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Full-length genome characterization of a new fusagravirus hosted by the spring orange peel fungus *Caloscypha fulgens*

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Summary. – Mycoviruses widely exist in diverse lineages of fungi, yet there are only a few studies on mycovirus infection in uncultivated fungi. We here report the presence of a dsRNA mycovirus in saprotrophic spring orange peel fungus *Caloscypha fulgens*. A novel dsRNA virus, named "Caloscypha fulgens fusagravirus 1" (CfFV1), was isolated from a single ascocarp of *C. fulgens*, and its molecular features were revealed. The full-length cDNA of CfFV1 comprises 9,548 nucleotides with a calculated GC content of 47.9% and two discontinuous open reading frames (ORF 1 and 2). A-1 ribosomal frameshift region with two distinctive elements, including a canonical slippery heptanucleotide (AAAAAAC) and a pseudoknot structure, predicted as a Recoding Stimulatory Element, was detected in the junction region of ORF1 and ORF2. The deduced amino acid sequence of ORF1 and ORF2 showed the highest similarity to the putative structural protein and RNA-dependent RNA polymerase (RdRp) of Rosellinia necatrix fusagravirus 4 (RnFGV4). Genome organization, sequence similarity, and phylogenetic analysis indicate that this virus belongs to a new member of the proposed family *Fusagraviridae*. This is the first report of the presence of a mycovirus in the spring orange peel fungus *C. fulgens*.

Keywords: mycovirus; dsRNA; proposed Fusagraviridae; uncultivated fungi; Caloscypha fulgens

Introduction

Mycoviruses specifically infect members of the kingdom fungi and propagate in fungal cells. They are ubiquitously present in fungal species from various lineages, including, phytopathogenic, mycorrhizal, and saprotrophic fungi (Ghabrial *et al*., 2015; Hillman *et al*., 2018; Sahin and Akata, 2018; Sutela *et al*., 2019). The majority of the known mycoviruses have either a double-stranded RNA (dsRNA) or positive sense, single stranded RNA (+ssRNA) genomes. However, negative-sense, single-stranded RNA (-ssRNA) and single-stranded circular DNA mycoviruses have also been reported in filamentous fungi (Yu *et al*., 2010; Liu *et al*., 2014; Donaire *et al*., 2016). According to the current ICTV Master species list 2019, mycoviruses with a dsRNA genome are classified into eight families, whose members have different numbers of genome segments, including *Amalgaviridae* (1 segment), *Chrysoviridae* (4–5 segments), *Megabirnaviridae* (2 segments), *Partitiviridae* (2 segments), *Polymycoviridae* (4 segments), *Quadriviridae* (4 segments), *Reoviridae* (11–12 segments) and *Totiviridae* (1 segment) (https://talk.ictvonline.org/taxonomy/). Along with the development of viral metagenomic approaches, the number of studies on viral diversity, particularly

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Abbreviations: aa = amino acid; cDNA = complementary DNA; CfFV1 = Caloscypha fulgens fusagravirus 1; dsRNA = double-stranded RNA; HTS = high-throughput sequencing; ICTV = International Committee on Taxonomy of Viruses; nt = nucleotide; ORF = open reading frame; RdRp = RNA dependent RNA polymerase; RLM-RACE = RNA ligase-mediated rapid amplification of cDNA ends; RnFGV4 = Rosellinia necatrix fusagravirus 4; rPCR = random polymerase chain reaction; ssRNA = single-stranded RNA; UTR = untranslated region

those on unveiling new viruses, is rapidly increasing (Gilbert *et al*., 2019; Starr *et al*., 2019; Myers *et al*., 2020). Some lately discovered mycoviruses have been shown to exhibit molecular and biological characteristics that are significantly different from other known viruses, and new virus families such as *Yadokariviridae* and *Megatotiviridae* have been proposed to accommodate these unassigned mycoviruses (Zhang *et al*., 2016; Arjona-Lopez *et al*., 2018; Hisano *et al*., 2018; Sahin *et al*., 2020). In this sense, the family *Fusagraviridae* is one of the recently proposed virus families and its members have mostly been identified in fungi (Wang *et al*., 2016). The members of this family are characterized by having monopartite, linear dsRNA genomes that often exceed 9 kbp and comprise two discontinuous ORFs harboring a putative slippery heptanucleotide in their junction region. A relatively large 5'-UTR sequence and a Phytoreo S7 domain residing downstream of the RdRp domain are other characteristics of the family. On the other hand, some other mycoviruses that are closely related to the *Fusagraviridae* members, but lacking one or more common characteristics of the family, such as putative slippery heptanucleotide, Phytoreo S7 domains, and long 5'-UTR sequences (De Wet *et al*., 2011; Abreu *et al*., 2015), have also been regarded as members of this family (Arjona-Lopez *et al*., 2018).

