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Characterization of a novel alternavirus infecting the fungal pathogen *Fusarium solani*

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ABSTRACT

A novel dsRNA mycovirus was found in *Fusarium solani* (*F. solani*) strain NW-FVA 2572. The fungus was originally isolated from a root, associated with stem collar necrosis of *Fraxinus excelsior* L. The viral genome is composed of four segments, which range from around 3.5 kbp to 1.7 kbp (RNA 1: 3522 bp; RNA 2: 2633 bp; RNA 3: 2403 bp; RNA 4: 1721 bp). The segments share a conserved and capped 5′-terminus and their 3′-termini are polyadenylated. Protein sequencing showed that the viral RdRP is encoded on segment 1. The virus clusters together with Aspergillus mycovirus 341 (AsV341), Aspergillus heteromorphus alternavirus 1 (AheAV1), Aspergillus foetidus virus-fast (AfV-F) and Cordyceps chanhua alternavirus 1 (CcAV1). As highest value, the RdRP showed 61.50% identical amino acids with P1 of the AfV-F. The capsid protein is encoded on segment 3, the proteins encoded on RNA 2 and RNA 4 are of unknown function. Segment 4 harbors large UTRs (186 nts at the 5′-terminus and 311 nts at the 3′-terminus).

Based on its genome organization and phylogenetic position, the virus is suggested to be a new member of the proposed family Alternaviridae and was therefore named Fusarium solani alternavirus 1 (FsAV1). This is the first report of an Alternavirus infecting a fungus of the *F. solani* species complex (FSSC).

Members of the species-rich *Fusarium solani* species complex (FSSC) (O'Donnell, 2000) are ubiquitous in soils, on plant debris and on various vegetable and animal tissues (Booth, 1971). These species have a very broad host range and were subdivided previously into *formae speciales* (Coleman, 2016) and phylogenetic clades and subclades (Nalim et al., 2011). Because the morphological concept of FSSC has a wide range and most of the pathogenic FSSC miss latin binomials, a multilocus haplotype nomenclatural system was invented by Chang et al. (2006) and O'Donnell et al. (2008) based on polymorphisms in three genes: the nuclear large-subunit rRNA (ITS, D1 and D2), the translation elongation factor 1 alpha gene (EF-1α), and the second largest subunit of the RNA polymerase II gene (RPB2). The studied sequence types could be assigned to three strongly supported clades designated 1, 2, and 3 by O'Donnell et al. (2008) confirming the results of Zhang et al. (2006). FSSC clade 1 taxa are restricted to host plants in New Zealand and FSSC clade 2 include taxa associated with host plants from South America. Most of the studied sequence types, with specimens from a wide host range (plants, animals, and humans) cluster in FSSC clade 3, including in the *Fusarium ensiforme* subclade sensu Nalim et al. (2011).

Several FSSC taxa are associated with woody tissues of trees (Chehri et al., 2015) or are causing trunk cankers, for example *Neocosmospora perseae* (Guarnaccia et al., 2018). They were also associated with woody tissues of *Fraxinus excelsior* L. diseased by ash dieback and having stem collar rots, respectively (Langer et al., 2015b; Langer, 2017; Meyn et al., 2019).

According to Li et al. (2019), mycoviruses have been reported in 13 *Fusarium* species to date. While most of the infections remain cryptic, several mycoviruses are known to cause hypovirulence to their host, for instance: Fusarium graminearum virus-China 9 (Darissa et al., 2012), Fusarium graminearum hypovirus 2 (Li et al., 2015) and Fusarium oxysporum f. sp. dianthi mycovirus 1 (Lemus-Minor et al., 2015). Especially in forest ecosystems, the use of fungicides may have offsite targets at beneficial organisms (Prospero et al., 2021). Hence, the use of mycovirus-induced hypovirulence can represent a sustainable approach to fight fungal contagions. However, there is no report about a mycovirus causing hypovirulence in *Fusarium solani* (*F. solani*) yet.

