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LETTER TO THE EDITOR

Development of RT-PCR for rapid detection of ssRNA ambi-like mycovirus in a root rot fungi (*Armillaria* spp.)

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Mycoviruses are viruses that infect fungi mainly from hosts in phyla *Ascomycota* and *Basidiomycota*. Although the majority of mycovirus infections appear to be cryptic or symptomless, some viruses affect fungal growth or reduce the virulence of their hosts (*1*). These days, mycoviruses are intensively studied because of their possible use in biological control against serious fungal diseases (*2*). Though mycoviruses are widely distributed throughout the fungal kingdom, until recently there was only one report describing mycoviruses infecting fungi of the genus *Armillaria* (*3*). Fungal viruses with ambisense genome described in several pathogenic fungi including *Armillaria* spp. form the virus group temporarily named ambiviruses (*4, 5*).

Fungi in the genus *Armillaria* are known as important pathogens of forest trees. *Armillaria ostoyae* is a highly pathogenic parasite of coniferous stands in the Palearctic region (*6*). An extensive survey of *Armillaria* viruses in the Czech Republic was carried out in 2019–2020 using the bioinformatic approach. However, analyses of virus sequences using NGS methods are time-consuming and costly. Therefore, the aim of this work was to develop a specific RT-PCR assay for easy identification of these mycoviruses and to speed up the detection of ambi-like viruses (AlVs), which seem to be abundant in *Armillaria* spp.

The *Armillaria* strains were collected from different places in the Czech Republic. They were cultivated on malt extract agar and growing mycelium was used for RNA isolation. In this experiment we analyzed 9 samples/strains collected in nature (7 *A. ostoyae*, 1 *A. gallica* and 1 *A. cepistipes*) and three fungal cultures obtained from CCBAS – *A. tabescens* CCBAS213, *A. ostoyae* CCBAS215 and CCBAS325, respectively. The specific virus primers AmbiF (5'-GCTATGGCTGACTCCTCGTC-3') and AmbiR (5'-ACAGGGCAATCATTGGAGGG-3') were designed based on the assembled contigs. This primer pair has been able to detect the major ORF region, corresponding to the hypothetical protein ORFA of the AlVs, deposited in the NCBI GenBank database.

Total RNA was isolated using SPLIT RNA Extraction Kit (Lexogen GmbH, Vienna, Austria) and used as a template for RT-PCR. The complementary DNA (cDNA) was synthesized using the ImProm-II Reverse Transcription System (Promega, Madison, USA) according to the manufacturer's protocol. The AmbiF/AmbiR primers (0.5 µl each) were mixed with 3 µl of isolated RNA, filled to 5 µl with water, incubated for 5 min at 70°C and then cooled on ice for 5 min. After a short centrifugation, 15 µl reverse transcription reaction mix containing 4 µl reverse transcriptase buffer, 1 µl dNTP mix (0.5 mM), 0.5 µl RNAse inhibitor, 4.8 µl of $MgCl_2$, 1 µl InProm-II RT (reverse transcriptase) was added and subsequently incubated at 25°C for 5 min, at 42°C for 1 h, and at 70°C for 15 min. The PCR reaction mixture (20 µl

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Abbreviations: AlV(s) = ambi-like virus(es); AlV2 = ambi-like virus strain 2; AlV3 = ambi-like virus strain 3; CCBAS = Culture Collection of Basidiomycetes (Prague, Czech Republic); NGS = next generation sequencing; RT-PCR = reverse transcription PCR

