Complete genome sequence of a novel ourmia-like mycovirus infecting the phytopathogenic fungus *Botryosphaeria dothidea*

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**Abstract**

Here, we describe the full-length genome sequence of a novel ourmia-like mycovirus, tentatively named “Botryosphaeria dothidea ourmia-like virus 1” (BdOLV1), isolated from the phytopathogenic fungus *Botryosphaeria dothidea* strain 8A, associated with apple ring rot in Shanxi province, China. The complete BdOLV1 genome is comprised of a 2797-nucleotide positive-sense (+) single-stranded RNA (ssRNA) with a single open reading frame (ORF). The ORF putatively encodes a 642-amino-acid polypeptide with conserved RNA-dependent RNA polymerase (RdRp) motifs related to those of viruses of the family Botourmiaviridae. Phylogenetic analysis based on RdRp amino acid sequences showed that BdOLV1 is grouped with unclassified oomycete-infecting viruses closely related to members of the genus Botoulivirus in the family Botourmiaviridae. This is the first report of a novel (+)ssRNA virus in *B. dothidea* related to members of the genus Botoulivirus in the family Botourmiaviridae.

*Botryosphaeria dothidea* is a notorious canker pathogen that infects a wide range of trees worldwide [1]. This fungus is the principal causal agent of apple ring rot in China and is distributed across almost every apple-planting region [2]. *B. dothidea* causes cankerous lesions on stems and brown rings on leaves and fruits, thereby ultimately reducing the apple yield and quality [3]. While fungicides are regularly applied for controlling this disease, the indiscriminate use of chemicals negatively impacts the environment and poses a threat to human health [3, 4]. These concerns necessitate the development of alternative, environmentally friendly management strategies for preventing apple ring rot.

Mycoviruses are viruses that infect fungi and are present in members of all major fungal taxa [5]. Mycoviruses are predicted to lack an extracellular phase, with their transmission occurring either vertically through conidia or spores, or horizontally via hyphal fusion followed by cytoplasmic mixing between compatible fungal strains [5]. Mycovirus genomes primarily consist of single- or double-stranded RNAs (ssRNA or dsRNA), although the recent discovery of circular ssDNA mycoviruses has increased their diversity [5, 6].

In general, mycoviruses cause asymptomatic infections in their hosts, although some can diminish host virulence upon infection [7]. These viruses have the potential to be used as “virocontrol” agents for managing fungal diseases of plants. Mycovirus-infected debilitated strains can be introduced into a field to undergo hyphal fusion with their virulent counterparts, making them hypovirulent upon virus transmission. The first successful example of such mycovirus-mediated biocontrol was the use of Cryphonectria hypovirus 1 (CHV1) to control chestnut blight caused by *Cryphonectria parasitica* [7]. Several other mycoviruses have since been experimentally proven capable of conferring hypovirulence on their host fungi [5]. To explore mycoviral diversity in *B. dothidea*, extensive virus hunting in this pathogen has been conducted by numerous research groups. Such expeditions have discovered several novel viruses in this fungus, including members of the families *Mitoviridae, Chrysoviridae, Totiviridae, Partitiviridae*, and *Botourmiaviridae* as well as the proposed family "Fusariviridae" [8–13].

*Botourmiaviridae* is a recently established family of linear positive-sense (+) ssRNA viruses comprising six
recognized genera: *Ourmiavirus*, *Botoulivirus*, *Scleroulivirus*, *Magoulivirus*, *Penoulivirus*, and *Rhizoulivirus* [14]. The genus *Ourmiavirus* consists of plant-infecting viruses with encapsidated trisegmented genomes, where each segment separately encodes a movement protein, capsid protein, and RNA-dependent RNA polymerase (RdRp) [15]. In contrast, viruses belonging to the other five genera specifically infect fungi and oomycetes and are monosegmented with a single open reading frame (ORF) encoding an RdRp [14].

In this study, we report a novel (+) ssRNA ourmia-like mycovirus from *B. dothidea* strain 8A, which is associated with apple ring rot in China. Sequence comparisons and phylogenetic analysis suggested that this virus is related to members of the genus *Botoulivirus* in the family *Botourmiaviridae*, and it has been provisionally named "Botryosphaeria dothidea ourmia-like virus 1" (BdOLV1).

**Provenance of the virus in *B. dothidea***

*B. dothidea* strain 8A was originally isolated from an infected apple tree in Shanxi province, China. Upon establishing a pure culture, the strain was maintained on potato dextrose agar (PDA) at 25°C under dark conditions. Strain identification was performed by internal transcribed spacer (ITS) sequencing as described by Xu and colleagues [16]. Total dsRNA (the replicative form of the virus) was extracted from a three-day-old mycelial culture grown on cellophane-overlaid PDA as described by Eusebio-Cope and Suzuki and visualized by 1% agarose gel electrophoresis in 1x TAE buffer [17].