Caloscypha, including single species *Caloscypha fulgens* (Pers.) Boud., is a monotypic genus, belonging to the family *Caloscyphaceae* within the order *Pezizales* (Ascomycota), which is also known as the spring orange peel fungus, grows under hardwood and coniferous trees, on mossy ground, leaf litters or needle mats in spring and early summer (Kirk, 2008). Sessile to short stalk, up to 40 mm broad, deeply cup-shaped apothecia with dark yellow to olivaceous orange outside, pale yellow to bright orange interior are characteristics of the species (Hansen, 2000). Although it is not poisonous, there is no information about the consumption of *Caloscypha fulgens* in Turkey.

Currently, there is little information on viruses hosted by uncultivated or wild fungi. Studying viruses of fungal species with no apparent economic importance is also crucial in the sense of obtaining a more objective view of the diversity, evolution, and ecology of mycoviruses. According to the literature, no viruses have hitherto been reported from the species of the genus *Caloscypha*. In this study, we investigated the possible presence of viral infections in a single isolate of *C. fulgens* with the help of high-throughput sequencing (HTS). By combining HTS and RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE), we were able to identify and characterize the complete genome sequences of a novel mycovirus belonging to the proposed family *Fusagraviridae*.

Materials and Methods

Sampling of C. fulgens ascocarp. A single ascocarp of *C. fulgens* ANK Akata 7325 (Fig. 1a) was sampled under an individual of a natural population of *Abies nordmanniana* subsp. equitrojani (Trojan fir) located in the Ilgaz Mountain of Kastamonu province, North-Western Turkey (Lat/Long: N 41° 05' - E 33° 45'; Alt: 1600 m) during a field study conducted on the 26th of April 2020. The surface of the ascocarp specimen was sterilized by consecutive treatment with 2% (w/v) sodium hypochlorite for 2 minutes and 70% ethanol for 10 s. After surface-sterilization, the ascocarp specimen was rinsed with sterile distilled water.

dsRNA enrichment, cDNA synthesis, and PCR amplification. Double-stranded RNA was enriched from the hymenium layer of the ascocarp using a cellulose-column-based Double-RNA Viral dsRNA Extraction Kit (iNtRON Biotechnology). The isolated dsRNA was consecutively subjected to DNase I and S1 nuclease treatment according to the manual of the manufacturer (Promega). The nuclease-treated dsRNA sample was purified using a GeneJET PCR Purification Kit (Thermofisher) and its integrity was evaluated on a 1% agarose gel (Fig. 1b). The purified dsRNA sample was reverse transcribed into cDNA using the primer-dN6 (5'-CCTGAATTCGGATCCTCCNNNNNN-3') and Transcriptor First Strand cDNA Synthesis Kit (Roche). Briefly, reverse transcription was carried out in a 10 µl volume containing 1X Transcriptor RT Reaction Buffer (Roche), 0.5 mM dNTPs, 0.5 mM of primer-dN6, 10 units of Transcriptor Reverse Transcriptase, and 1 µl of heat-denatured dsRNA (50-100 ng). Reverse transcription was performed with an initial annealing step at 25°C for 5 min, followed by incubation at 55°C for 30 min and a final enzyme denaturation step at 85°C for 5 min. The synthesized cDNA was used as a template for random PCR (rPCR), which was carried out as described elsewhere (Darissa *et al*., 2010). Briefly, PCR amplification was performed in a 50 µl reaction volume containing 1× Easy-A High-Fidelity PCR Master Mix (Agilent Technologies), 1 mM rPCR primer (5'-CCTGAATTCGGATCCTCC-3'), and 1 µl of cDNA. The thermal cycling was performed with 1 cycle at 95°C for 2 min, 65°C for 1 min, and 72°C for 1 min, then 35 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min followed by a final extension step at 72°C for 7 min. rPCR amplicon was purified using a GeneJET PCR Purification Kit (Thermofisher). Five hundred nanograms of the purified amplicon was delivered to the Novogene (Cambridge, United Kingdom) for library construction, and high-throughput sequencing of 150 bp long paired ends with a sequencing depth of ≥ 100X on a Novaseq 6000 platform (Illumina).

