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LETTER TO THE EDITOR

Molecular characterization of a ssRNA mycovirus isolated from the forest pathogenic fungus *Armillaria ostoyae*

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Species of the genus *Armillaria* are members of the soil-borne fungal community that induce root rot and are contributing to the decline of Norway spruce and other coniferous stands in the northern hemisphere (*1*). Fungi of *Armillaria* spp. are highly destructive forest pathogens that infect economically important conifers and cause huge economic losses. Since the first description of fungal virus in cultivated mushrooms (*2*), dozens of new mycoviruses were identified and placed into a taxonomic system of viruses (*3*). Mycovirus infections are symptomless and persist inside their host for decades (*4*). However, instead of classical molecular methods for the identification of mycoviruses in fungal genomes, the transcriptome approaches have become more popular. Using the HTS, several mycoviruses with ambisense genome were described recently in *Armillaria* spp. from Finland, Russia and South Africa (*5*). Together with Cryphonectria parasitica ambivirus, Rhizoctonia solani ambiviruses (*6*), Tulasnella ambiviruses and Ceratobasidium ambivirus they established a new group of viruses temporarily named ambiviruses (*7*).

In the present study, we describe a new strain of the fungal virus with ambisense genome isolated from *Armillaria ostoyae* and, to our knowledge, this is the first report of ssRNA mycovirus found in this species of serious forest pathogen.

Strains/species of *Armillaria* spp. were isolated from rhizomorphs (mycelial cords) collected at different locations in the Czech Republic. Fungal mycelia were cultivated on malt extract agar (ME agar). Total RNA was isolated using PureLink RNA Mini Kit (Invitrogen) according to the manufacturer's procedures. Pooled total RNA sample of 13 *Armillaria* strains/species (10 *A. ostoyae*, 2 *Armillaria gallica* and 1 *Armillaria cepistipes*) was delivered to SEQme (Dobříš, Czech Republic) for further processing of the sample, including RNA quality control and library preparation (NEBNext Ultra II Directional RNA Library Prep Kit for Illumina, NEB Inc.). The host rRNA was depleted from the pooled sample using NEBNext rRNA Depletion Kit (Human/Mouse/Rat) (NEB Inc.). An Illumina platform Novaseq 6000 was used to generate 150 bp paired-end reads. After quality trimming, reads were assembled *de novo* with SPAdes. The assembled contigs longer than 1 kb were compared (yielding e-values ≤ 1e^{-5}) with the NCBI virus database, and with the RefSeq non-redundant database using blastn and blastx, respectively. A sample of each total RNA included in the pool was converted to cDNA using LunaScript RT SuperMix Kit (NEB Inc.) according to the manufacturer's instructions. Identification of virus-host sample was performed using RT-PCR with virus-specific

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Abbreviations: aa = amino acids; AlV = ambi-like virus; AbAlV2 = *Armillaria borealis* ambi-like virus 2; AAlV3 = *Armillaria* ambi-like virus 3; AoAlV = *Armillaria ostoyae* ambi-like virus; CDD = Conserved domain database; HTS = high throughput sequencing; nt = nucleotides; ORF = open reading frame; RdRP = RNA dependent RNA polymerase; RT-PCR = reverse transcription PCR primers based on the ORFs found in assembled virus contig (Table 1).

Table 1. Primer sets of AoAlV partial ORFs

Primer name	Primer sequence 5'–3'	Position (nt)
AoA1F	GCTATGGCTGACTCTTCATC	1025–1080
AoA1R	ACAGGGCATTCATTGGAGGG	
AoA2F	CTTCTCTCCCCGCGTATGAC	2625–3240
AoA2R	CGCATGTGCCATATATCGCG	