The partial cDNA sequence of BdOLV1 was initially obtained through RNA deep sequencing of ribosomal-RNA-depleted total RNA from strain 8A. A cDNA library for next-generation sequencing was constructed using a NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, Inc.) and sequenced on an Illumina HiSeq4000 platform (Illumina, Inc.). After the removal of adapter sequences and low-quality bases (PHRED quality scores ≤ 5), the raw reads were trimmed using the Trimmomatic package with default parameters that discarded the truncated reads smaller than 35 nt. The remaining high-quality reads were then assembled de novo using Trinity (http://trinityrnaseq.sourceforge.net/) with a K-mer value of 25. The full-length cDNA sequence of BdOLV1 was then obtained by amplifying its terminal regions adopting a 3' RNA-ligase-mediated rapid amplification of cDNA ends (3' RLM-RACE) method. Briefly, a linker primer, PC3-T7-loop (5'-p-GGATCCCGGGAATTCGTAATACGACTCACTATATTATTATAGTGAGTCGTTATTA-OH-3'), was ligated to the 3' ends of heat-denatured (95°C for 4 min) viral dsRNA at 4°C for 24 h using T4 RNA ligase (Takara) following the manufacturer's instructions. The loop-primer-linked purified dsRNA was then subjected to first-strand cDNA synthesis using SuperScript™ III Reverse Transcriptase (Invitrogen) with linker primer PC2 (5'-CCGAATTCCGATCC-3'), complementary to the 5' side of the PC3-T7-loop primer. To amplify 5' and 3' viral terminal regions, the resulting cDNA was then amplified using 2×Es Taq MasterMix (CWBiO) with the complementary primer PC2 (5'-CCGAATTCGATCC-3') and the gene-specific primers 406R (5'-AAACCAGGGCGAAGCGACGAC-3') and 2546F (CGA ACTGCTGAGTCCGCGGTGAT), respectively. The PCR products were subsequently cloned using pGEM®-T Easy Vector System I (Promega). For each RACE reaction, a minimum of three recombinant plasmids were sequenced in both directions using the universal primers M13F and M13R.

The partial viral sequence and all terminal sequences were assembled and analyzed using DNAMAN version 9.0 (Lynnon Biosoft). The identity of BdOLV1 and its similarity to other viruses were determined via online BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The position of the ORF in the BdOLV1 genome and its corresponding putative polypeptide sequence were determined using the ORF Finder program (http://www.ncbi.nlm.nih.gov/orffig.cgi). Sequence alignments and phylogenetic analyses were performed using the MEGA version 10.1.7 software package [18]. The complete genome sequence of BdOLV1 was submitted to the GenBank database under accession no. MZ073729.

**Sequence properties**

The complete genome of BdOLV1 is 2797 nucleotides (nt) in length with a GC content of 54.06% (Fig. 1a). The 5' and 3' untranslated regions (UTRs) are 68 and 800 nt long, respectively (Fig. 1a). BdOLV1 contains a single ORF of 1929 nt, putatively encoding a 642-amino-acid polypeptide with a deduced molecular mass of 76.62 kDa (Fig. 1a).

A BLASTp analysis showed that this polypeptide is related to the RdRps of several ourmia-like viruses from oomycetes and fungi. BdOLV1 RdRp shared 98.91%, 56.94%, and 46.51% sequence identity with the corresponding regions of Botryosphaeria dothidea ourmia-like virus (BdOLV, unpublished partial genome sequence), Plasmopara viticola lesion associated ourmia-like virus 54, and Plasmopara viticola lesion associated ourmia-like virus 2, respectively [19]. Despite a lack of conserved domains in CD-Search, a multiple sequence alignment of the putative
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The RdRp region of BdOLV1 with the corresponding region of other *Boutourmiaviridae* members showed the presence of eight conserved RdRp motifs, including a highly conserved GDD signature (in motif VI) in the BdOLV1 polypeptide. Collectively, these findings suggest that BdOLV1 is a novel ourmia-like virus in the family *Boutourmiaviridae*.

Notably, a pairwise sequence alignment of the full-length RdRp amino acid sequences of BdOLV1 and Botrytis ourmia-like virus (species *Botrytis botoulivirus*), an exemplar strain of the genus *Botoulivirus* [20], showed only 34.85% sequence identity, far below the current species demarcation cutoff (≤ 90%) within this genus. Moreover, the complete RdRp sequences of members of different genera within the family *Boutourmiaviridae* differ by >70% [14]. At present, based on phylogenetic analysis, it is difficult to conclude whether BdOLV1 and its closely related viruses are novel members of the genus *Botoulivirus* or whether they constitute a new genus in the family *Boutourmiaviridae*.

In this study, we characterized BdOLV1 from an apple-infecting ascomycete fungus (*B. dothidea*) showing no apparent disease symptoms. BdOLV1 differs from the previously characterized ourmia-like virus Botryosphaeria...
dothidea botourmaviirus 1 (BdBOV-1), which was isolated from a hypovirulent pear-infecting B. dothidea strain [10]. A pairwise BLASTp analysis showed that BdOLV1 and BdBOV-1 shared 32.37% sequence identity with each other. While BdBOV-1 is phylogenetically related to members of the genus Magoulivirus, BdOLV1 is related to members of the genus Botoulivirus in the family Botourmaviiridae. Interestingly, BdOLV1 shares a close relationship to several ourmia-like viruses infecting the oomycete P. viticola, suggesting that such ourmia-like viruses might have been exchanged between fungi and oomycetes or that both of these organisms acquired such viruses from a common source.

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Declarations

Conflict of interest All authors declare that they have no conflicts of interest.

Fig. 2 A maximum-likelihood phylogenetic tree constructed using the LG model based on the amino acid sequences of the conserved RdRp regions of BdOLV1 (highlighted in red) and selected members of the family Botourmaviiridae (corresponding GenBank accession numbers are adjacent to virus names). A discrete gamma distribution was used to model evolutionary rate differences among the sites. The numbers next to each branch reflect the percentage of congruent clusters resulting from 500 bootstrap replicates. Bootstrap values ≤ 70% are not shown. The scale bar indicates a genetic distance of 0.5 amino acid substitutions per site. Red, blue, and green filled circles adjacent to virus names indicate the viral host (fungus, oomycete, and plant, respectively)

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

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