3 | 3/60 (5%) | 0/60 (0%) | 48/60 (80%) | 3 | 85% |
| | ph1113 | 58/60 (96%) | 3 | 0/60 (0%) | 0/60 (0%) | 60/60 (100%) | 3 | 100% |
| *C. naterciae* | C0754 | 30/40 (75%) | 2 | NT | | | | |
| *V. ceratosperma* | ASV53 | NT | | 0/60 (0%) | 18/60 | 0/60 | 3 | 0% |

* In each assay 20 isolates were tested.

** CnCV1 was unstable and occasionally eliminated during subsequent hyphal fusion with *C. nitschkei* E16, whereas CHV1 was stably maintained.

*** NT denotes “not tested.”

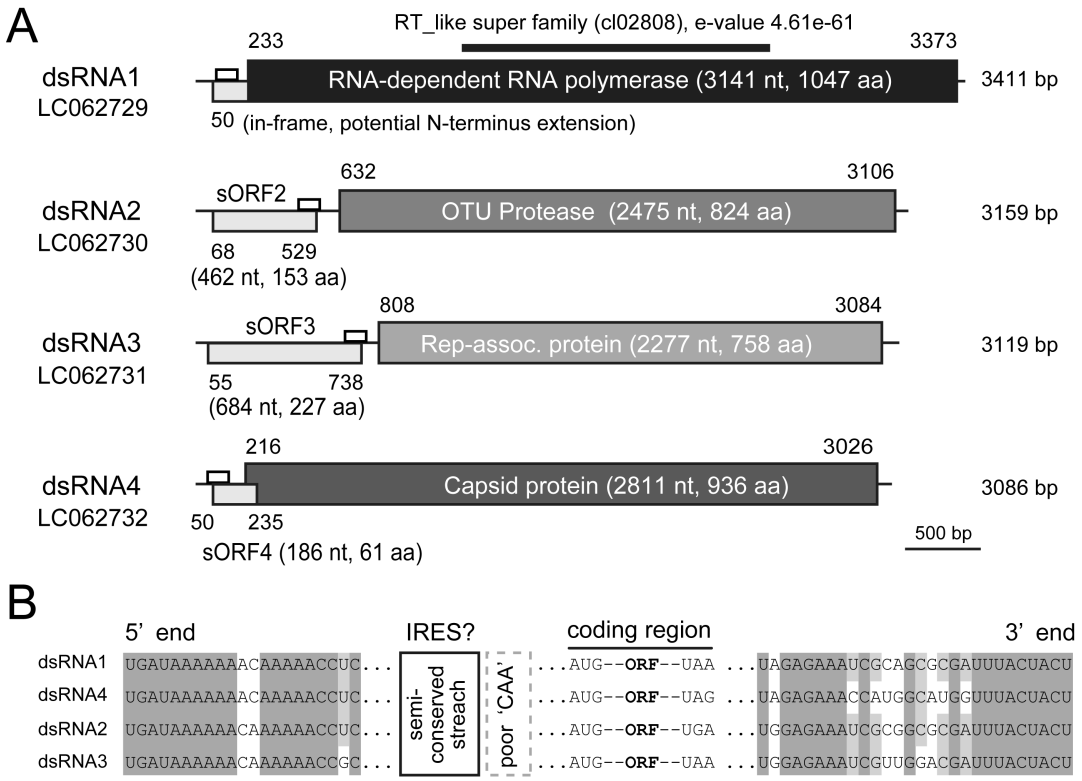
**Table 3. Horizontal transfer of CnCV1 via anastomosis**

| Fungal species | Fungal strain (Recipients) | Hyphal fusion (Donor: CN105/CnCV1) (transmission rate) | |
|---|---|---|---|
| | | With $ZnCl_2$ | Without $ZnCl_2$ |
| *C. nitschkei* | EP-E24 | **1/15 (6.7%)** | **0/15 (0%)** |
| | E16 | **0/15 (0%)** | **0/15 (0%)** |
| *C. parasitica* | EP155 | **0/15 (0%)** | **0/15 (0%)** |
| | Δ*dcl2* | **0/15 (0%)** | **0/15 (0%)** |
| *C. radicalis* | DR1 | **1/15 (6.7%)** | **0/15 (0%)** |
| | ph1113 | **0/15 (0%)** | **0/15 (0%)** |

**Table 4. Experimental introduction of CnCV1 via virion transfection.**

| Fungal species | Fungal strain | No. of experiments* | No. of isolates (positive/tested) | Transfection rate |
|---|---|---|---|---|
| *C. nitschkei* | LEP-E24 | **4** | **10/40** | **25%** |
| | E16 | **2** | **10/10** | **100%** |
| *C. radicalis* | ph1113 | **2** | **1/16** | **6.3%** |
| | DR1 | **2** | **2/16** | **12.5%** |
| *C. parasitica* | EP155 | **4** | **0/40** | **0** |

* Five to ten transfectants were examined in each experiment.


A
RT_like super family (cl02808), e-value 4.61e-61
233
3373
dsRNA1
LC062729
RNA-dependent RNA polymerase (3141 nt, 1047 aa)
3411 bp
50 (in-frame, potential N-terminus extension)
632
3106
sORF2
dsRNA2
LC062730
OTU Protease (2475 nt, 824 aa)
3159 bp
68
529
(462 nt, 153 aa)
808
3084
sORF3
dsRNA3
LC062731
Rep-assoc. protein (2277 nt, 758 aa)
3119 bp
55
738
(684 nt, 227 aa)
216
3026
dsRNA4
LC062732
Capsid protein (2811 nt, 936 aa)
3086 bp
50
235
sORF4 (186 nt, 61 aa)
500 bp
B
5' end
IRES?
coding region
3' end
dsRNA1
UGAUAAAAAAACAAAAAACCUC...
semi-conserved streach
poor 'CAA'
...AUG--ORF--UAA ...UAGAGAAAUCGCAGCGCGAUUUACUACU
dsRNA4
UGAUAAAAAAACAAAAAACCUC...
...AUG--ORF--UAG ...UAGAGAAACCAUGGCAUGGUUUACUACU
dsRNA2
UGAUAAAAAAACAAAAAACCUC...
...AUG--ORF--UGA ...UGGAGAAAUCGCGGCGCGAUUUACUACU
dsRNA3
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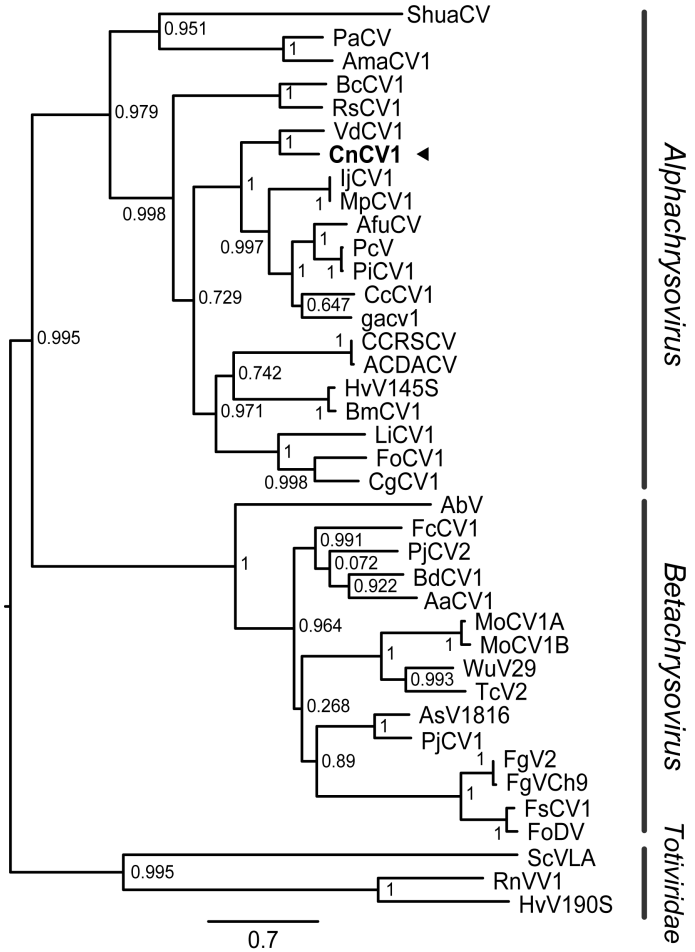
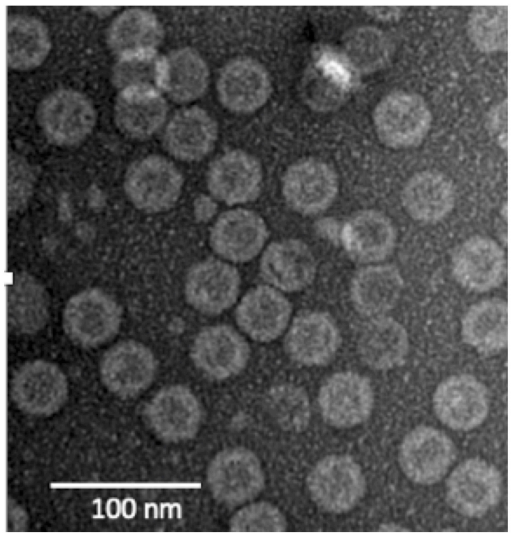
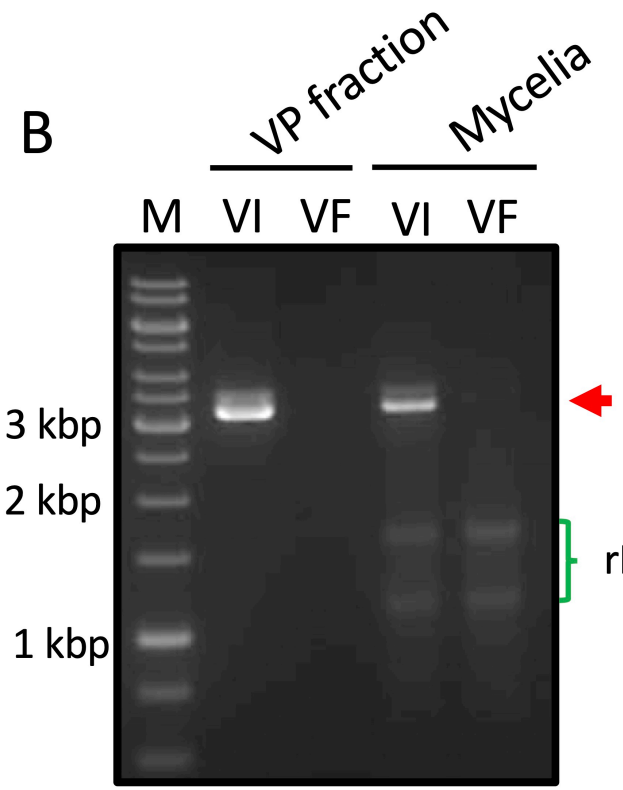
...AUG--ORF--UAA ...UGGAGAAAUCGUUGGACGAUUUACUACU