Thirteen *Armillaria* strains from the Czech Republic representing three species (*A. ostoyae*, *A. gallica* and *A. cepistipes*) were included in the HTS analysis. The RNA-Seq library comprised ~113 millions paired-end reads. After quality trimming and *de novo* assembly, we obtained 8809 contigs longer than 1 kb. Among viral contigs, we found one sequence that resembled the ambi-like virus strain 3 described from *Armillaria borealis* and *A. cepistipes*, respectively. RT-PCR analysis of cDNA performed on each of the strains included in the pooled sample revealed that ambi-like virus was found only in the isolate representing *A. ostoyae* strain Harta. The complete sequence comprises 4523 nt, encoding three ORFs (Fig. 1). The ORF1 and ORF2 are overlapped in 2 nucleotides. A blastp search of predicted proteins against a non-redundant database confirmed strong sequence similarity to ORFs of previously described ambi-like viruses deposited in the GenBank database. The ORF1 was predicted to encode a putative protein of 712 amino acids (2139 nt). Blastp analysis showed that ORF1 had a high amino acid similarity to the hypothetical proteins of AAlV3 (94.20–98.46%) and AbAlV2 (71.59–73.19%). Notably, the GDD triad is included in all these longest ORFs, which is considered to be a mark of the RdRP (Fig. 2).

The smallest ORF2 encoded sequence of 217 aa (654 nt) and shared at least 62% similarity with hypothetical protein of AbAlV2, and between 94.47 and 99.08% with hypothetical proteins of AAlV3. The ORF3 was 407 aa (1224 nt) long and shared 96.42–98.53% similarity with the hypothetical proteins of AAlV3 and at least 62% with predicted ORF3 of AbAlV2. Both, ORF2 and ORF3, separated by an intergenic region of 33 nt in length, are read in the opposite direction of the ORF1.

To define the relationships within the group of ambiviruses, a phylogenetic tree was derived based on the nucleotide sequences of previously described ambiviruses and ambi-like viruses. Three distant main clades, ambi-like virus 1, 2 and 3, separated from ambivirus sequences were detected. The results showed that AoAlV formed a clade with a bootstrap value of 100% with other AAlVs3, and ambi-like virus 1 and 2 made individual clusters with highly supported bootstrap values (Fig. 3). The sequenced genome of the virus infecting *A. ostoyae* was deposited in the NCBI GenBank database (Accession no. OL863120). Based on the similarity with up-to-date described ambi-like viruses of several *Armillaria* spp. we designated this virus as *Armillaria ostoyae* ambi-like virus.

A new group tentatively named ambiviruses comprises new putative viruses recently described in mycorrhizal fungi using HTS (7). Similar viruses were also found to infect *C. parasitica* and *R. solani* (6). Though viruses from this group seem to be very common in basidiomycetes, there are no data on their biological or population structure. There is nothing known about the relationship between virus and host as well as virus-virus interaction even though many descriptions of new viruses were reported to infect forest fungal pathogens in recent years (*4; 8; 9*). The ambi-like viruses were described to infect members of the genus *Armillaria* worldwide but not *A. ostoyae*. These mycoviruses seem to be abundant and are distributed throughout the area where *Armillaria* fungi are found (*5*). All these ambi-like viruses remain unclassified and are not included in the virus taxonomy. Last year, many viruses with ambisense genome have been described to infect the pathogenic fungi (*5, 6*). The ambi-like virus strain delimitation is based on the nucleotide and amino acid sequence identities (*5*). Although no other criteria are available for this new group of viruses, similarity in sequences revealed that AoAlV shared homology with the ambi-like virus 3, which is strongly supported with the phylogenetic analysis based on multiple alignment of the nucleotide sequences of ambi-like viruses.

Although the GDD motif is present in aa sequence of the ORF1, no conserved domains have been detected in AoAlV. The GDD motif is considered as a marker of RdRP but this gene has not been described either in ambi-like

Fig. 1

Schematic representation of the *Armillaria ostoyae* ambi-like virus genome organization
Three ORFs encoding three hypothetical proteins are depicted. Note the orientation of the ORF1, ORF2 and 3, respectively. A black line illustrates the genome. Numbers represent position of ORFs.