Bioinformatic analyses of the sequence data. Raw reads obtained from HTS were assembled *de novo* by using the CLC Genomic Workbench version 20.0.2 (Qiagen) software to generate contigs. Assembly parameters were adjusted as word size of 26, default bubble size of 50, automatic paired distance estimation, and a minimum contig length of 200 nt. Assembled

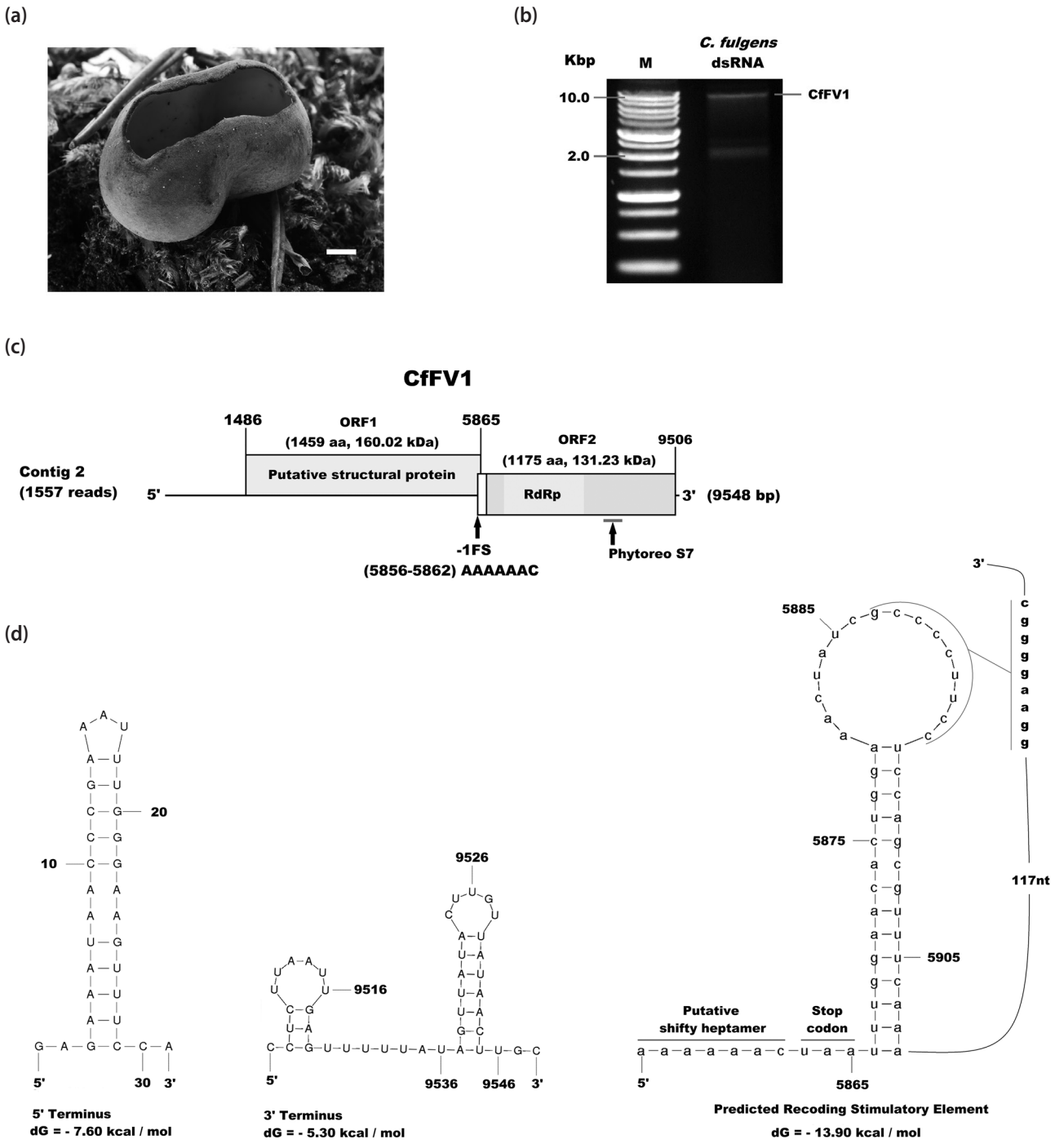


Fig. 1

Molecular analyses of Caloscypha fulgens fusagravirus 1 (CfFV1)