ShuaCV
PaCV
AmaCV1
BcCV1
RsCV1
VdCV1
CnCV1
IjCV1
MpCV1
AfuCV
PcV
PiCV1
CcCV1
gacv1
CCRSCV
ACDACV
HvV145S
BmCV1
LiCV1
FoCV1
CgCV1
AbV
FcCV1
PjCV2
BdCV1
AaCV1
MoCV1A
MoCV1B
WuV29
TcV2
AsV1816
PjCV1
FgV2
FgVCh9
FsCV1
FoDV
ScVLA
RnVV1
HvV190S
0.951
0.979
0.998
0.997
0.729
0.647
0.995
0.742
0.971
0.998
0.991
0.072
0.922
0.964
0.993
0.268
0.89
0.995
0.7
Alphachrysovirus
Betachrysovirus
Totiviridae

A

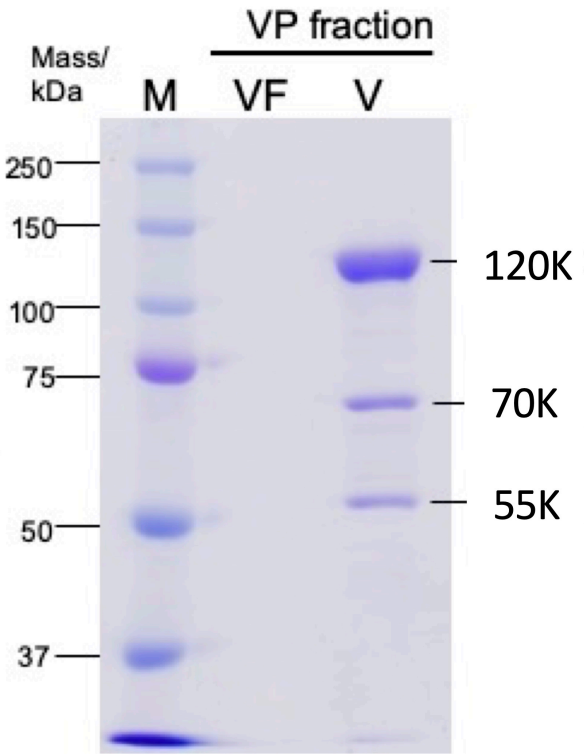



B

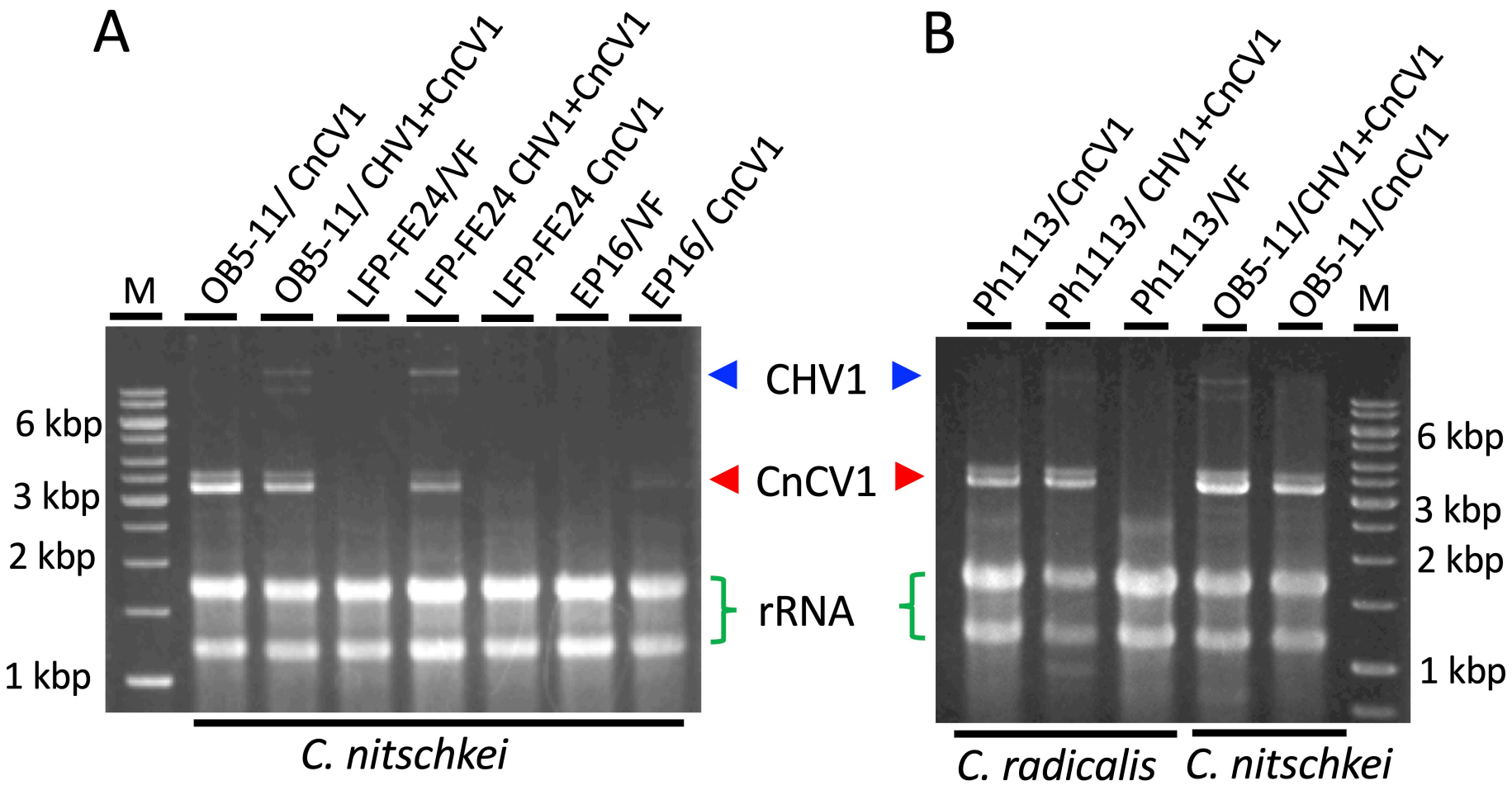



EtBr staining

C




CBB staining


A
M
OB5-11/ CnCV1
OB5-11/ CHV1+CnCV1
LFP-FE24/VF
LFP-FE24 CHV1+CnCV1
LFP-FE24 CnCV1
EP16/VF
EP16/ CnCV1
6 kbp
3 kbp
2 kbp
1 kbp
CHV1
CnCV1
rRNA
C. nitschkei
B
Ph1113/CnCV1
Ph1113/ CHV1+CnCV1
Ph1113/VF
OB5-11/CHV1+CnCV1
OB5-11/CnCV1
M
6 kbp
3 kbp
2 kbp
1 kbp
C. radicalis
C. nitschkei

**SUPPLEMENTARY DATA FOR**

# Cryphonectria nitschkei chrysovirus 1 with unique molecular features and a very narrow host range

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Short title: Alphachrysovirus with a narrow host range

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Supplementary tables: 8

Supplementary figures: 4

**Supplementary Table S1.** List of primers used in this study.

**Supplementary Table S2.** BLASTP search with CnCV1-RdRp (dsRNA1).

**Supplementary Table S3.** BLASTP search with CnCV1-OTU-Pro (dsRNA2).

**Supplementary Table S4.** BLASTP search with CnCV1-Rep-Assoc (dsRNA3).

**Supplementary Table S5.** BLASTP search with CnCV1-CP (dsRNA4).

**Supplementary Table S6.** BLASTP search with a protein encoded by CnCV1-potential small ORF2 (dsRNA2).

**Supplementary Table S7.** BLASTP search with a protein encoded by CnCV1-potential small ORF3 (dsRNA3).

**Supplementary Table S8.** List of viruses used in the phylogenetic analysis of chrysovirus RdRPs.

**Legend of Supplementary Figures**

**Supplementary Fig. S1.** Neighbor joining (NJ) phylogenetic tree based on the nucleotide sequence of the internal transcribed spacer (ITS) of selected members of the genus *Cryphonectria*. The accession number (in parentheses) following each fungal strain name is indicated. The sequences obtained from this study are shown in red. The ITS sequences form representative fungal strains belonging to the genus *Cryphonectria* are included in this analysis (Gryzenhout et al., 2006). *Endothia gyrosa*, another species in the family Cryphonectriaceae, was used as an outgroup taxon.