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                                1       10         20         30         40         50         60
Consensus                       MIFWGVCVAR SILLLEIPEH SQVVLRRAAE ERMHTRTDLK ELFSLVYPEY RTVIRTLSRL
DAD54839 Armillaria mellea AlV2 .......... .......... .......... .......... .......... ..........
QUD20382 Armillaria AlV3        .......... .......... .......... .......... .......... ..........
QUD20376 Armillaria AlV3        .......... .......... .......... .......... .......... ..........
QUD20379 Armillaria AlV3        .......... .......... .......... .......... .......... ..........
OL863120 Armillaria ostoyae AlV .......... .......... .......... .......... .......... ..........
                                        70         80         90        100        110        120
Consensus                       PLSARNLLIK XCQAQSWPXP DSFSTLRZMI EQHPDXYXHD PYPXIIXFSN FAPXFNLRLH
DAD54839 Armillaria mellea AlV2 .......... A.......A. .......E.. .....V.V.. ...D..K... ...Y......
QUD20382 Armillaria AlV3        .......... S.......S. .......Q.. .....V.I.. ...E..P... ...H......
QUD20376 Armillaria AlV3        .......... S.......R. .......Q.. .....V.V.. ...E..P... ...H......
QUD20379 Armillaria AlV3        .......... S.......R. .......Q.. .....V.V.. ...E..P... ...H......
OL863120 Armillaria ostoyae AlV .......... S.......R. .......Q.. .....I.V.. ...E..P... ...H......
                                       130        140        150        160        170        180
Consensus                       DPLESAILLD IARGJPLPSI YDVXEAQSAS WSKEIRLXHG VKREAAVNWD VFFXEGCQHL
DAD54839 Armillaria mellea AlV2 .......... ....L..... ...L...... .......V.. .......... ...V......
QUD20382 Armillaria AlV3        .......... ....I..... ...L...... .......L.. .......... ...V......
QUD20376 Armillaria AlV3        .......... ....I..... ...F...... .......L.. .......... ...E......
QUD20379 Armillaria AlV3        .......... ....I..... ...F...... .......L.. .......... ...E......
OL863120 Armillaria ostoyae AlV .......... ....I..... ...F...... .......L.. .......... ...E......
                                       190        200        210        220        230        240
Consensus                       XIADGVXPNP QMARRIHHQL XXFREXXRGX RFXPRATHKN MSRXTRRDFE RQTGXSLEXI
DAD54839 Armillaria mellea AlV2 P.....D... .......... KR...KI..K ..Y....... ...G...... ....L...E.
QUD20382 Armillaria AlV3        P.....E... .......... RR...SI..R ..F....... ...S...... ....Q...G.
QUD20376 Armillaria AlV3        P.....D... .......... KR...SV..R ..F....... ...S...... ....H...G.
QUD20379 Armillaria AlV3        P.....D... .......... KR...SI..R ..F....... ...S...... ....H...G.
OL863120 Armillaria ostoyae AlV S.....D... .......... KK...SI..R ..F....... ...S...... ....H...G.
                                       250        260        270        280        290        300
Consensus                       PIFGQDNWGA HYHKTGXKLG GXSEMRQKWY HXGAKPRTYF AMGGEAYEXC RFLQDFFTXJ
DAD54839 Armillaria mellea AlV2 .......... ......V... .P........ .S........ ........G. ........EL
QUD20382 Armillaria AlV3        .......... ......V... .S........ .H........ ........A. ........EI
QUD20376 Armillaria AlV3        .......... ......K... .S........ .H........ ........A. ........DI
QUD20379 Armillaria AlV3        .......... ......K... .S........ .H........ ........A. ........DI
OL863120 Armillaria ostoyae AlV .......... ......K... .S........ .H........ ........A. ........DI
                                       310        320        330        340        350        360
Consensus                       VDFFMPTNHK TRLQPDRLFL SSXYDKEDPH FRIYDLSNFT SNMSEQSRCL XGLXXFMEGV
DAD54839 Armillaria mellea AlV2 .......... .......... ..R....... .......... .......... R..TK.....
QUD20382 Armillaria AlV3        .......... .......... ..R....... .......... .......... K..ER.....
QUD20376 Armillaria AlV3        .......... .......... ..K....... .......... .......... K..EK.....
QUD20379 Armillaria AlV3        .......... .......... ..R....... .......... .......... K..EK.....
OL863120 Armillaria ostoyae AlV .......... .......... ..K....... .......... .......... K..EK.....