(a) A single ascocarp of *Caloscypha fulgens* Scale bar: 5 mm **(b)** Agarose gel image of dsRNA enriched from *C. fulgens* ANK Akata 7325. Lane M, DNA ladder. The genome segment belonging to CfFV1 is indicated with red lines. **(c)** The schematic depiction of the genome organization of CfFV1. Numbers indicate total lengths of the CfFV1 genome segment and positions of start and stop codons. The positions of 5'- and 3'-UTR regions, the slippery heptanucleotide sequence, and the RdRp and Phytoreo S7 domains are also shown. **(d)** Predicted secondary structures of the 5'- and 3'-UTR regions (left and middle) and the recoding stimulatory element (right) of the coding strand of CfFV1. Numbers indicate nucleotide positions. For the predicted secondary structure of the recoding stimulatory element, the putative slippery heptanucleotide sequence of AAAAAAC and the stop codon are indicated with blue and red colors, respectively.

contigs and their deduced amino acid sequences were analyzed by Batch BLASTx and BLASTp respectively to identify virus-associated contigs (e-value < 1). The Pfam protein family database (https://pfam.xfam.org/) was utilized to determine virus-related domains including RNA-dependent RNA polymerase.

Deduced amino acid sequences of virus-associated contigs were aligned using ClustalW in MEGA X software (Kumar *et al*., 2018) and analyzed to find the best amino acid substitution model. Maximum likelihood (ML) phylogenetic trees were constructed by using the Nearest-Neighbor-Interchange (NNI) algorithm for the ML Heuristic method by applying 1000 bootstrap replicates.

Sequencing of the 5'- and 3'-terminals using RLM-RACE. To determine the sequences of 5'- and 3'-terminals, the 3' ends of the purified dsRNA were ligated to the oligonucleotide RLO (5'p-CATGGTGGCGACCGGTAG-NH23') at 37°C for 6 h followed by overnight treatment at 12°C with T4 RNA ligase 1 (New England Biolabs). Oligo-ligated dsRNA was purified using a GeneJET PCR Purification Kit (Thermo Scientific) and reverse transcribed into cDNA using the primer RTP (5'-CTACCGGTCGCCAC CATG-3'), which is complementary to the RLO oligonucleotide, and a Transcriptor First Strand cDNA Synthesis Kit (Roche) as described above. The 5'- and 3'-terminal sequences were PCR amplified with the sequence-specific reverse and forward primers, CfV5 (5'-GCAGACAAAAGAAAACGGACAGA-3') and CfV3 (5'-TGCTCAGGTCCATTCAAGCTC-3'), respectively, each of which was coupled with the RTP primer in the PCR step. The amplicons were cloned into pGEM-T Easy Vector (Promega) and directly sequenced with Sanger sequencing method using the universal M13 primers at the laboratory of Eurofins Genomics (Germany).