**Supplementary Fig. S2.** RT-PCR analyses of recipients obtained via protoplast fusion. Protoplasts of *C. nitschkei* strain OB5/11 doubly infected by CnCV1 and CHV1 (OB5/11/CnCV1+CHV1) were fused with those of *C. parasitica* strain 3K/87 (A), *C. parasitica* strain GH2 (B) or *V. ceratosperma* strain AVC53 (C). Five subcultures from each protoplast fusion experiment were subjected to one-step colony RT-PCR for detection of CnCV1 and CHV1 as described in Materials and Methods. Primer pairs used in RT-PCR are shown in D. The virus donor strain OB5/11/CnCV1+CHV1 and original virus-free strains used as a recipient in each fusion assay were also tested in parallel. Amplified PCR fragments were electrophoresed on a 1.0% agarose gel as described in the legend of Fig. 2 and stained with ethidium bromide. M refers to DNA ladders used as size standards (Thermo Fisher Scientific., Inc., Waltham, MA, USA).

**Supplementary Fig. S3.** CnCV1 accumulation in *C. radicalis* and *C. naterciae*.
CnCV1 dsRNA accumulation in *C. radicalis* strains DR1 (A) and *C. naterciae* strain C0754 (B). Total RNA fractions were purified from respective fungal strains infected singly by CnCV1 or doubly by CnCV1 and CHV1. The original CnCV1-infected *C. nitschkei* strain OB5/11 and respective virus free fungal strains were treated in parallel. Total RNAs were electrophoresed on a 1.0% agarose gel as described in the legend of Fig. 3 and stained with ethidium bromide. The blue and red arrows, and green brackets indicate the migration positions of the CHV1 replicative form dsRNA and CnCV1 genomic dsRNA, and rRNAs, respectively. DNA ladders were used as size standards (M).

**Supplementary Fig. S4.** Colony morphology of the virus-infected and virus-free isogenic fungal strains *C. nitschkei* and *C. radicalis*. CnCV1 was transferred to four fungal strains, two *C. nitschkei* strains LFP-E24 and E16, and two *C. radicalis* strains ph1113 and DR1, and one strain C0754 of *C. naterciae*. Five sets of CnCV1-infected and -free isogenic strains were grown for 8 days on the benchtop and photographed. The original CnC1-infected OB5/11 was used as a reference.

**Supplementary Table S1.** List of primers used in this study.

| primer name | nt sequence (5'-3') | source sequence (accession) | nt position in the CnCV1 genome (accession, segment) |
|---|---|---|---|
| 105-1A_F1 | CTGTTTGCAATAGTGATGC | CnCV1 clone 1A (DQ865185) | 266-284 (LC062729, dsRNA1) |
| 105-1A_F2 | GGCCATGGCAAGACTCATTA | CnCV1 clone 1A (DQ865185) | 290-309 (LC062729, dsRNA1) |
| 105-1A_F3 | TGCGGCACCGCATATGTAC | *an RT-PCR product* | 736-754 (LC062729, dsRNA1) |
| 105-1A_R | GGTGGCCAAACTTCTTTGCG | CnCV1 clone 1A (DQ865185) | 310-325 (LC062729, dsRNA1)<br>*including mismatch |
| 105-1A_R2 | CTCTATTCGTAAGCCCGT | *an RT-PCR product* | 669-686 (LC062729, dsRNA1) |
| 105-1B_F | GTTAACATACTGGATCAGGC | CnCV1 clone 1B (DQ865186) | 2552-2571 (LC062729, dsRNA1) |
| 105-1B_F2 | GATGATAGGATATTCGACG | *an RT-PCR product* | 3020-3038 (LC062729, dsRNA1) |
| 105-1B_R1 | ACATATCTGGCACCTGCTC | CnCV1 clone 1B (DQ865186) | 2762-2780 (LC062729, dsRNA1) |
| 105-1B_R2 | GATTATCACTCGTGGCATAC | CnCV1 clone 1B (DQ865186) | 2676-2695 (LC062729, dsRNA1) |
| 105-1B_R3 | TTATGCCGATTACCGTCC | *an RT-PCR product* | 2158-2175 (LC062729, dsRNA1) |
| 105-2_F1 | AGCTACAGAAGCTCGTCATG | CnCV1 clone 2 CP (DQ865187) | 595-614 (LC062732, dsRNA4) |
| 105-2_F2 | CATGCACGAGTCGCTAGTC | CnCV1 clone 2 CP (DQ865187) | 611-629 (LC062732, dsRNA4) |
| 105-2_F3 | TGCATGGCTAGCGCCTAC | *an RT-PCR product* | 966-983 (LC062732, dsRNA4) |
| 105-2_F4 | ACATCTCTCGGCTATACTGG | *an RT-PCR product* | 1535-1554 (LC062732, dsRNA4) |
| 105-2_F5 | AGGAAGGAATCACAGATAGC | *an RT-PCR product* | 2514-2533 (LC062732, dsRNA4) |
| 105-2_R1 | ACGGCTGGGATAGGAATTCG | CnCV1 clone 2 CP (DQ865187) | 446-465 (LC062732, dsRNA4) |
| 105-2_R2 | CGGTGGCTTTTGAGATATCC | CnCV1 clone 2 CP (DQ865187) | 662-681 (LC062732, dsRNA4) |
| 105-3_F | AGGCTGGCTGTTGGAATGAC | CnCV1 clone 3 OUT-Pro (DQ865188) | 2582-2601 (LC062730, dsRNA2) |
| 105-3_F2 | GGCTTTTCACTGCCAGCC | CnCV1 clone 3 OUT-Pro (DQ865188) | 796-813 (LC062730, dsRNA2) |
| 105-3_F3 | CGGAAGGGTATGAACGAAGG | CnCV1 clone 3 OUT-Pro (DQ865188) | 824-843 (LC062730, dsRNA2) |
| 105-3_F4 | GGACATCGTCGACTTCAG | CnCV1 clone 3 OUT-Pro (DQ865188) | 1207-1224 (LC062730, dsRNA2) |

| | | | |
|---|---|---|---|
| 105-3_R | GGAGACTTTGTCACCGCTTG | CnCV1 clone 3 OUT-Pro (DQ865188) | 879-898 (LC062730, dsRNA2) |
| 105-3_R2 | TGCGACCACTGTCTAGG | *an RT-PCR product* | 344-360 (LC062730, dsRNA2) |
| 105-3_R3 | TTCGTGCGCACGTCTATTGG | CnCV1 clone 3 OUT-Pro (DQ865188) | 2654-2673 (LC062730, dsRNA2) |
| 105-3_R4 | CTTGCCATCCAAGACGTAG | CnCV1 clone 3 OUT-Pro (DQ865188) | 2623-2641 (LC062730, dsRNA2) |
| 105-3_R5 | CATAGTCCTTAATCGCAC | CnCV1 clone 3 OUT-Pro (DQ865188) | 1938-1955 (LC062730, dsRNA2) |
| 105-3_R6 | CAACTTTCCACGCGATAAC | CnCV1 clone 3 OUT-Pro (DQ865188) | 406-424 (LC062730, dsRNA2) |
| 105-4_F | TCGAATCTGCTTACCACTCC | CnCV1 clone 4 RepAssoc (DQ865189) | 1765-1784 (LC062731, dsRNA3) |
| 105-4_F2 | TGATCTCGAATCTGCTTACC | CnCV1 clone 4 RepAssoc (DQ865189) | 1760-1779 (LC062731, dsRNA3) |
| 105-4_F3 | GATAGGATGTACTTCTACAGC | CnCV1 clone 4 RepAssoc (DQ865189) | 1150-1170 (LC062731, dsRNA3) |
| 105-4_F4 | GATGACAGGATCTACATTC | *an RT-PCR product* | 2730-2748 (LC062731, dsRNA3) |
| 105-4_F5 | TCAGCCAGCGATATGCAGAG | CnCV1 clone 4 RepAssoc (DQ865189) | 1076-1095 (LC062731, dsRNA3) |
| 105-4_R | ATCGTGTCCCAGTCTGATCG | CnCV1 clone 4 RepAssoc (DQ865189) | 1403-1422 (LC062731, dsRNA3) |
| 105-4-R2 | AACGCCCAGATAAGCTAAGC | CnCV1 clone 4 RepAssoc (DQ865189) | 1202-1221 (LC062731, dsRNA3) |
| 105-4-R3 | TCAGTTGTCAGGTCATCTAC | CnCV1 clone 4 RepAssoc (DQ865189) | 1027-1046 (LC062731, dsRNA3) |
| 105-4-R4 | CAGTACAATGTATCCTAACC | *an RT-PCR product* | 281-300 (LC062731, dsRNA3) |
| 105-4-R5 | CTTCGTCTGACTCGTAGG | *an RT-PCR product* | 2198-2215 (LC062731, dsRNA3) |
| 105-4-R6 | GATGTCCTGTTCTGGTGCC | *an RT-PCR product* | 1907-1925 (LC062731, dsRNA3) |