Even though mycoviruses exist which encode their genes from positive single-stranded RNA (+ssRNA), negative single-stranded (-ssRNA)

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or single-stranded DNA (ssDNA), viruses with a double-stranded RNA (dsRNA) genome are the most distributed among fungi. To date, dsRNA mycoviruses are classified into seven families (*Totiviridae, Partitiviridae, Megabirnaviridae, Chrysoviridae, Quadriviridae, Endornaviridae* and *Reoviridae*) and one genus (*Botybirnavirus*) (Kotta-Loizou and Coutts, 2017). Aoki et al. (2009) isolated a novel dsRNA virus from the fungus *Alternaria alternata* (Fr.) Keissl. (Fr.) and named it Alternaria alternata virus 1 (AaV1). Its genome is composed of four segments and the 3′-termini are polyadenylated. Phylogenetic analysis revealed that this new virus did not fit in any of the families and genera described before. It clustered together with the Aspergillus mycovirus 341 (AsV341) (Hammond et al., 2008) between the families *Chrysoviridae* and *Totiviridae*. Later, Kozlakidis et al. (2013) published a new virus, isolated from *Aspergillus foetidus* (AfV-F), which was phylogenetically related to AaV1. This finding let to the proposal of the new family Alternaviridae with the type species AaV1. Members of this family possess a genome consisting of three or four monocistronic dsRNA segments ranging from 3.6 kbp (dsRNA 1) to 1.4 kbp (dsRNA 4), and a poly(A) tail at the 3′-termini (Aoki et al., 2009; Gilbert et al., 2019; Hammond et al., 2008; He et al., 2018; Kozlakidis et al., 2013; Osaki et al., 2016; Zhang et al., 2019). While for AaV1 a cap-structure at the 5′-termini was confirmed (Wu et al., 2021), it is not known, if other viruses belonging to that family are also capped.

Today, complete sequences of several approved or putative alternaviruses are available; however, the knowledge beyond is partly low. It was shown by *in silico* sequence analysis that the viral RdRP is encoded on segment 1 and the viral particle was experimentally proven to be built of subunits of the protein encoded on segment 3 (Aoki et al., 2009; Wu et al., 2021). While there is no data about the protein encoded on segment 2, segment 4 was discussed to be a satellite-like RNA, since it vanished after subculturing (Wen et al., 2021). An altered colony morphology including abnormally enlarged vesicles on a microscopic scale was observed for AaV1 and growth with reduced aerial mycelium was monitored for Fusarium oxysporum alternavirus 1 (FoAV1) (Aoki et al., 2009; Wen et al., 2021). Until now, no alternavirus has been reported to induce hypovirulence to its original host.

In our study, we provide the complete genome organization and sequence analysis of a novel quadripartite dsRNA virus isolated from *F. solani*. Additionally, we give a deeper insight into the virus particle composition and biological properties. Based on BLASTp and phylogenetic analysis, it is supposed to be a new member within the proposed family of Alternaviridae. Therefore, we named it Fusarium solani alternavirus 1 (FsAV1).

The *Fusarium solani* strain NW-FVA 2572 (GenBank accession ID: OM921008, OM925485 and OM974589) was isolated from a necrotic trunk disk of a 17 year old *Fraxinus excelsior* L. which was diseased by ash dieback and had a stem collar necrosis. This trunk was collected by Markus Pfeffer in 2015 in the forest district Hils-Vogler-Ost, Mark Einbeck Stroit, Forest department Südniedersachsen, Lower Saxony, Germany (51°53′49″N, 9°51′54″E). In this area, ash dieback was observed since 2007. In 2014, the infection rate of *F. excelsior* trees by *Hymenoscyphus fraxineus* was about 96%, and collar necroses were observed on 6,8% of the trees (Langer et al., 2015b). This forest stand is a first afforestation of arable land in an agricultural landscape on a slope inclined to the north-east. Isolation and identification as a member of the FSSC was performed as described by Langer (2017) and Meyn et al. (2019). Briefly, genomic DNA was extracted using a modified method of Izumitsu et al. (2012) and the fungal strain was identified by ITS sequencing (Gardes and Bruns, 1993; Rehner and Samuels, 1994; Vilgalys and Hester, 1990; White et al., 1990). Additionally, a partial sequence of the EF-1α was amplified (Carbone and Kohn, 1999; O'Donnell et al., 1998). The primer sequences used are summarized in Supplementary Table 1.

