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ANNOTATED SEQUENCE RECORD

Complete nucleotide sequence of TaV1, a novel totivirus isolated from a black truffle ascocarp (*Tuber aestivum* Vittad.)

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Introduction

New viruses associated with fungi are discovered frequently and have been reported since the early 1960s in members of numerous fungal taxa [8, 9, 12–14, 16]. Here, we report the complete genome sequence of the first virus infecting an ectomycorrhizal fungus. Mycoviruses, most of which have double-stranded (ds) RNA genomes, are currently not known to have any natural vectors. They are transmitted intracellularly by anastomosis of the hyphae or by heterokaryosis (lateral transmission), or they are spread via spores (serial transmission) [6, 7]. Cytoplasmatic exchange in different periods of the fungal life-cycle allows highly efficient virus transmission via different types of fungal propagules, such as sexually and asexually produced spores. Virus-infected fungi may show symptoms such as abnormal colony morphology or sporocarps [7] but in most cases remain symptomless. In contrast, mycoviruses can be responsible for attenuation and enhancement of fungal virulence itself (hypo- and hypervirulence), especially in the well-studied group of phytopathogenic fungi [2, 7, 15]. The commercial importance of several saprophytic and phytopathogenic fungi, such as *Cryphonectria parasitica*, which causes chestnut blight; *Ustilago maydis*, which causes corn smut, *Botrytis cineria*; which causes spoilage of strawberries and grapes; and the commonly important white mushroom, *Agaricus bisporus*, has consequently focused research on commercially relevant fungi to develop biocontrol agents to prevent massive loss of crop yields [10, 15, 19, 21]. In contrast, the mycorrhizas, which have a profound impact on the colonization of terrestrial life, have so far been screened only rudimentarily for the presence of viruses. Considering the extraordinary number of mycorrhizal fungi, it seems odd that ectomycorrhizal macrofungi, which are present in almost any global forest ecosystem, have received much less attention as a source of viruses than other organisms. Here, we report the entire genome sequence of a putatively new totivirus. The virus reported here was isolated from the ascocarp of the hypogeous ectomycorrhizal fungus *Tuber aestivum*, which is commonly known as black noble truffle, and the name Tuber aestivum virus 1 (TaV1) is proposed. Accordingly, these findings provide the first evidence for the presence of mycoviruses in ectomycorrhizal fungi belonging to the largest fungal phylum (*Ascomycota*). However, Bai et al. [1] were the first to isolate a smaller dsRNA of 2.2 kbp from the ectomycorrhizal basidomycete *Hebeloma circinans* (*Cortinariaceae*). The genomes of members of the family *Totiviridae* typically contain two open reading frames (ORFs) that can be translated as a single fusion-protein by a −1 frameshift. The predicted proteins encoded by ORF1 and ORF2 show sequence motifs characteristic of viral coat proteins (CP) and viral RNA-dependent RNA polymerases (RdRp), respectively.

Provenance of the virus material

Ascocarps of *Tuber aestivum* Vittad. were collected with the help of trained truffle dogs in a mixed beech forest in northeastern Hungary in the region of the Bükk mountains. The gleba tissue (inner part) of the black truffle ascocarp than served as the source for dsRNA isolation [18]. Agarose gel electrophoresis of the dsRNA revealed one fragment ca. 4.5 kbp in size, which represented the genome

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RNA of a totivirus [3, 14, 16]. The dsRNA fragment was purified and used as template for amplification of the complete genome. Primary clones were generated by applying a random RT-PCR approach [5] in which the products were cloned into the pGEM T vector (Promega) and sequenced in the forward and reverse directions at Eurofins MWG GmbH (Martinsried, Germany). The remaining gaps in the sequence were filled in by amplification with sequence-specific primers. The 5′- and 3′-ends of the genomic RNA were determined by RACE-PCR based on terminal deoxynucleotide transferase poly(C)- or poly(G)-tailed cDNA. PCR amplicons were sequenced directly. Sequence data were analyzed using Vector NTI 11 (Invitrogen, USA). The genome sequence has been deposited in the INSDC database under accession number HQ158596. Amino acid sequences of the predicted CP and RdRp were aligned using the EMBL-EBI MAFFT server (http://www.ebi.ac.uk/Tools/mafft/) using the standard setup, and phylogenetic trees were inferred using MEGA version 4 [11, 17].