**Supplementary Table S2.** BLASTP search with CnCV1-RdRp (dsRNA1).

| Description (virus [isolate]) | Description (protein) | Max score | Total score | Query cover | E value | Identity | Accession |
|---|---|---|---|---|---|---|---|
| Cryphonectria nitschkei chrysovirus 1 BS122 | RNA dependent RNA polymerase | 1870 | 1870 | 85% | 0 | 99.11% | YP_009507942.1 |
| Cryphonectria nitschkei chrysovirus 1 bs131 | RNA dependent RNA polymerase | 1866 | 1866 | 85% | 0 | 98.66% | ACT79256.1 |
| Cryphonectria nitschkei chrysovirus 1 BS321 | RNA dependent RNA polymerase | 1865 | 1865 | 85% | 0 | 98.66% | ACT79258.1 |
| Verticillium dahliae chrysovirus 1 | RNA-dependent RNA polymerase | 1521 | 1521 | 100% | 0 | 64.79% | YP_009507948.1 |
| Macrophomina phaseolina chrysovirus 1 | RNA-dependent RNA polymerase | 1145 | 1145 | 100% | 0 | 50.57% | YP_009667008.1 |
| Penicillium roseopurpureum chrysovirus 1 | RNA-dependent RNA polymerase | 1110 | 1110 | 100% | 0 | 49.57% | AYP71812.1 |
| Penicillium raistrickii chrysovirus 1 | RNA-dependent RNA polymerase | 1096 | 1096 | 100% | 0 | 49.38% | AZT88567.1 |
| Penicillium chrysogenum virus | RNA-dependent RNA polymerase | 1086 | 1086 | 100% | 0 | 49.48% | YP_392482.1 |
| Aspergillus fumigatus chrysovirus | RNA-dependent RNA polymerase | 1083 | 1083 | 99% | 0 | 49.33% | YP_009508104.1 |
| Penicillium italicum chrysovirus | RNA-dependent RNA polymerase | 1081 | 1081 | 100% | 0 | 49.09% | QCZ35876.1 |
| Chrysothrix chrysovirus 1 | RNA-dependent RNA polymerase | 1071 | 1071 | 100% | 0 | 47.56% | QGR26538.1 |
| Beauveria bassiana chrysovirus 1 | RNA-dependent RNA polymerase | 1061 | 1061 | 99% | 0 | 48.66% | AZT88571.1 |
| Isaria javanica chrysovirus 1 | RNA-dependent RNA polymerase | 1061 | 1061 | 99% | 0 | 48.76% | YP_009337840.1 |
| Wuhan insect virus 30 | hypothetical protein | 1055 | 1055 | 100% | 0 | 47.82% | APG76049.1 |
| Grapevine associated chrysovirus-1 | putative RNA-dependent RNA polymerase | 1008 | 1008 | 91% | 0 | 48.95% | ADO60926.1 |
| Bipolaris maydis chrysovirus 1 | RNA-dependent RNA polymerase | 758 | 758 | 99% | 0 | 39.22% | ARM36035.1 |
| Helminthosporium victoriae 145S virus | RNA-dependent RNA polymerase | 744 | 744 | 99% | 0 | 39.12% | YP_052858.1 |
| Amasya cherry disease associated chrysovirus | putative RNA-dependent RNA polymerase | 713 | 713 | 99% | 0 | 37.46% | YP_001531163.1 |
| Colletotrichum gloeosporioides chrysovirus 1 | RNA-directed RNA-polymerase | 707 | 707 | 99% | 0 | 37.09% | YP_009667012.1 |
| Raphanus sativas chrysovirus 1 | putative RNA-dependent RNA polymerase | 703 | 703 | 98% | 0 | 36.91% | YP_009667003.1 |
| Brassica campestris chrysovirus 1 | putative RNA-dependent RNA polymerase | 699 | 699 | 98% | 0 | 36.80% | YP_009667006.1 |
| Colletotrichum gloeosporioides chrysovirus 1 | RdRp | 681 | 681 | 95% | 0 | 36.88% | QCY49458.1 |

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|---|---|---|---|---|---|---|---|
| Fusarium oxysporum chrysovirus 1 | putative RNA polymerase | 605 | 605 | 81% | 0 | 38.24% | YP_009665200.1 |
| Lepraria chrysovirus 1 | putative RNA-dependent RNA polymerase | 576 | 576 | 76% | 0 | 38.10% | QGR26536.1 |
| Persea americana chrysovirus | putative RNA-dependent RNA polymerase | 461 | 461 | 99% | 1.00E-140 | 30.51% | YP_009666328.1 |
| Zea mays chrysovirus | RNA-dependent RNA polymerase | 437 | 437 | 89% | 6.00E-132 | 31.82% | QJC70223.1 |
| Anthurium mosaic-associated virus | putative RNA-dependent RNA polymerase | 432 | 432 | 99% | 3.00E-130 | 29.75% | YP_009667023.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerase | 427 | 427 | 89% | 2.00E-128 | 31.30% | YP_009551655.1 |
| Cryphonectria nitschkei chrysovirus 1OB5-11 | RNA-dependent RNA polymerase | 313 | 313 | 14% | 7.00E-97 | 98.68% | ABI20755.1 |
| Hubei chryso-like virus 1 | RdRp | 332 | 332 | 99% | 3.00E-93 | 26.16% | ASA47309.1 |
| Shuangao chryso-like virus 1 | RdRp | 326 | 326 | 99% | 6.00E-91 | 26.73% | ASA47445.1 |
| Barley aphid RNA virus 8 | RNA dependent RNA polymerasee | 326 | 326 | 97% | 7.00E-91 | 27.01% | BBV14774.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerasee | 315 | 315 | 53% | 1.00E-90 | 34.91% | AYD75748.1 |
| Hubei chryso-like virus 1 | RNA-dependent RNA polymerasee | 324 | 324 | 99% | 2.00E-90 | 26.18% | BBQ05099.1 |
| Shuangao chryso-like virus 1 | RdRpe | 315 | 315 | 99% | 5.00E-87 | 26.72% | QDC23188.1 |
| Hubei chryso-like virus 1 | hypothetical proteine | 303 | 303 | 79% | 3.00E-84 | 27.89% | APG76013.1 |
| Salado virus | RdRpe | 303 | 303 | 99% | 6.00E-83 | 25.83% | QHA33836.1 |
| Shuangao chryso-like virus 1 | hypothetical proteine | 300 | 300 | 83% | 1.00E-82 | 27.80% | APG76016.1 |
| Broome chryso-like virus 1 | RNA-dependent RNA polymerasee | 277 | 349 | 89% | 6.00E-74 | 27.60% | QLJ83488.1 |
| Shuangao chryso-like virus 1 | hypothetical proteine | 271 | 271 | 68% | 4.00E-73 | 27.84% | APG76009.1 |
| Eskilstorp virus | RNA-dependent RNA polymerasee | 259 | 327 | 73% | 3.00E-68 | 28.96% | QGA70949.1 |
| Hubei chryso-like virus 2 | hypothetical proteine | 252 | 312 | 71% | 3.00E-65 | 27.81% | APG76028.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerasee | 203 | 203 | 28% | 2.00E-54 | 37.67% | AYD75750.1 |
| Penicillium janczewskii chrysovirus 1 | 127 kDa proteine | 177 | 177 | 49% | 3.00E-41 | 27.39% | YP_009182332.1 |
| Penicillium janczewskii chrysovirus 2 | ORF1e | 171 | 171 | 49% | 3.00E-39 | 27.57% | YP_009667018.1 |
| Aspergillus thermomutatus chrysovirus 1 | RNA-dependent RNA polymerasee | 170 | 170 | 55% | 5.00E-39 | 27.52% | AWC67507.1 |

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| Aspergillus fumigatus chrysovirus | putative RNA-dependent RNA polymerase | 166 | 166 | 63% | 1.00E-37 | 25.95% | BBC45614.1 |
| Coniothyrium diplodiella chrysovirus 1 | RNA-dependent RNA polymerase | 164 | 164 | 47% | 5.00E-37 | 28.79% | QDB74971.1 |
| Aspergillus coremiiformis | viral RdRp-domain-containing protein | 162 | 162 | 56% | 2.00E-36 | 27.05% | KAE8348079.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerase | 158 | 158 | 48% | 2.00E-36 | 27.10% | AYD75744.1 |
| Botryosphaeria dothidea chrysovirus | RNA-dependent RNA polymerase | 162 | 162 | 79% | 2.00E-36 | 22.43% | AJD14830.1 |
| Aspergillus mycovirus 1816 | putative RNA-dependent RNA polymerase | 161 | 161 | 50% | 2.00E-36 | 27.94% | ABX79996.1 |
| Botryosphaeria dothidea chrysovirus 1 | putative RNA-dependent RNA polymerase | 160 | 160 | 79% | 7.00E-36 | 22.64% | YP_009353026.1 |
| Hubei chryso-like virus 1 | RNA-dependent RNA polymerase | 152 | 152 | 33% | 3.00E-35 | 27.93% | AWR88277.1 |
| Tolypocladium cylindrosporum virus 2 | RNA dependent RNA polymerase | 144 | 144 | 57% | 6.00E-31 | 25.20% | CBY84993.1 |
| Wuhan insect virus 29 | hypothetical protein | 137 | 137 | 51% | 7.00E-29 | 25.87% | APG76052.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerase | 124 | 124 | 19% | 3.00E-28 | 33.82% | AYD75746.1 |
| unidentified | putative RNA-dependent RNA polymerase | 120 | 120 | 10% | 9.00E-28 | 46.96% | AHB33556.1 |
| Neofusicoccum parvum chrysovirus 1 | RNA-dependent RNA polymerase | 133 | 133 | 47% | 1.00E-27 | 26.37% | QDB74975.1 |
| White button mushroom virus 1 | RNA-directed RNA polymerase | 132 | 132 | 44% | 3.00E-27 | 24.76% | T00494 |
| La France disease virus | ORF | 125 | 125 | 44% | 4.00E-25 | 24.37% | 2211445A |
| Spissistilus festinus virus 1 | RNA-directed RNA polymerase | 115 | 115 | 50% | 4.00E-22 | 24.22% | YP_003800001.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerase | 111 | 111 | 34% | 6.00E-22 | 25.93% | AYD75747.1 |
| Chrysothrix chrysovirus 1 | hypothetical protein | 113 | 113 | 36% | 2.00E-21 | 25.06% | QGR26540.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerase | 108 | 108 | 34% | 5.00E-21 | 26.19% | AYD75751.1 |
| Rosellinia necatrix megabirnavirus 1/W779 | RNA-dependent RNA polymerase | 110 | 110 | 52% | 2.00E-20 | 23.87% | YP_003288763.1 |
| Persimmon latent virus | RNA-dependent RNA polymerase | 107 | 107 | 46% | 2.00E-19 | 25.15% | YP_009025166.1 |
| Penicillium raistrickii chrysovirus 1 | hypothetical protein PrCV1_s3gp1 | 105 | 105 | 37% | 6.00E-19 | 24.69% | AZT88569.1 |
| Macrophomina phaseolina chrysovirus 1 | P3 | 103 | 103 | 35% | 3.00E-18 | 22.34% | YP_009667009.1 |
| Penicillium chrysogenum virus | hypothetical protein | 102 | 102 | 35% | 5.00E-18 | 24.40% | YP_392484.1 |