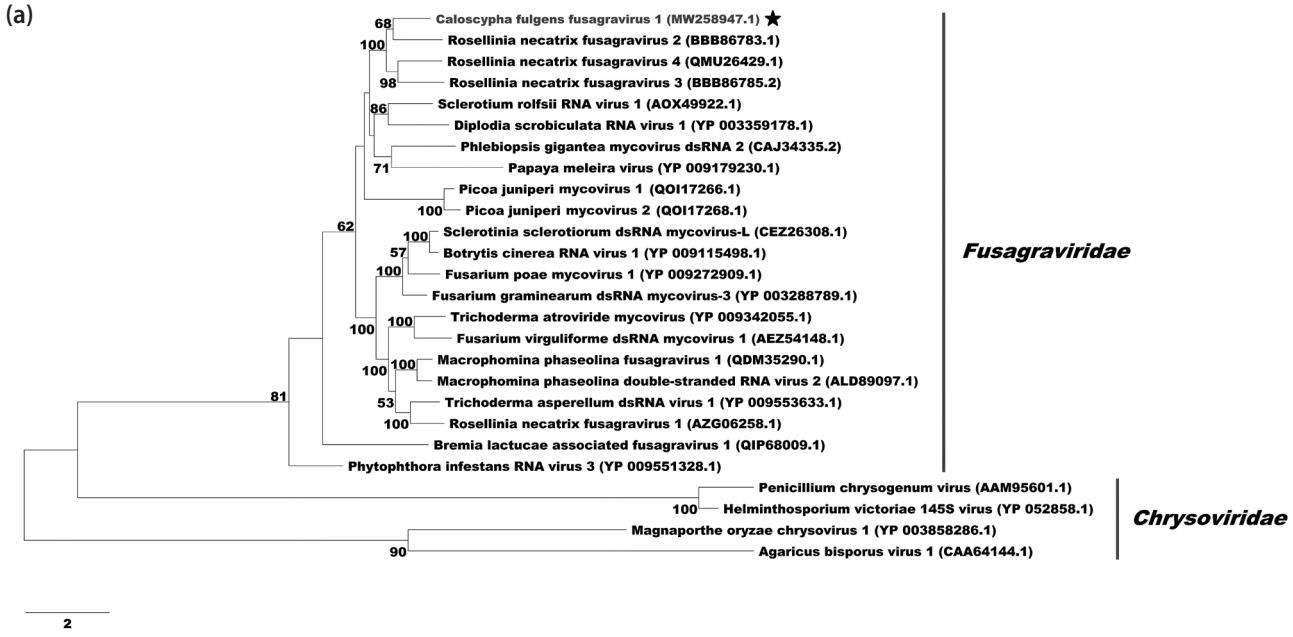
RdRp and Phytoreo S7 sequence alignment. The most conserved motifs found in the palm subdomain of the identified RdRps (Motifs A, B, and C) and Phytoreo S7 domain of CfFV1 were identified by taking the relevant RdRp and Phytoreo S7 domains of different mycoviruses as references. The conserved motifs of both identified and representative RdRps and Phytoreo S7 domains were then aligned using Clustal Omega online tool and AliView software, respectively, (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Larsson, 2014) by using the default parameters to reveal their amino acid sequence identities and variations.

Results and Discussion

Among the assembled contigs, other than those belonging to the host RNA sequence of *C. fulgens*, one long contig (contig 2 with 1557 reads) with a size of 8469 nucleotides (nt) that encodes two distinct proteins, related to the proteins encoded by fusagraviruses, was identified. We thereby considered this contig as a partial genomic sequence of a mycovirus, which was designated as Caloscypha fulgens fusagravirus 1 (CfFV1). Finally, by using RLM-RACE approach, we obtained the complete genome sequence of CfFV1 and submitted it to the GenBank database under the accession number MW258947.1. The complete genome of CfFV1 contains a dsRNA with a length of 9548 nt that comprises two discontinuous ORFs, designated as ORF1 (nt 1486–5865) and ORF2 (nt 5979–9506) (Fig. 1c). The complete cDNA sequence of the CfFV1 genome and the amino acid sequences of the putative structural and RdRp proteins encoded by the ORF1 and ORF2 of CfFV1 genome are given in Supplementary Fig. 1.

The length of the 5'-UTR and 3'-UTR was 1485 nt and 42 nt, respectively (Fig. 1c). The sequences at the 5'- and 3'-termini of the coding strand of CfFV1 were predicted to form stem-loop structures, and their initial Gibbs free energy values were calculated as -7.60 kcal/mol and -5.30 kcal/mol, respectively (Fig. 1d). The ORF1 of CfFV1 encodes a putative structural protein comprising 1459 amino acids (aa), with a predicted molecular weight of 160.02 kDa. The BLASTp search of the aa sequence of the putative structural protein encoded by CfFV1 ORF1 showed that it shared the highest sequence identity (30.84%) with the putative structural protein of Rosellinia necatrix fusagravirus 4 (RnFGV4) (Chun and Kim, 2020a). Besides, a search for the presence of possible conserved domains using Pfam protein family database revealed that the ORF1-encoded putative structural protein does not contain any known conserved domains. Although it remains uncertain whether members of *Fusagraviridae* form rigid virions (Wang *et al*., 2016), electron microscopic studies performed with the purified preparations of a newly described fusagravirus Trichoderma atroviride mycovirus 1 (TaMV1) suggested that the ORF1 of TaMV1 encodes a structural/gag protein product to form isometric virion particles with a diameter of approximately 40 nm (Chun *et al*., 2020b).