Sequence properties

The complete genome of TaV1 was 4,587 bp in length, with a G + C content of 42%. The genome contains two ORFs, a common characteristic feature of members of the genus *Totivirus*. A typical, 30-nucleotide (nt)-long, 5′ untranslated region (UTR), is followed by the CP-encoding ORF1, ending at position 2,084. The 681-amino-acid (aa)-long CP had a calculated molecular mass of 76.2 kDa, with the highest degree of identity (39.1%) shared with Saccharomyces cerevisiae virus L-A (AAA50320.1). A −1 ribosomal frameshift close to the end of ORF1 (nt position

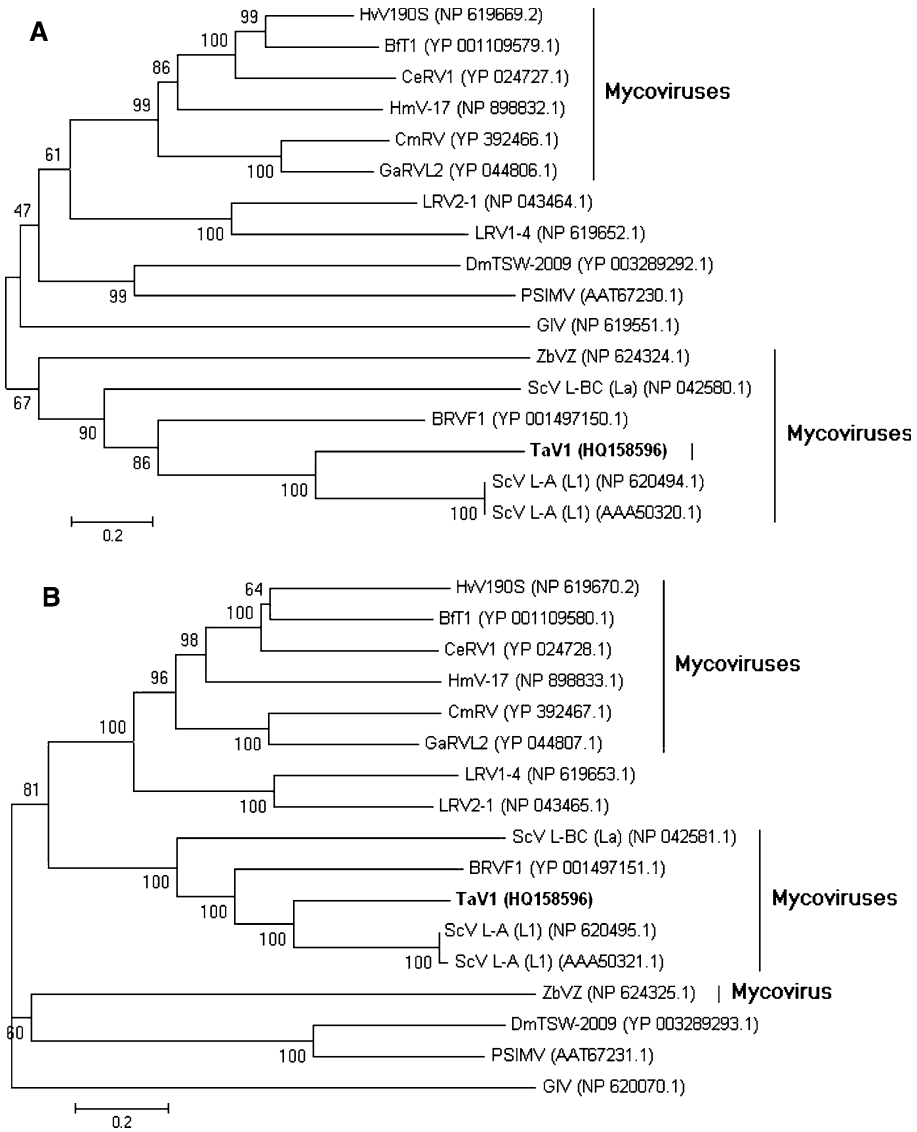

Fig. 1 Inferred neighbour-joining trees based on CP (**a**) and RdRp (**b**) amino acid sequences. Values at the nodes indicate bootstrap values obtained for 1,000 replicates (only values above 50% are shown). Accession numbers are given in *parentheses*. *BRVF*, black raspberry virus F; *BfT1*, *Botryotinia fuckeliana* totivirus 1; *CeRV1*, *Chalara elegans* RNA virus 1, *CmRV*, *Coniothyrium minitans* RNA virus; *DmTSW-2009*, *Drosophila melanogaster* totivirus SW-2009a; *GlV*, *Giardia lamblia* virus, *GaRVL2*, *Gremmeniella abietina* RNA virus; *HmV-17 Helicobasidium mompa* No.17 dsRNA virus; *HvV190S*, *Helminthosporium victoriae* virus 190S; *LRV1-4*, Leishmania RNA virus 1-4; *LRV2-1*, Leishmania RNA virus 2-1; *PSIMV*, Penaeid shrimp infectious myonecrosis virus; *ScV L-A L1*, *Saccharomyces cerevisiae* virus L-A L1; *ScV L-A* (*L1*), *Saccharomyces cerevisiae* virus L-A (L1); *ScV L-BC* (*La*), *Saccharomyces cerevisiae* virus L-BC (La); *ZbVZ*, *Zygosaccaromyces bailii* virus Z

Table 1 Percentage aa (ORF1-2) and nt (overall) sequence identities between TaV1 and other members of the family *Totiviridae*