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|---|---|---|---|---|---|---|---|
| Penicillium italicum chrysovirus | p3 | 101 | 101 | 35% | 7.00E-18 | 24.67% | QCZ35878.1 |
| Hubei toti-like virus 11 | hypothetical protein | 100 | 100 | 36% | 1.00E-17 | 26.11% | APG76021.1 |
| Rhizoctonia solani dsRNA virus 8 | RNA-dependent RNA polymerase | 99.4 | 99.4 | 46% | 5.00E-17 | 24.53% | QDW81320.1 |
| Rosellinia necatrix megabirnavirus 2-W8 | RNA-dependent RNA polymerase | 96.3 | 96.3 | 58% | 4.00E-16 | 23.61% | YP_009227124.1 |
| Fusarium pseudograminearum megabirnavirus 1 | RNA-dependent RNA polymerase | 95.1 | 95.1 | 55% | 1.00E-15 | 23.56% | AYJ09269.1 |
| Aedes camptorhynchus toti-like virus 1 | RdRp | 94.4 | 94.4 | 50% | 2.00E-15 | 24.08% | YP_009388611.1 |
| Aedes alboannulatus toti-like virus 1 | RdRp | 93.6 | 93.6 | 50% | 3.00E-15 | 24.08% | YP_009388609.1 |
| Circulifer tenellus virus 1 | RNA-directed RNA polymerase | 94 | 94 | 54% | 3.00E-15 | 23.31% | YP_003800003.1 |
| Atrato virus | RdRp | 93.6 | 93.6 | 47% | 3.00E-15 | 23.78% | QHA33708.1 |
| Totiviridae sp. | RNA-dependent RNA polymerase | 93.6 | 93.6 | 47% | 3.00E-15 | 24.02% | QKN88741.1 |
| Atrato virus | RdRp | 92.8 | 92.8 | 47% | 4.00E-15 | 23.78% | QHA33710.1 |
| Beauveria bassiana chrysovirus 1 | hypothetical protein BbCV1_s3gp1 | 91.7 | 91.7 | 35% | 1.00E-14 | 23.54% | AZT88573.1 |
| Sclerotinia sclerotiorum botybirnavirus 3 | RNA-dependent RNA polymerase | 89.7 | 89.7 | 37% | 6.00E-14 | 24.03% | QDF82045.1 |
| Sclerotium rolfsii mycovirus dsRNA 1 | RNA-dependent RNA polymerase | 89.4 | 89.4 | 22% | 7.00E-14 | 28.52% | AZF86106.1 |
| Barrymore virus | RNA-dependent RNA polymerase | 88.6 | 88.6 | 38% | 7.00E-14 | 24.53% | QED21514.1 |
| Sclerotinia sclerotiorum botybirnavirus 3 | cap-pol fusion protein | 89.4 | 89.4 | 37% | 7.00E-14 | 24.03% | AWY10943.1 |
| Botrytis porri botybirnavirus 1 | cap-pol fusion protein | 89.4 | 89.4 | 37% | 7.00E-14 | 24.03% | YP_006390636.1 |
| Phytophthora infestans RNA virus 3 | RNA-dependent RNA polymerase | 88.6 | 88.6 | 41% | 9.00E-14 | 23.84% | YP_009551328.1 |

**Supplementary Table S3.** BLASTP search with CnCV1-OTU-Pro (dsRNA2) (only the top 20 hits shown).

| Description (virus [isolate]) | Description (protein) | Max score | Total score | Query cover | E value | Identity | Accession |
|---|---|---|---|---|---|---|---|
| Cryphonectria nitschkei chrysovirus 1 BS122 | putative cysteine protease | 1677 | 1677 | 100% | 0 | 98.18% | YP_009507945.1 |
| Cryphonectria nitschkei chrysovirus 1 OB5-11 | OTU proteinase | 1554 | 1554 | 97% | 0 | 94.33% | ABI20757.1 |
| Verticillium dahliae chrysovirus 1 | ovarian tumor protease | 1049 | 1049 | 99% | 0 | 59.34% | YP_009507947.1 |
| Macrophomina phaseolina chrysovirus 1 | P4 | 560 | 560 | 99% | 0 | 35.11% | YP_009667011.1 |
| Wuhan insect virus 30 | hypothetical protein | 535 | 535 | 99% | 8.00E-175 | 36.02% | APG76050.1 |
| Grapevine chrysovirus | putative protease | 521 | 521 | 100% | 3.00E-169 | 35.96% | AFX73020.1 |
| Chrysothrix chrysovirus 1 | protease | 461 | 461 | 99% | 2.00E-146 | 32.16% | QGR26541.1 |
| Penicillium chrysogenum virus | hypothetical protein | 462 | 462 | 98% | 2.00E-146 | 31.54% | YP_392485.1 |
| Aspergillus fumigatus chrysovirus | hypothetical protein | 461 | 461 | 99% | 5.00E-146 | 31.79% | YP_009508106.1 |
| Penicillium italicum chrysovirus | p4 | 459 | 459 | 98% | 2.00E-145 | 31.54% | QCZ35879.1 |
| Penicillium roseopurpureum chrysovirus 1 | hypothetical protein | 450 | 450 | 98% | 7.00E-142 | 31.10% | AYP71815.1 |
| Isaria javanica chrysovirus 1 | putative protease | 448 | 448 | 98% | 2.00E-141 | 33.25% | YP_009337841.1 |
| Penicillium raistrickii chrysovirus 1 | hypothetical protein PrCV1_s4gp1 | 448 | 448 | 99% | 3.00E-141 | 31.83% | AZT88570.1 |
| Beauveria bassiana chrysovirus 1 | hypothetical protein BbCV1_s4gp1 | 444 | 444 | 91% | 1.00E-139 | 32.28% | AZT88574.1 |
| Helminthosporium victoriae 145S virus | Hv145SV-protein 3 | 306 | 306 | 95% | 1.00E-87 | 28.90% | YP_052860.1 |
| Bipolaris maydis chrysovirus 1 | protein 3 | 301 | 301 | 100% | 7.00E-86 | 27.87% | ARM36037.1 |
| Colletotrichum gloeosporioides chrysovirus 1 | PP | 288 | 288 | 99% | 3.00E-81 | 27.54% | QCY49460.1 |
| Colletotrichum gloeosporioides chrysovirus 1 | putative protease | 287 | 287 | 96% | 7.00E-81 | 28.44% | YP_009667013.1 |
| Amasya cherry disease associated chrysovirus | putative protease | 265 | 265 | 95% | 5.00E-72 | 26.56% | CAH03666.1 |
| Amasya cherry disease associated chrysovirus | putative protease | 264 | 264 | 95% | 5.00E-72 | 26.80% | YP_001531161.1 |

**Supplementary Table S4.** BLASTP search with CnCV1-Rep-Assoc (dsRNA3) (only the top 20 hits shown).

| Description (virus [isolate]) | Description (protein) | Max score | Total score | Query cover | E value | Identity | Accession |
|---|---|---|---|---|---|---|---|
| Cryphonectria nitschkei chrysovirus 1 BS122 | putative replication associated protein | 1405 | 1405 | 97% | 0 | 96.22% | YP_009507944.1 |
| Cryphonectria nitschkei chrysovirus 1 OB5-11 | unknown | 572 | 572 | 36% | 0 | 99.28% | ABI20758.1 |
| Verticillium dahliae chrysovirus 1 | unknown | 451 | 451 | 95% | 4.00E-144 | 36.40% | YP_009507949.1 |
| Macrophomina phaseolina chrysovirus 1 | P3 | 101 | 101 | 48% | 4.00E-18 | 27.12% | YP_009667009.1 |
| Chrysothrix chrysovirus 1 | hypothetical protein | 73.9 | 73.9 | 52% | 2.00E-09 | 21.72% | QGR26540.1 |
| Isaria javanica chrysovirus 1 | hypothetical protein | 68.6 | 68.6 | 43% | 1.00E-07 | 24.19% | YP_009337890.1 |
| Penicillium chrysogenum virus | hypothetical protein | 67 | 67 | 44% | 3.00E-07 | 24.73% | YP_392484.1 |
| Penicillium raistrickii chrysovirus 1 | hypothetical protein PrCV1_s3gp1 | 67 | 67 | 44% | 3.00E-07 | 22.43% | AZT88569.1 |
| Penicillium italicum chrysovirus | p3 | 66.6 | 66.6 | 44% | 3.00E-07 | 25.00% | QCZ35878.1 |
| Wuhan insect virus 30 | hypothetical protein | 60.8 | 60.8 | 47% | 2.00E-05 | 21.85% | APG76049.1 |
| Beauveria bassiana chrysovirus 1 | hypothetical protein BbCV1_s3gp1 | 55.5 | 55.5 | 41% | 0.001 | 22.88% | AZT88573.1 |
| Anthurium mosaic-associated virus | putative RNA-dependent RNA polymerase | 50.4 | 50.4 | 18% | 0.031 | 24.67% | YP_009667023.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerase | 49.7 | 49.7 | 18% | 0.041 | 24.83% | AYD75751.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerase | 49.3 | 49.3 | 18% | 0.059 | 24.83% | AYD75744.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerase | 48.9 | 48.9 | 18% | 0.061 | 24.83% | AYD75747.1 |
| Helminthosporium victoriae 145S virus | Hv145SV-protein 4 | 48.9 | 48.9 | 16% | 0.094 | 29.51% | YP_052861.1 |
| Bipolaris maydis chrysovirus 1 | protein 4 | 48.5 | 48.5 | 11% | 0.1 | 32.22% | ARM36038.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerase | 48.9 | 48.9 | 18% | 0.11 | 24.83% | AYD75736.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerase | 48.5 | 48.5 | 18% | 0.11 | 24.83% | AYD75731.1 |
| Zea mays chrysovirus 1 | putative RNA-dependent RNA polymerase | 48.5 | 48.5 | 18% | 0.12 | 24.83% | YP_009551655.1 |