CfFV1 genome has a putative slippery heptanucleotide sequence (5856AAAAAAC5862) located immediately upstream of the ORF1 stop codon (Fig. 1c). Additionally, a putative recoding stimulatory element (RSE) structure (an RNA pseudoknot located at nt 5865 to 6035) was predicted to reside immediately downstream of the ORF1 stop codon (Fig. 1d). These two functional cis-acting elements found in CfFV1 genome are suggested to regulate a -1 ribosomal frameshifting event that results in translation of the ORF2-encoded protein. The ORF2 of CfFV1 encodes an RNA-dependent RNA polymerase (RdRp) consisting of 1175 aa, with a predicted molecular weight of 131.23 kDa. A BLASTp search of the protein encoded by CfFV1 ORF2 revealed its best hit as the RdRp of RnFGV4 with a percent identity rate of 34.88%. A search of the conserved protein domains and multiple protein sequence alignment showed that the ORF2-encoded protein contains a conserved RdRp domain (RdRp_4, pfam02123) with



(b)

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                                  MOTIF A              MOTIF B              MOTIF C
Papaya meleira virus              [544]LMFDYSDFNINHSI[60]MRSLESGERGTSFINTFLSN[23]WLQGDDVFA[487]
Bremia lactucae associated fusagravirus 1  [392]LAWDYADFNLNHEL[54]SRSMMTGTRGTAFTNTLLNG[23]TGRGDDMIS[402]
Trichoderma atroviride mycovirus  [466]LMWDYADFNLNHTF[61]VRSLQSGERGTSSINSDSNE[23]DHAGDDAFE[622]
Macrophomina phaseolina fusagravirus  [522]LMWDFSDFNINHTF[61]VRSLQSGERGTSFVNSMRNH[23]DKQGDDVFL[622]
Fusarium poae mycovirus 1         [614]CMWDYADFNINHTF[61]ARSLQSGERGTSFTNTLRSN[23]DKTGDDVFL[614]
Sclerotinia sclerotiorum dsRNA mycovirus-L  [608]FMWDFADFNINHTF[61]KRSLQSGERATMWVNTLRNN[23]HKTGDDVFL[604]
Botrytis cinerea RNA virus 1      [608]FMWDFADFNINHTF[61]KRSLQSGERATMWVNTLRNN[23]DKTGDDVFL[604]
Picoa juniperi mycovirus 1        [181]VMWDYSDFNINHTF[63]ARSMQSGERMTSFINTFSSR[31]YHLGDDVWA[529]
Phlebiopsis gigantean mycovirus dsRNA 2  [433]LMWDFSDFNINHQK[61]LRCLASGERATSFTNTILSR[23]SQQGDDVFA[594]
Diplodia scrobiculata RNA virus 1  [433]FMWDYADFNINHSR[59]VRSLASGERATSFVNTVLSR[23]DHQGDDVFA[553]
Rosellinia necatrix fusagravirus 4  [425]LMWDYSDFNINHSV[65]VRSLQSGERLTSTTNTYLNR[23]YHQGDDAFE[641]
Rosellinia necatrix fusagravirus 3  [408]LMWDYADFNINHTF[73]VRSLQSGQRSTSFTNTVLNR[23]FHLGDDVFL[638]
Caloscypha fulgens fusagravirus 1  [431]LMWDYSDFNINHSF[61]VRSLQSGERATSTVNTFLSR[23]DHQGDDVWA[618]
                                        :*::***:**       *.: :* * *   *:  .        ***
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Fig. 2

Phylogeny and sequence comparison of the RdRp encoded by CfFV1

(a) Unrooted phylogenetic tree of the RdRp domain of CfFV1 and related fusagraviruses and chrysoviruses constructed by a maximum-likelihood method. Several viruses from the proposed family *Fusagraviridae* including Fusarium poae mycovirus 1 were included in the analysis. The numbers at the branches show bootstrap values (>50%). The bar (lower left) represents a genetic distance of 2. **(b)** Sequence comparison of the RdRp catalytic palm subdomains (motifs A, B, and C) of CfFV1 and related viruses from the proposed the family *Fusagraviridae*.

all seven conserved motifs (G, F, A, B, C, D, and E from N terminal to C terminal) that are characteristic of the viral RdRps (Ganeshpurkar, 2019). Moreover, detailed sequence analysis of the ORF2-encoded protein revealed the presence of a conserved Phytoreovirus S7 domain (S7; pfam07236) located downstream of the RdRp domain. The identified Phytoreo S7 domain of CfFV1 is 104 aa long (aa 736 to 839), and polypeptides exhibiting sequence homology to Phytoreo S7 domain were also reported from other fusagraviruses and related mycoviruses (Wang *et al*., 2016).

