Molecular characterization of a novel alternavirus infecting the entomopathogenic fungus *Cordyceps chanhua*

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Abstract

In this study, a novel double-stranded (ds) RNA mycovirus, named *Cordyceps chanhua alternavirus 1* (CcAV1), was detected in the entomogenous fungus *Cordyceps chanhua* in China and characterized. The complete genome of CcAV1 is composed of three dsRNA segments: dsRNA 1 (3,512 bp), dsRNA 2 (2,655 bp), and dsRNA 3 (2,415 bp). Each of the three dsRNAs possesses a single open reading frame (ORF). dsRNA 1 encodes a putative RNA-dependent RNA polymerase (RdRp), and dsRNA 2 and dsRNA 3 encode hypothetical protein 1 (HP 1) and hypothetical protein 2 (HP 2), respectively. The predicted amino acid sequences of the putative RdRp, HP 1, and HP 2 had the highest identity of 66.99%, 49.30%, and 56.91%, respectively, to those of *Aspergillus foetidus* dsRNA mycovirus. A maximum-likelihood phylogenetic tree based on RdRp amino acid sequences showed that CcAV1 clustered with members of the proposed family “*Alternaviridae*”. Hence, we propose that *Cordyceps chanhua alternavirus 1* is a novel member of the proposed family “*Alternaviridae*”.

Most of the mycoviruses identified so far have been found to have double-stranded (ds) RNA genomes [1]. The International Committee on Taxonomy of Viruses (ICTV) (https://talk.ictvonline.org/) has classified dsRNA mycoviruses into eight families, including *Amalgaviridae*, *Chrysoviridae*, *Megabirnaviridae*, *Partitiviridae*, *Polymycoviridae*, *Quadrioviridae*, *Reoviridae*, and *Totiviridae*, and the genus *Botybirmavirus*. The establishment of another family, “*Alternaviridae*”, is still awaiting approval by the ICTV. Members of the proposed family “*Alternaviridae*” have a genome consisting of at least three dsRNA segments, the largest of which encodes an RNA-dependent RNA polymerase. In 2013, *Aspergillus foetidus* dsRNA mycovirus was described as a putative member of the proposed genus “*Alternavirus*” in the proposed family “*Alternaviridae*” [2]. To date, six mycoviruses have been suggested to be members of this family. However, it has been pointed out that *Fusarium graminearum* alternavirus 1 (FgAV1) [3] should be regarded as a strain of *Fusarium poae* alternavirus 1 (FpAV1) [4] because these two isolates infect members of the same genus and have a high degree of similarity in their RdRp sequences, and a similar relationship has been observed between *Aspergillus foetidus* dsRNA mycovirus (AfVF) and *Aspergillus mycovirus 341* (AsV341) [5]. Therefore, the proposed family “*Alternaviridae*” currently has four members: *Alternaria alternata* virus 1 (AaV1) [6], AfVF, FpAV1, and *Fusarium incarnatum* alternavirus 1 (FiAV1) [7, 8]. Despite being grouped together, the number of RNA segments differs between AaV1 (4 segments), AfV-F (4 segments), FiAV1 (3 segments), and FgAV1 (3 segments).

*Cordyceps chanhua* is a precious traditional medicinal and insecticidal fungus with uses in both medicine and food in China. Taxonomically, because its teleomorph was unknown, this species was long referred to as "*Isaria cicadae*". With the recent discovery of the teleomorph of this taxon, the name *Cordyceps chanhua* [9] was proposed and accepted based on molecular data and the principle of one name for one fungus [10]. Over the last three decades, more than 100 *Cordyceps chanhua* strains from investigative studies of entomopathogenic fungi in China have been isolated and deposited at the Research Center for Entomogenous Fungi of Anhui Agricultural University (RCEF). Here, we report the complete genome sequence of a dsRNA virus with
three dsRNA genome segments, derived from the *Cordyceps chanhua* isolate RCEF6000 and designated as "Cordyceps chanhua alternavirus 1" (CcAV1). Phylogenetic analysis based on RdRp sequences showed that CcAV1 is a novel member of the proposed family "*Alternaviridae*".

Strain RCEF6000 was isolated from cicada in Anhui Province of China and was identified as *Cordyceps chanhua* based on its morphological features and molecular data (the sequences of its ITS region and translation elongation factor 1-α gene). The strain was incubated on SDAY medium (1% w/v peptone, 4% w/v dextrose, 0.2% w/v yeast, and 1.5% w/v agar) at 25 °C for 5 days [11]. Approximately 0.5 g of fresh mycelium of each isolate cultured on SDAY was harvested and ground into powder in liquid nitrogen, and the dsRNA was extracted by CF-11 cellulose (Sigma) chromatography [12]. *C. chanhua* strain RCEF6000 was found to harbor five distinct dsRNAs, approximately 3.5, 2.6, 2.4, 1.8, and 1.6 kb in length, which were named dsRNA 1-5, respectively (Fig. 1A). All gel-purified dsRNAs were sequenced on an Illumina HiSeq 2500 platform at BGI (Shenzhen, China), the clean reads were assembled into approximately 9,500 contigs, and sequence analysis was performed by searching the GenBank database using BLASTx. BLAST and RT-PCR results indicated that strain RCEF6000 was infected by two different dsRNA mycoviruses: a partitivirus (dsRNA 4, dsRNA 5) and an alternavirus (dsRNA 1, dsRNA 2, and dsRNA 3). A cDNA library was constructed using the specific primer 5'-GATCCACTA