                                       370        380        390        400        410        420
Consensus                       EVEXVDERXG PXXXTMDXLL XXYQESCVER PXVSLERYXG XDTSEGDXHD SIPHMVASLL
DAD54839 Armillaria mellea AlV2 ...Y....F. .VAT...M.. GE........ .P......K. T......L.. ..........
QUD20382 Armillaria AlV3        ...I....F. .ITI...V.. SD........ .S......K. V......F.. ..........
QUD20376 Armillaria AlV3        ...I....Y. .LMV...M.. GE........ .S......R. I......F.. ..........
QUD20379 Armillaria AlV3        ...I....Y. .LMV...M.. GD........ .S......R. I......F.. ..........
OL863120 Armillaria ostoyae AlV ...I....Y. .LMV...M.. GD........ .S......R. I......F.. ..........
                                       430        440        450        460        470        480
Consensus                       GIFGNLMSCT XAHYLXVSPV VRDEEEVNVA GDDGILPEDA XNPXPVXRVI DLVGSCAMEK
DAD54839 Armillaria mellea AlV2 .......... F....M.... .......... .......... T..E..R... ..........
QUD20382 Armillaria AlV3        .......... L....I.... .......... .......... S..E..R... ..........
QUD20376 Armillaria AlV3        .......... L....I.... .......... .......... C..D..K... ..........
QUD20379 Armillaria AlV3        .......... L....I.... .......... .......... D..D..K... ..........
OL863120 Armillaria ostoyae AlV .......... L....I.... .......... .......... S..D..R... ..........
                                       490        500        510        520        530        540
Consensus                       TFRSDEESAI ALKRPIWEDL PHLHTXXNII PPSVVRCVQS ICSXFXDPRY PPLPEPYTLN
DAD54839 Armillaria mellea AlV2 .......... .......... .....SF... .......... ...T.Q.... ..........
QUD20382 Armillaria AlV3        .......... .......... .....MY... .......... ...T.K.... ..........
QUD20376 Armillaria AlV3        .......... .......... .....TY... .......... ...S.K.... ..........
QUD20379 Armillaria AlV3        .......... .......... .....MY... .......... ...S.K.... ..........
OL863120 Armillaria ostoyae AlV .......... .......... .....TY... .......... ...S.K.... ..........
                                       550        560        570        580        590        600
Consensus                       EGLNXVGKDL LRFLRSAYLR RXXDVXRLXX VIXGYEXLVX XJSRXXPXPG TKGTQGYTWP
DAD54839 Armillaria mellea AlV2 ....V..... .......... .FY..D..GM ..D...R..N RI..VT.K.. ..........
QUD20382 Armillaria AlV3        ....I..... .......... .YQ..E..SE ..S...K..K TL..VR.S.. ..........
QUD20376 Armillaria AlV3        ....I..... .......... .YQ..E..SE ..S...K..K TI..VR.T.. ..........
QUD20379 Armillaria AlV3        ....I..... .......... .YQ..E..SE ..S...K..K TL..VK.T.. ..........
OL863120 Armillaria ostoyae AlV ....I..... .......... .YQ..E..SE ..S...K..K TI..IK.T.. ..........
                                       610        620        630        640        650        660
Consensus                       XXPXXYDFLD APPLTVLSIX FAPKXLWTAK LEXRPVYXXX DLYXGLXFES NSXPKLKMLE
DAD54839 Armillaria mellea AlV2 VN.LA..... .........H ....L..... ..K....IDT ...A..E... ..N.......
QUD20382 Armillaria AlV3        MR.EG..... .........N ....R..... ..K....RET ...C..S... ..S.......
QUD20376 Armillaria AlV3        VK.EG..... .........N ....R..... ..R....QES ...A..S... ..S.......
QUD20379 Armillaria AlV3        MR.EG..... .........N ....R..... ..R....RES ...T..S... ..S.......
OL863120 Armillaria ostoyae AlV MR.EG..... .........N ....R..... ..R....RES ...A..S... ..S.......
                                       670        680        690        700  712
Consensus                       XYGYXEKEEV XXLLXDXXVL XFXXXXXXPX HIPXVYXYSV VRDVPEXFQG WV
DAD54839 Armillaria mellea AlV2 S...L..... KV..K.YE.. D.LGMLAA.I ...V..T... ......A... ..
QUD20382 Armillaria AlV3        A...V..... ME..E.FR.. G.PRNVSC.C
QUD20376 Armillaria AlV3        A...V..... ME..E.FR.. D.LGMLAA.V ...I..S... ......T... ..
QUD20379 Armillaria AlV3        A...V..... ME..E.FR.. D.LGMLAA.V ...I..S... ......A... ..
OL863120 Armillaria ostoyae AlV A...V..... ME..E.FR.. D.LGMLAA.V ...V..S... ......A... ..
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Fig. 2