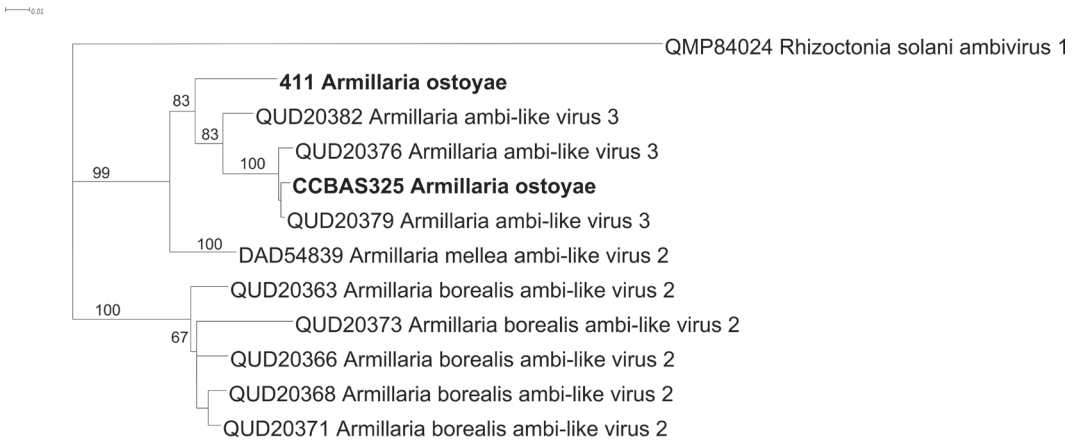

Fig. 1

A neighbor-joining tree of the AlVs was generated from sequences of the hypothetical protein encoded by the ORFA aligned with MUSCLE

The tree was inferred with Rhizoctonia solani ambivirus 1 as an outgroup. Bootstrap values above 50% from 1000 replicates are indicated for the corresponding branches. The length of branches is proportional to the number of base changes indicated by the scale.

total volume) containing 10 µl of PPP Master Mix (Top-Bio, Czech Republic), 1 µl of each primer (AmbiF/R) and 4 µl of the water was supplemented with 4 µl of cDNA. PCR reaction consisted of denaturation at 94°C for 2 min., followed by 25 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. Final PCR products were analyzed by gel electrophoresis and positive samples were sequenced in an external laboratory (SEQme, Czech Republic).

The isolated RNA from fungal mycelium was subjected to RT-PCR assay to investigate AlV sequences in different *Armillaria* strains/species. Two positive samples, both isolated from *A. ostoyae*, marked as 411 and CCBAS325 and producing 776 bp amplicon, were sequenced and blasted against the NCBI nucleotide database. The results indicated that CCBAS325 shared 81.97% sequence identity with the AlV2 (BK014421) and sequence identity between 89.4–96.31% with AlV3 (MW423811, MW423812, MW423813). The amplicon of sample 411 shared 83.04% sequence identity with AlV2 (BK014421) and between 84.58 and 85.35 % sequence identity with strain 3 (MW423812, MW423813) at the nucleotide level. Thus, AlV sequences amplified with our designed primers were detected in the mycelium of both *A. ostoyae* samples. The sample 411 was collected in a spruce stand in 2019 and is cultivated in the laboratory. The CCBAS325 strain was isolated and deposited in culture collection in 1964 and is maintained there since then, obviously infected with the ambi-like virus for decades.

The positive nucleotide sequences were then searched against NCBI non-redundant database using blastx. The results showed that the amino acid identity of the CCBAS325 and Armillaria AlV3 hypothetical protein (ORFA) was greater than 94%, while the identity of the hypothetical protein (ORFA) of sample 411 and ORFA of Armillaria AlV3 was below 91%. The amino acid sequences of both samples had identity below 91% (75–90%) with hypothetical proteins (ORFA) encoded in AlV genomes of *A. borealis* (Fig. 1).

We acknowledge that little is currently known about the ecology and/or host interactions of the AlVs. Despite the fact that there is also little known about the molecular and biological properties of this new putative group of viruses, our assay can now be used as a simple and fast RT-PCR test that will encourage the study of these fungal viruses. The RT-PCR method described here is rapid, reliable, and cost-effective for screening for AlV infection in *Armillaria* fungi. This method provides a useful tool for the investigation of mycoviruses within a host fungus. It is likely that with the specific detection of the AlV developed in the present work, a greater opportunity to study this group of mycoviruses will emerge.

In conclusion, we demonstrated a novel detection method for AlVs using specific RT-PCR amplification. We have verified our method through successful detection of the *Armillaria* AlV sequences from growing mycelium. This work includes only preliminary results, which can prompt studies to examine the presence of AlVs in *Armillaria* spp., to investigate the specific host-virus or virus-virus interactions as well as to further the description of incredible virus diversity.

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