![Fig. 1](image-url) (A) Electrophoresis of a purified dsRNA extract from *C. cicadae* strain RCEF6000 in a 1.5% agarose gel. M, DNA marker; lane 1, dsRNA. (B) Schematic representation of the CcAV1 genome structure. (C) Conserved sequences of 5' and 3' termini of dsRNA 1, dsRNA 2, and dsRNA 3 of CcAV1. "∗" indicates a conserved nucleotide.
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GTCTAGAGCGGC -3', and the 5' and 3' ends of these three dsRNA elements were determined using RNA-ligase-mediated rapid amplification of cDNA ends (RLM-RACE) [13]. The primers for amplification of each dsRNA segment are shown in Supplementary data 1 and 2. Each of the PCR products was cloned into the vector pMD18-T (Takara Bio Inc., Kusatsu, Shiga) and sequenced at least three times. The results showed that contig 8618 (3,465 bp) corresponded to dsRNA 1, with the highest percent identity (66.99%) to *Aspergillus foetidus* dsRNA mycovirus, while contig 3757 (2,607 bp) corresponded to dsRNA 2 and contig 3831 (2,359 bp) corresponded to dsRNA 3, with 48.61% and 56.91% amino acid sequence identity, respectively, to *Aspergillus foetidus* dsRNA mycovirus. Thus, dsRNA 1, dsRNA 2, and dsRNA 3 appear to be the genome segments of a potentially novel dsRNA mycovirus, designated as "*Cordyceps chanhua* alternavirus 1" (CcAV1) [2, 14].

The complete genome sequence of CcAV1 was deposited in the GenBank database under accession numbers OK481552, OK481553, and OK481554. The putative ORFs of these three dsRNAs were predicted using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/) (Fig. 1B), and the amino acid sequence of the putative RdRp of CcAV1 was aligned with those of other dsRNA viruses using the Multiple Alignment using Fast Fourier Transform (MAFFT) program [15]. A phylogenetic tree was constructed by the maximum-likelihood (ML) method with the LG+G+I+F model and 1000 bootstrap replicates, using MEGA X [16]. The resulting phylogenetic tree was exported to Figtree 1.4.4.

The complete genome of CcAV1 is composed of three dsRNA segments: dsRNA 1 (3512 bp), dsRNA 2 (2,655 bp), and dsRNA 3 (2,415 bp) (Fig. 1B). The G+C content of dsRNA 1, dsRNA 2, and dsRNA 3 is 55.4%, 57.1%, and 59.7%, respectively. Each dsRNA contains a single ORF, with dsRNA 1 encoding a 126.41-kDa protein of 1,127 amino acids (aa), which is a putative RNA-dependent RNA polymerase (RdRp), dsRNA 2 encoding a 90.73-kDa hypothetical protein of 831 aa (HP 1), and dsRNA 3, encoding a 78.68-kDa hypothetical protein of 731 aa (HP 2). A conserved domain database (CDD; NCBI) search using the RdRp amino acid sequence of CcAV1 as a query sequence
confirmed the presence of the expected eight conserved domains that are found in viral RdRp proteins. Further analysis showed that the triad within domain VI of RdRp has an alanine (ADD) instead of the nearly universally conserved glycine (GDD), supporting the previous suggestion that ADD is a typical feature of this family [17].

The 5’ untranslated regions (5’-UTRs) of dsRNA 1, 2, and 3 are 48, 51, and 53 nt in length, respectively. Sequence alignment of the 5’ UTRs of the three dsRNA segments of CcAV1 demonstrated that all three dsRNA shared a conserved sequence (GGCTGACGCTGAGTGGTGNCCCT AATCNANTACNACCAGCTGTGC) (Fig. 1C). A poly(A) sequence present in all three dsRNA segments ranges from 23 to 46 nt in length (Fig. 1B), and the nucleotide sequence identity among these 3’-UTRs is 27.27%, excluding the poly(A) tails (Fig. 1C). A BLASTp search showed that the RdRp, HP 1, and HP 2 of CcAV1 had the highest sequence similarity to the corresponding proteins of AfVF (66.99%, 49.30%, and 56.91%, respectively). In order to determine the taxonomic position of CcAV1, an ML phylogenetic tree based on RdRp amino acid sequences of 21 mycoviruses, including 10 totiviruses, five chrysoviruses, and four alternaviruses was constructed with two partitiviruses as outgroups. The phylogenetic tree showed that CcAV1 grouped in the proposed family “Alternaviridae” with AfVF (Fig. 2). In summary, we propose that CcAV1 is a new member of the proposed family “Alternaviridae” with AfVF (Fig. 2).

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Declarations

Conflict of interest The authors have no conflict of interest.

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

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