**Supplementary Table S5.** BLASTP search with CnCV1-CP (dsRNA4) (only the top 20 hits shown).

| Description (virus [isolate]) | Description (protein) | Max score | Total score | Query cover | E value | Identity | Accession |
|---|---|---|---|---|---|---|---|
| Cryphonectria nitschkei chrysovirus 1 BS321 | capsid protein | 1840 | 1840 | 96% | 0 | 98.12% | ACT79254.1 |
| Cryphonectria nitschkei chrysovirus 1 BS122 | capsid protein | 1840 | 1840 | 96% | 0 | 98.12% | YP_009507943.1 |
| Cryphonectria nitschkei chrysovirus 1 bs131 | capsid protein | 1783 | 1783 | 96% | 0 | 95.25% | ACT79252.1 |
| Verticillium dahliae chrysovirus 1 | capsid protein | 647 | 862 | 99% | 0 | 50.75% | YP_009507946.1 |
| Cryphonectria nitschkei chrysovirus 1 OB5-11 | capsid protein | 325 | 325 | 16% | 4.00E-102 | 100.00% | ABI20756.1 |
| Macrophomina phaseolina chrysovirus 1 | capsid protein | 350 | 350 | 98% | 2.00E-101 | 27.59% | YP_009667010.1 |
| Beauveria bassiana chrysovirus 1 | capsid protein | 312 | 312 | 98% | 1.00E-87 | 27.44% | AZT88572.1 |
| Aspergillus fumigatus chrysovirus | capsid protein | 311 | 311 | 98% | 1.00E-87 | 27.95% | YP_009508105.1 |
| Wuhan insect virus 30 | hypothetical protein | 300 | 300 | 64% | 1.00E-84 | 30.54% | APG76051.1 |
| Isaria javanica chrysovirus 1 | capsid protein | 297 | 297 | 59% | 2.00E-82 | 32.09% | YP_009337889.1 |
| Chrysothrix chrysovirus 1 | capsid protein | 293 | 293 | 84% | 5.00E-81 | 28.80% | QGR26539.1 |
| Penicillium raistrickii chrysovirus 1 | capsid protein | 289 | 289 | 98% | 2.00E-79 | 27.02% | AZT88568.1 |
| Penicillium roseopurpureum chrysovirus 1 | capsid protein | 287 | 287 | 67% | 3.00E-79 | 29.59% | AYP71813.1 |
| Penicillium chrysogenum virus | major capsid protein | 283 | 283 | 66% | 3.00E-77 | 29.23% | YP_392483.1 |
| Penicillium italicum chrysovirus | major capsid protein | 280 | 280 | 97% | 3.00E-76 | 26.32% | QCZ35877.1 |
| Helminthosporium victoriae 145S virus | putative capsid protein | 273 | 273 | 56% | 4.00E-74 | 30.60% | YP_052859.1 |
| Bipolaris maydis chrysovirus 1 | putative capsid protein | 270 | 270 | 56% | 4.00E-73 | 30.41% | ARM36036.1 |
| Colletotrichum gloeosporioides chrysovirus 1 | coat protein | 249 | 249 | 58% | 5.00E-66 | 28.93% | YP_009667014.1 |
| Amasya cherry disease associated chrysovirus | putative coat protein | 214 | 214 | 54% | 1.00E-53 | 25.98% | YP_001531162.1 |
| Amasya cherry disease associated chrysovirus | putative coat protein | 213 | 213 | 54% | 3.00E-53 | 26.17% | CAH03665.1 |

**Supplementary Table S6.** BLASTP search with a protein encoded by CnCV1-potential small ORF2 (dsRNA2) (only the top 10 hits shown).

| Description (virus [isolate]) | Description (protein) | Max score | Total score | Query cover | E value | Identity | Accession |
|---|---|---|---|---|---|---|---|
| *Bacillus* sp. P16(2019) | aminotransferase class I/II-fold pyridoxal phosphate-dependent enzyme | 43.9 | 43.9 | 42% | 0.11 | 36.36% | WP_143847740.1 |
| *Bacteroides stercoris* | L-rhamnose/proton symporter RhaT | 42.4 | 42.4 | 47% | 0.26 | 31.51% | WP_118940055.1 |
| *Bacteroides stercoris* | L-rhamnose/proton symporter RhaT | 42.4 | 42.4 | 47% | 0.27 | 31.51% | WP_117985973.1 |
| *Bacteroides stercoris* | L-rhamnose/proton symporter RhaT | 42.4 | 42.4 | 47% | 0.28 | 31.51% | WP_005652803.1 |
| *Bacteroides stercoris* | L-rhamnose/proton symporter RhaT | 42.4 | 42.4 | 47% | 0.28 | 31.51% | WP_117906676.1 |
| *Bacteroides stercoris* | L-rhamnose/proton symporter RhaT | 42.4 | 42.4 | 47% | 0.29 | 31.51% | WP_117741581.1 |
| *Porphyromonadaceae bacterium* | TPA: rhamnose/proton symporter RhaT | 40 | 40 | 47% | 0.32 | 28.77% | HBT84875.1 |
| *Dysgonomonas* sp. 216 | L-rhamnose/proton symporter RhaT | 42.4 | 42.4 | 47% | 0.34 | 28.77% | WP_163266912.1 |
| *Bacteroidales bacterium* St1 | L-rhamnose-proton symporter | 41.2 | 41.2 | 49% | 0.73 | 27.63% | KAA6300534.1 |
| *Bacteroides sp. CAG:1076* | l-rhamnose-proton symport protein (RhaT) | 40.8 | 40.8 | 47% | 0.85 | 27.40% | CCY92405.1 |

**Supplementary Table S7.** BLASTP search with a protein encoded by CnCV1-potential small ORF3 (dsRNA3).

| Description (virus [isolate]) | Description (protein) | Max score | Total score | Query cover | E value | Identity | Accession |
|---|---|---|---|---|---|---|---|
| Plasmopara viticola associated mymonavirus 1 | putative nucleocapsid | 88.6 | 88.6 | 64% | 3.00E-18 | 34.46% | QHD64781.1 |
| Tobacco leaf enation phytoreovirus | unknown | 47.4 | 47.4 | 64% | 0.022 | 28.48% | AAT97064.1 |
| *Drosophila busckii* | CG12869 | 42.4 | 42.4 | 47% | 1.1 | 27.52% | ALC41780.1 |
| *Drosophila busckii* | liver carboxylesterase 2 | 41.6 | 41.6 | 47% | 1.9 | 27.52% | XP_017835970.1 |
| Macrophomina phaseolina chrysovirus 1 | RNA-dependent RNA polymerase | 40.4 | 40.4 | 65% | 5.6 | 21.94% | YP_009667008.1 |

**Supplementary Table S8.** List of viruses used in the phylogenetic analysis of chrysovirus RdRPs**.**

| virus name | accession* | host (Class, Division) | abbreviation |
|---|---|---|---|
| ***viruses listed in the phylogenetic analysis in the 10th ICTV report for the family*** **Chrysoviridae** | | | |
| Agaricus bisporus virus 1 | X94361 | Agaricomycetes, Basidiomycota | AbV |
| Alternaria alternata chrysovirus 1 | LC350277 | Dothideomycetes, Ascomycota | AaCV1 |
| Amasya cherry disease associated chrysovirus | AJ781166 | (suspected fungal etiology) | ACDACV |
| Anthurium mosaic-associated virus | FJ899675 | Alismatales (Monocot plant) | AmaCV |
| Aspergillus fumigatus chrysovirus | FN178512 | Eurotiomycetes, Ascomycota | AfuCV |
| Aspergillus mycovirus 1816 | EU289896 | Eurotiomycetes, Ascomycota | AsV1816 |
| Bipolaris maydis chrysovirus 1 | KY489954 | Dothideomycetes, Ascomycota | BmCV1 |
| Botryosphaeria dothidea chrysovirus 1 | KF688736 | Dothideomycetes, Ascomycota | BdCV1 |
| Brassica campestris chrysovirus 1 | KP782031 | (Dicotyledonae, - ) | BcCV1 |
| Cherry chlorotic rusty spot associated chrysovirus | AJ781397 | (suspected fungal etiology) | CCRSCV |
| Colletotrichum fructicola chrysovirus 1 | MG425969 | Sordariomycetes, Ascomycota | FcCV1 |
| Colletotrichum gloeosporioides chrysovirus 1 | KT581957 | Sordariomycetes, Ascomycota | CgCV1 |
| Cryphonectria nitschkei chrysovirus 1 | LC062729 | Sordariomycetes, Ascomycota | CnCV1 |
| Fusarium graminearum dsRNA mycovirus-2 | HQ343295 | Sordariomycetes, Ascomycota | FgV2 |
| Fusarium graminearum mycovirus China-9 | HQ228213 | Sordariomycetes, Ascomycota | FgV-Ch9 |
| Fusarium oxysporum chrysovirus 1 | EF152346 | Sordariomycetes, Ascomycota | FoCV1 |
| Fusarium oxysporum f. sp. Dianthi virus | KP876629 | Sordariomycetes, Ascomycota | FoDV |
| grapevine associated chrysovirus 1 | GU108588 | (suspected fungal etiology) | gacv1 |
| Helminthosporium victoriae 145S virus | AF297176 | Dothideomycetes, Ascomycota | HvV145S |
| Isaria javanica chrysovirus 1 | KX898416 | Sordariomycetes, Ascomycota | IjCV1 |
| Macrophomina phaseolina chrysovirus 1 | KP900886 | Dothideomycetes, Ascomycota | MpCV1 |