An additional specimen of virus free *F. solani*, NW-FVA-3168 (GenBank accession ID: MH191236, MH220420) from a different tree, within the same area was collected by M. Pfeffer and R. Meyn in 2016 and used for control reactions. It was identified as *F. solani* agg. (Mart.) Sacc. and was placed in the *Fusarium ensiforme* subclade sensu Nalim et al. (2011) within the FSSC (Meyn et al., 2019).

Cultivation of mycelium was performed at room temperature on solid complete medium (CM_S) as stated in Leach et al. (1982). Mycelium was harvested by the separation of the mycelium from the CM_S by a cellophane sheet. Conidia were floated off from mycelium with distilled H_2O. Virus-like particles (VLPs) were enriched according to Lutz et al. (2021) and examined by transmission electron microscopy (LEO 906E, Zeiss, Germany) with 2% (w/v) uranyl acetate contrasting. Sixty-five VLPs were measured by means of ImageJ (imagej.nih.gov, version: 1.8.0_172) and the molecular weight of viral proteins was estimated by a 10% (w/v) SDS-PAGE visualized by Coomassie-Brilliant Blue staining. Peptides were sequenced with LC-MS/MS by a nano-liquid chromatography system (Dionex UltiMate 3000 RSLCnano, ThermoFisher Scientific, Waltham, MA, USA) and analyzed by means of the Proteome Discoverer 2.0 (ThermoFisher Scientific) by the Universitätsklinikum Hamburg-Eppendorf (UKE, Hamburg, Germany).

Nucleic acids were extracted either from VLPs or from mycelium using the Double-RNA – Viral dsRNA Extraction Kit (iNtRON Biotechnology, Seongnam-Si, South Korea) and were analyzed by 1% (w/v) agarose gel electrophoresis. The presence of a cap-structure was determined following the procedure of Wu et al. (2021).

Isolated dsRNA was submitted to Next-Generation Sequencing. The libraries were prepared according to Nextera XT DNA Library Preparation Kit (Illumina Inc., San Diego, CA, USA) and run on a NextSeq 2000 (Illumina Inc., San Diego, CA, USA) instrument at the Leibniz Institute DSMZ (Braunschweig, Germany) as pair-end reads (2 × 151). *De novo* assembly and contigs were analyzed using Geneious Prime software (Biomatters, New Zealand, version 2021.2.2). The 5′- and 3′-termini were determined by single-primer amplification technique (SPAT) using an oligonucleotide with a phosphorylated 5′-terminus and a 2′,3′-dideoxyC-group (23ddC) at the 3′-terminus as a blocker to prevent self-ligation (5′-PO_4-TCTCTTCGTGGGCTCTTGCG-23ddC-3′) RT and PCR. Sequences of further primers used are displayed in Supplementary Table 1. Amplicons were cloned into pGEM®-T Vector (Promega Corporation, Madison, WI, USA) and were sequenced. Nucleic acid sequences and ORFs were analyzed by SnapGene Viewer (GSL Biotech, San Diego, CA, USA) and BLAST on the NCBI website (Altschul et al., 1997).

Sequence alignments and phylogenetic analysis were performed using MEGA X (version 10.2.4) and Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). A bootstrap test was conducted with 1000 replicates for the construction of a Maximum-Likelihood Tree using the model by Le and Gascuel (2008) with amino-acid frequencies and a gamma distribution of 5 (LG+*G*+*F*) (Kumar et al., 2018). Phylogenetic analysis was carried out after sequence alignment of P1 of FsAV1 with P1 of putative or approved alternaviruses found by BLASTp with an E-value of 0.0. As an outgroup, RdRPs of the Magnaporthe oryze chrysovirus 1 D/B (MoCV1-D/B) of the *Chrysoviridae* family were added. Figures were generated and edited by Unipro UGENE (ugene.net, version 1.32.0) and INKSCAPE (inkscape.org, version 1.1).

The viral genome consists of four viral dsRNA segments (Fig. 1A) with sizes ranging from 3522 bp (dsRNA 1) to 1721 bp (dsRNA 4), and a GC content between 57% and 61%. Each segment encodes a single open reading frame (Fig. 2A). A conserved nonamer region at the 5′-termini (5′-GGCTAGCAG −3′) (Supplementary Figure 1) and a poly(A) tail on the 3′-termini and a cap-structure, respectively, were determined (Supplementary Figure 2). The complete genome of FsAV1 was deposited in the GeneBank database (accession ID: OM326757-OM326760).