Alignment representing amino acids encoded by ORF1 of representative ambi-like viruses of strains 2 and 3

Highlighted is GDD motif, which is a hallmark of RdRP. Points indicate identical sites. Letters reveal differences in aa sequence. AmAlV2 = *Armillaria mellea* ambi-like virus 2, AlV3 = ambi-like virus 3, *Armillaria ostoyae* AlV = *Armillaria ostoyae* ambi-like virus.

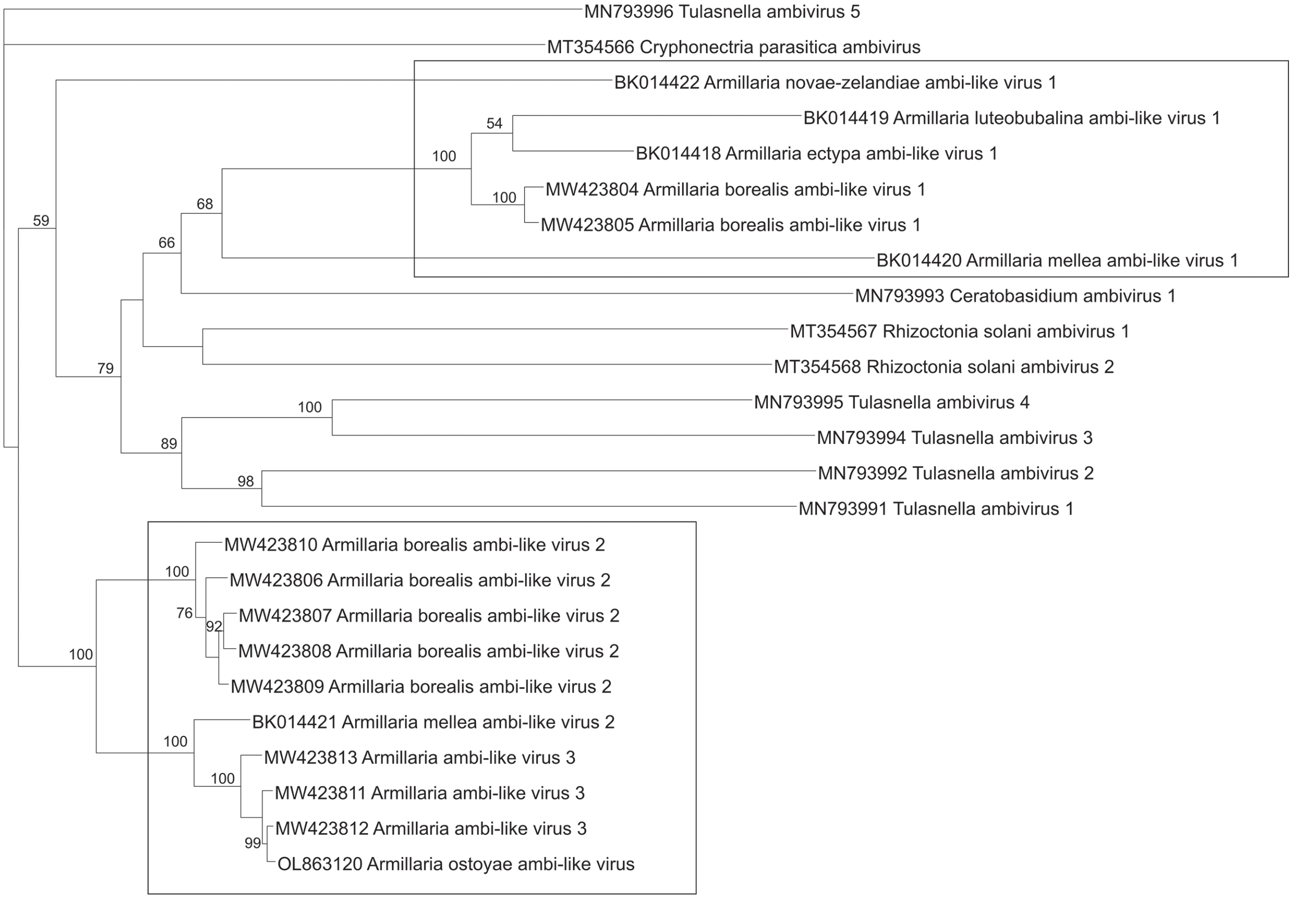

Fig. 3

Phylogenetic tree of a putative group of ambiviruses inferred using maximum likelihood method
The complete nt sequences were aligned in Geneious 8.1.9 with MUSCLE and the tree was constructed using GTR+F+R4 model (best-fit model according to ModelFinder) and 1000 bootstrap repetitions. The bootstrap values are shown on the branches. The ambi-like viruses, including AoAlV OL863120, are highlighted.

viruses nor ambiviruses infecting *C. parasitica* or other macromycetes (*4, 5, 6*).

Based on genomic analysis, phylogenetic relationships, similarity of nucleotide sequences, amino acid translation and host properties, this is a first report of the complete sequence of ambi-like virus isolated from *A. ostoyae* as well as the Czech Republic. The rod-shaped virus-like particles have been described in *Armillaria mellea* 50 years ago (*10*). However, this description of virus cannot be verified as it is unclear whether observed particles represent known viruses or not. Further research on the dsRNA viruses in *Armillaria* spp. proceeded in the Czech Republic with no success (11) and that is in agreement with later studies showing that viruses infecting *Armillaria* belong to the ssRNA viruses (*4, 5*).