| | | | |
|---|---|---|---|
| Magnaporthe oryzae chrysovirus 1-A | AB560761 | Sordariomycetes, Ascomycota | MoCV1A |
| Magnaporthe oryzae chrysovirus 1-B | AB824667 | Sordariomycetes, Ascomycota | MoCV1B |
| Penicillium chrysogenum virus | AF296439 | Eurotiomycetes, Ascomycota | PcV |
| Penicillium janczewskii chrysovirus 1 | KT601115 | Eurotiomycetes, Ascomycota | PjCV1 |
| Penicillium janczewskii chrysovirus 2 | KT950836 | Eurotiomycetes, Ascomycota | PjCV2 |
| Persea americana chrysovirus | KJ418374 | (Dicotyledonae, - ) | PaCV |
| Raphanus sativus chrysovirus 1 | JQ045335 | (Dicotyledonae, - ) | RsCV1 |
| Shuangao chryso-like virus | MF176340 | (suspected insect etiology) | ShuaCV |
| Tolypocladium cylindrosporum virus 2 | FR750563 | Sordariomycetes, Ascomycota | TcV2 |
| Verticillium dahliae chrysovirus 1 | HM004067 | Sordariomycetes, Ascomycota | VdCV1 |
| Wuhan insect virus 29 | KX882987 | (suspected insect etiology) | WuV29 |
| ***additional chrysoviruses from recent reports*** | | | |
| Chrysothrix chrysovirus 1 | MN625832 | Arthoniomycetes, Ascomycota | CcCV1 |
| Fusarium sacchari chrysovirus 1 | MN295964 | Sordariomycetes, Ascomycota | FsCV1 |
| Lepraria chrysovirus 1 | MN393162 | Lecanoromycetes, Ascomycota | LiCV1 |
| Penicillium crustosum chrysovirus 1 | not available | Eurotiomycetes, Ascomycota | PcCV1 |
| Penicillium italicum chrysovirus 1 | MK214380 | Eurotiomycetes, Ascomycota | PiCV1 |
| ***outgroup* Totiviridae *viruses*** | | | |
| Saccharomyces cerevisiae virus L-A | J04692 | Saccharomycetes, Ascomycota | ScV-LA |
| Rosellinia necatrix victorivirus 1 | AB742454 | Sordariomycetes, Ascomycota | RnVV1 |
| Helminthosporium victoriae 190S virus | U41345 | Dothideomycetes, Ascomycota | HvV190S |

*those in bold letters are sequence of ICTV-approved definitive viruses

1

# Fig. S1

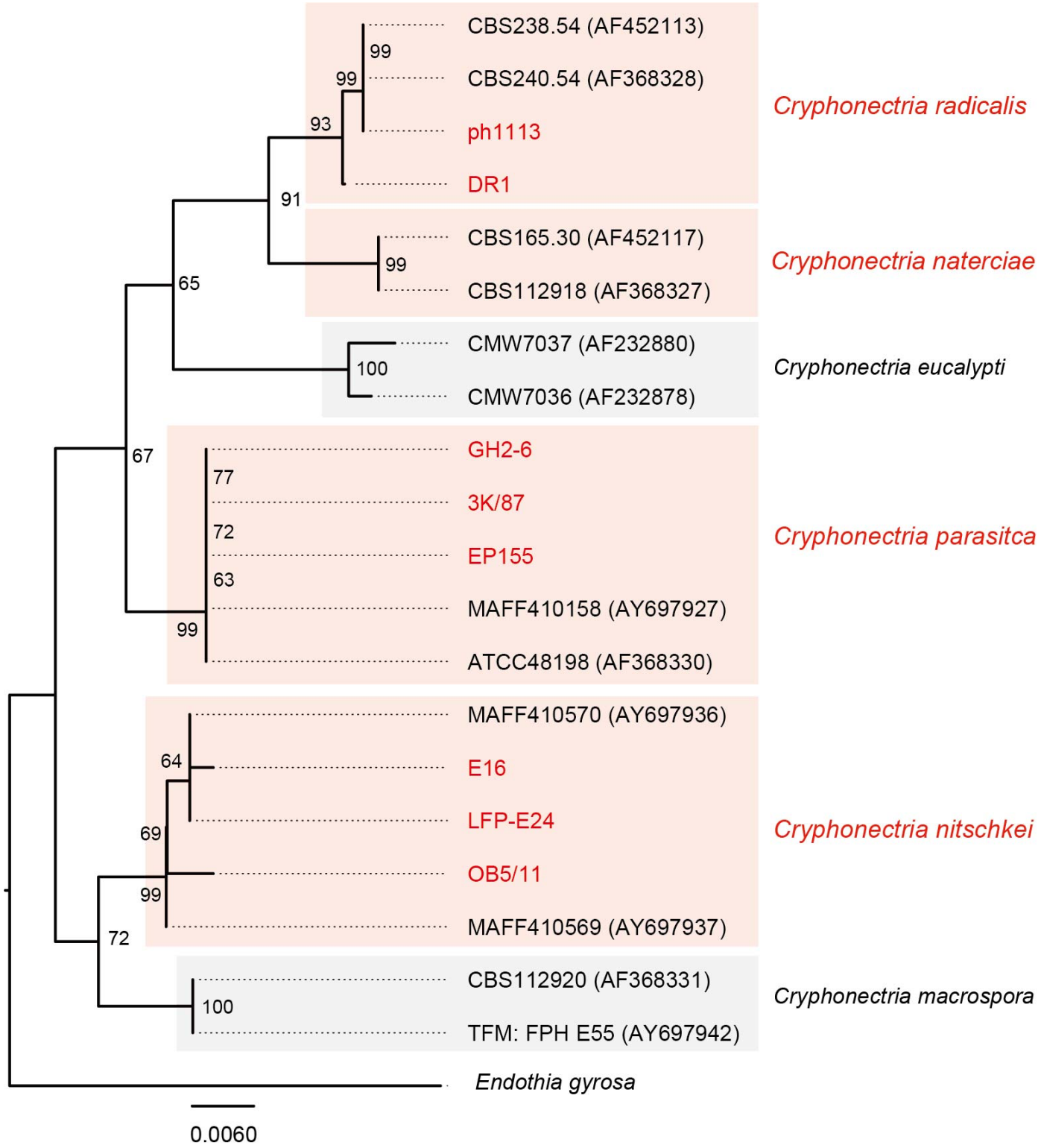
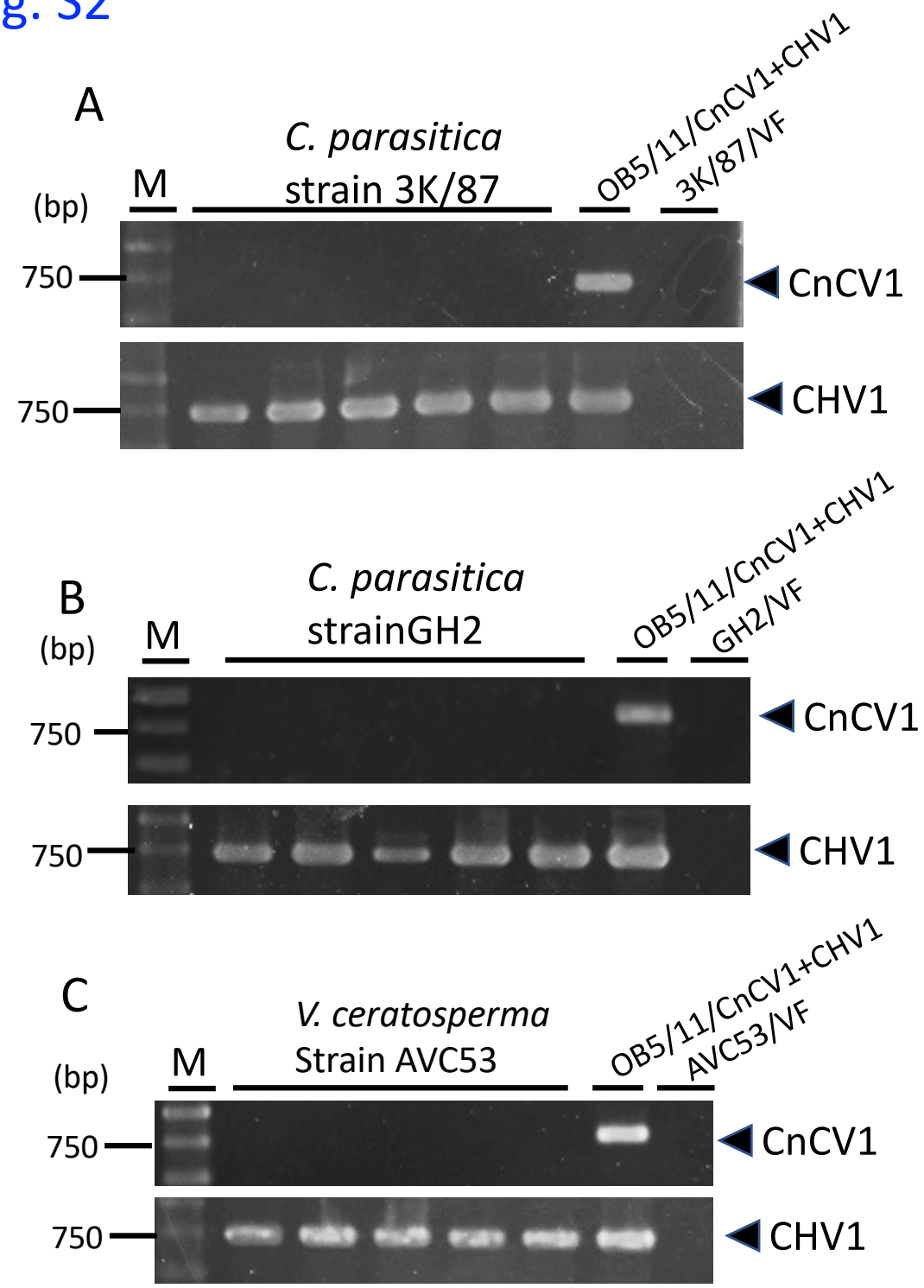