To reveal the relationship between CfFV1 and other mycoviruses, we conducted a phylogenetic analysis using the RdRp of CfFV1 and RdRps of 25 other selected dsRNA viruses (Fig. 2a). As a result, CfFV1 clustered in a well-supported clade including 21 other unassigned dsRNA mycoviruses that are suggested to be members of the proposed family *Fusagraviridae*. However, CfFV1 and its closely related dsRNA viruses were found to be distantly related to the clade consisting of members of the family *Chrysoviridae* (Fig. 2a). Multiple sequence alignment of the RdRp domains obtained from 13 different members of the family *Fusagraviridae* revealed that RdRp of CfFV1 possesses all seven conserved motifs (A-G) including the three most conserved RdRp motifs (motifs A, B, and C)

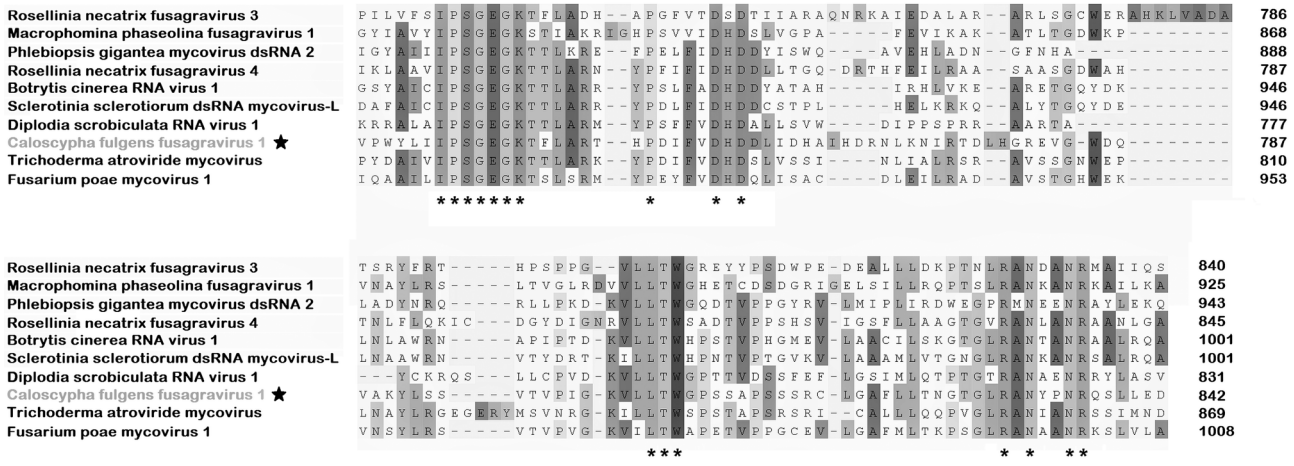

Fig. 3

Multiple sequence alignment of the Phytoreo S7 domains of CfFV1 and various related fusagraviruses
The default color scheme for ClustalW alignment in the AliView program was used. The conserved amino acid residues are indicated with asterisks. The positions of the amino acids are also indicated for each row.

found in the catalytic palm subdomain (Fig. 2b). Both magnesium coordinating aspartate residues in motifs A and C, and rNTP binding asparagine residue in motif B of CfFV1 were aligned with those of RdRps from other fusagravirus members (Fig. 2b). Besides, while the function of Phytoreo S7 domains in dsRNA viruses other than phytoreoviruses is not known and yet to be addressed, multiple sequence alignment of the Phytoreo S7 domains of CfFV1 and other related viruses revealed that the sequences are presumably conserved among different fusagraviruses (Fig. 3). CfFV1 possesses all of the features that are characteristics of the fusagraviruses including a genome size of >9 kbp, relatively long 5' UTR sequence, slippery heptanucleotide, and Phytoreo S7 domain. Therefore, CfFV1 is a potential new member of the proposed family *Fusagraviridae*.

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Supplementary information is available in the online version of the paper.

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