In conclusion, our study describes virus with ssRNA genome that can be classified in the tentative group of ambi-like viruses. Members of this group share highly similar single-stranded genome encoding 2–3 ORFs in ambisense orientation. They also share a similar host range. To date, in addition to AoAlV, only fifteen other ambi-like viruses have been identified, infecting exclusively fungi of the genus *Armillaria* and also *Heterobasidion parviporum*. Thus, these results together with previously discovered viruses indicate that there is huge undescribed diversity of fungal viruses in forest macromycetes. Moreover, as ambi-like viruses seem to be hosted by *Armillaria* fungi and other serious fungal pathogens, it will be important to determine biological and ecological properties of this emerging viruses in more detail. The effect of these viruses on *Armillaria* spp. or their hosts in general remains unknown and needs to be investigated in further research.

Here, the sequencing results revealed a virus with an ambisense genome infecting *A. ostoyae* and we named it *Armi-*

llaria ostoyae ambi-like virus. In the future, biological function of AoAlV will be under investigation due to the possible biological control of honey fungus and further research also can expand our knowledge of the diversity and taxonomy of these mycoviruses as well as the host virus relationships. Description of the new virus may provide new insights into the taxonomy of fungal viruses. To our knowledge, this is the first report of the naturally mycovirus-infected fungal species *A. ostoyae*, the major and serious pathogen of the coniferous stands in Palearctic region.

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