Species/isolate	CP (aa)	RdRp (aa)	Overall (nt)	INSDC acc. no.
Chalara elegans RNA virus 1	12.7	7.5	41.8	NC_005883.1
Zygosaccaromyces bailii virus *Z*	6.3	9.9	34.0	NC_003874.1
Helicobasidium mompa No.17 dsRNA virus	12.4	18.1	41.9	NC_005074.1
Helminthosporium victoriae virus 190S	11.3	18.8	42.6	NC_003607.2
Saccharomyces cerevisiae virus L-A (L1)	39.1	48.7	54.8	SCSL1A
Saccharomyces cerevisiae virus L-A L1	39.1	46.4	54.9	NC_003745.1
Saccharomyces cerevisiae virus L-BC (La)	15.7	25.6	47.6	NC_001641.1
Black raspberry virus F	22.7	37.1	48.0	NC_009890.1
Drosophila melanogaster totivirus SW-2009a	6.6	8.6	32.7	NC_013499.1
Botryotinia fuckeliana totivirus 1	12.8	16.5	41.9	NC_009224.1
Coniothyrium minitans RNA virus	12.6	16.3	43.5	NC_007523.1
Leishmania RNA virus 2-1	12.3	16.2	42.5	NC_002064.1
Gremmeniella abietina RNA virus	12.0	17.4	43.4	NC_005965.1
Leishmania RNA virus 1-4	11.0	15.7	42.5	NC_003601.1
Giardia lamblia virus	9.4	6.0	35.8	NC_003555.1
Penaeid shrimp infectious myonecrosis virus	12.1	12.3	30.8	AY570982.1

1,959), identified as a GGGTTTA motif, is identical to the −1 frameshift slippery site heptamer in Chalara elegans RNA virus 1 (NC_005883) [4]. The heptamer motif is also present with a slight modification (GGGTTTT) in Helicobasidium mompa No.17 dsRNA virus [HmV-17] (NC_005074), *Saccharomyces cerevisiae* virus L-A (L1) [ScVLA (L1)] (NC_003745), and Ustilago maydis virus H1 (GGGTTTC, NC_003823). ORFs 1 and 2 are separated by a 92-nt intergenic region. The start codon of ORF2 is predicted at position 2,169, while the stop codon is located at position 4,554. Accordingly, the predicted molecular mass of the ORF2-encoded, 795-aa-long RdRp is 89.8 kDa. A comparison with selected sequences (Table 1) showed that the putative TaV1 CP shared the highest degree of sequence identity (39.1%) with the ScVLA (L1) ORF1-encoded putative CP, while the RdRp shared 48.7% identity to the predicted ScVLA (L1) RdRp. The phylogenetic basal position of the *Saccharomycotina* placed adjacently to the *Pezizomycotina* suggests an ancient evolutionary origin of the highly similar ScVLA (L1) and TaV1 during fungal evolution. The phylogenetic trees (Fig. 1) based on the amino acid sequences of CPs (a) and RdRps (b) have similar topologies. Mykitavirus, leishmaniavirus, and a group of viruses not yet assigned to a virus genus (i.e., HmV-17), which infect filamentous fungi, each form a distinct clade with an identical topology in both phylogenetic trees that is highly supported by the backbone of the RdRp tree (100%). A similar result was also obtained by Maejima et al. [13]. The phylogenetic position of the genus *Totivirus*, which includes TaV1, is paraphyletic within the family *Totiviridae*, which is also reflected by differences of the CP and RdRp trees. The high bootstrap support (81%) of the RdRp tree indicates that the genus *Totivirus*, including all ScV isolates, TaV1 and black raspberry virus F (BRVF), is nested close to the genera *Leishmaniavirus*, *Mykitavirus* and the clade of unassigned viruses (i.e., HmV-17). The BRVF isolate originating from raspberry but nested among the viruses infecting fungi is thus most likely a virus infecting an endophytic filamentous fungus or yeast. Members of the genus *Giardiavirus* that infect protists such as vertebrate intestine parasites, i.e., Giardia lamblia virus (GIV), as well as penaeid shrimp infectious myonecrosis virus (PSMIV) and Drosophila melanogaster totivirus SW-2009 (DmTSW-2009), which infect vertebrates and invertebrates, respectively, are nested within both topologies, isolated from the virus genera whose members infect fungi, with the exception of *Zygosaccharomyces bailii* virus Z and the protist-infecting *Leishmania* viruses. Further studies on PSMIV and DmTSW-2009-like viruses might eventually lead to the proposal of a new genus name for these vertebrate- and invertebrate-infecting viruses, which has also been suggested by Wu et al. [20].

In conclusion, this is the first report of the complete nucleotide sequence of a totivirus (TaV1) infecting black truffles. It might call for future initiatives to study the interaction of viruses with these prized and precious fungi. Moreover, ecological highly important and diverse organisms like ectomycorrhizal fungi constitute a vast black box of probably hundreds of new virus taxa—a new emerging field of research for collaborations between virology and mycology.

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