CBS238.54 (AF452113)
CBS240.54 (AF368328)
ph1113
DR1
Cryphonectria radicalis
CBS165.30 (AF452117)
CBS112918 (AF368327)
Cryphonectria naterciae
CMW7037 (AF232880)
CMW7036 (AF232878)
Cryphonectria eucalypti
GH2-6
3K/87
EP155
MAFF410158 (AY697927)
ATCC48198 (AF368330)
Cryphonectria parasitca
MAFF410570 (AY697936)
E16
LFP-E24
OB5/11
MAFF410569 (AY697937)
Cryphonectria nitschkei
CBS112920 (AF368331)
TFM: FPH E55 (AY697942)
Cryphonectria macrospora
Endothia gyrosa
0.0060

Fig. S2

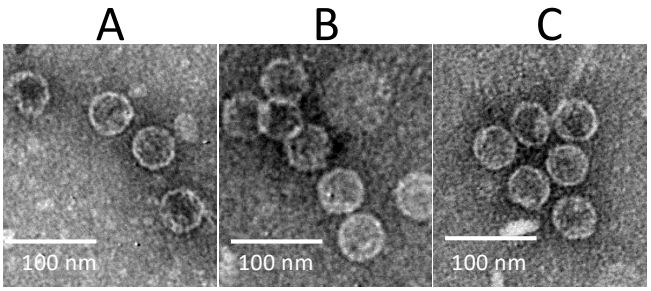



D

| Primer | Sequence 5’ - 3’ | Direction | Remarks |
|---|---|---|---|
| CHV1-3185-F | GATAGGATTAAGTCACGAGGC | Forward | CHV1 RT-PCR |
| CHV1-3925-R | CAAGAAATCCCCAGGTAGCC | Reverse | |
| CnCV1-Rd-F | ACCGAGTTTATCCATGTCGT | Forward | CnCV1 RT-PCR |
| CnCV1-Rd-R | ATCTCCAGTTATTCACCCGTG | Reverse | |

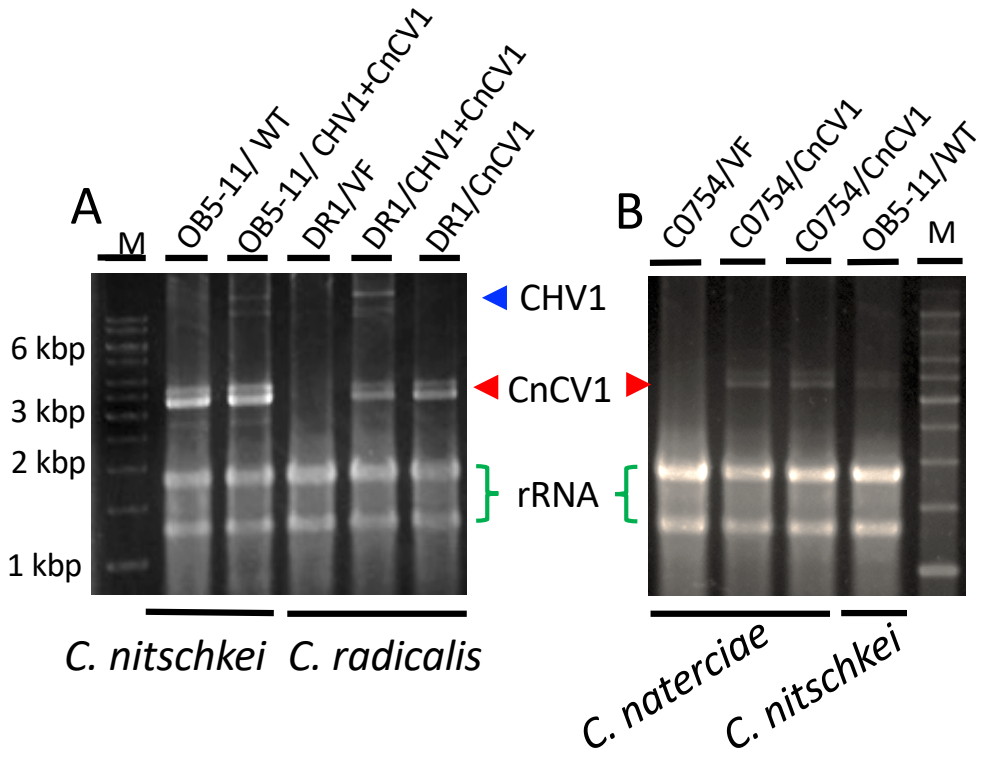
# Fig. S3

CnCV1 particles

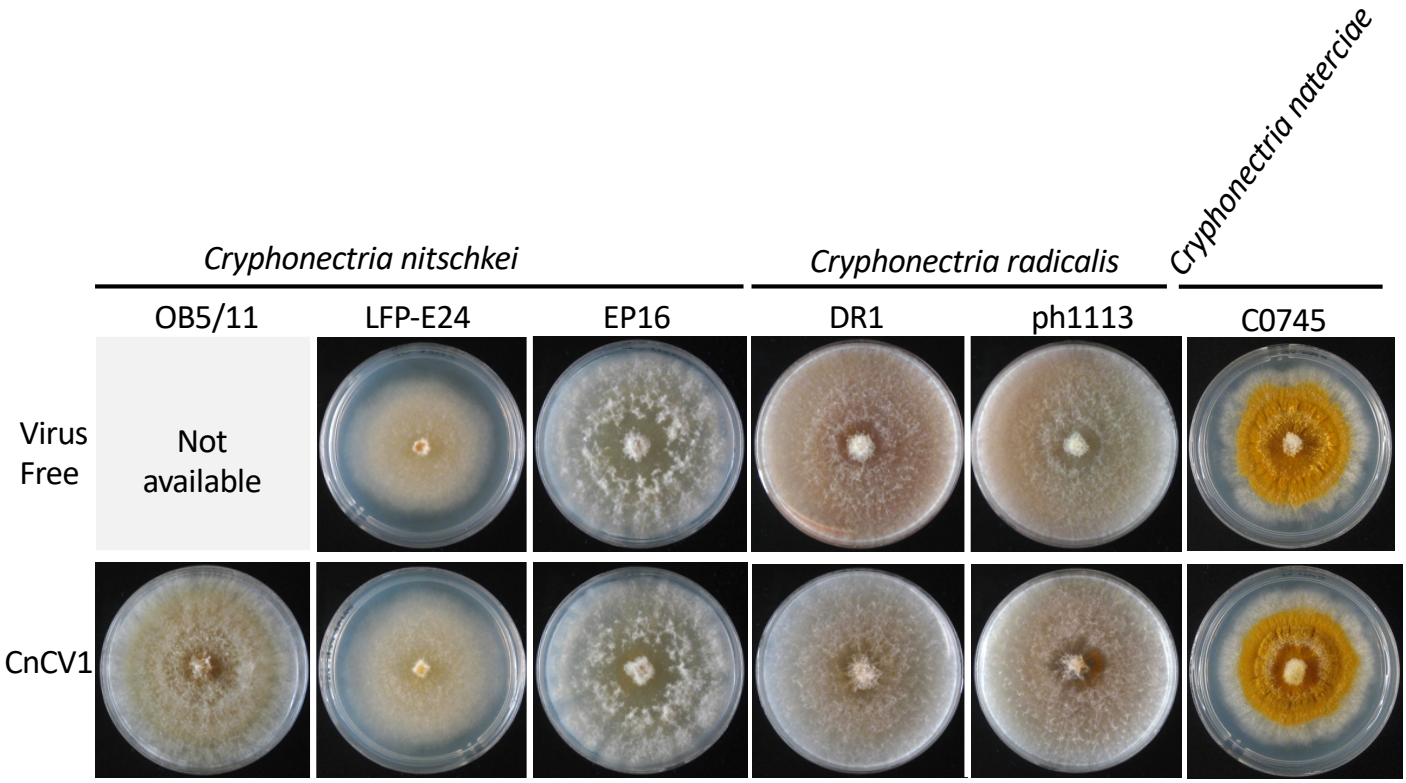



A: *C. radicalis* strain ph1113
B: *C. radicalis* strain DR1
C: *C. naterciae* strain C0754

Fig. S4


A
M
OB5-11/ WT
OB5-11/ CHV1+CnCV1
DR1/VF
DR1/CHV1+CnCV1
DR1/CnCV1
6 kbp
3 kbp
2 kbp
1 kbp
CHV1
CnCV1
rRNA
C. nitschkei
C. radicalis
B
C0754/VF
C0754/CnCV1
C0754/CnCV1
OB5-11/WT
M
C. naterciae
C. nitschkei

# Fig. S5


Cryphonectria nitschkei
Cryphonectria radicalis
Cryphonectria naterciae
OB5/11
LFP-E24
EP16
DR1
ph1113
C0745
Virus Free
Not available
CnCV1