The complete sequence of dsRNA 1 (segment 1) is 3522 bp in length and its ORF is initiated at nucleotide position 54 and terminated at position 3440. The ORF encodes a protein (P1) containing 1128 aa with a calculated molecular weight of 126.34 kDa. According to the homology search on BLASTp, P1 of FsAV1 shares similarities to RdRPs of nine approved or putative alternaviruses, respectively, with an E-value of 0.0. The highest value (61.50% identical aa) was shared with P1 of AfV-F (accession ID: YP_007353985.1) and the lowest value (36.86%

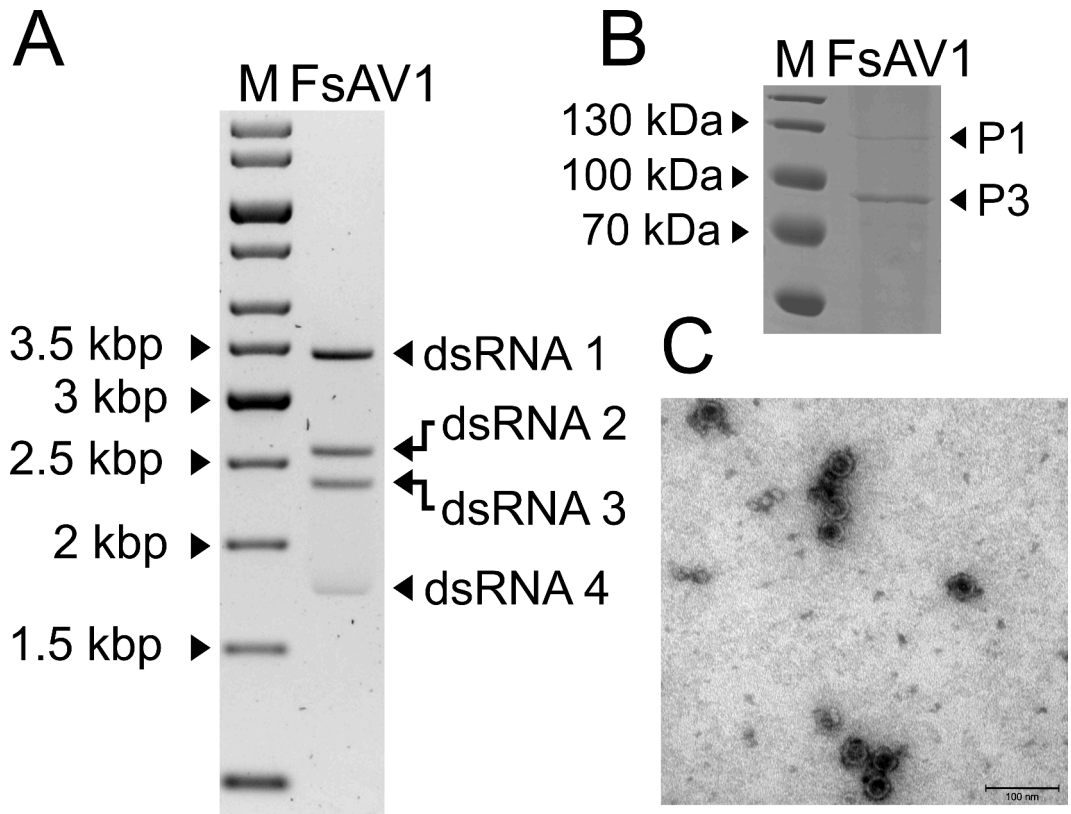

Fig. 1. **A**: Agarose gel electrophoresis (1% w/v) of dsRNA of FsAV1 extracted from isolated VLPs from *F. solani* NW-FVA-2572. M, GeneRuler 1 kb DNA ladder (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Segment 1 to segment 4 of FsAV1 range from around 3.5 kbp to 1.7 kbp. **B**: VLPs separated by SDS-PAGE (10% w/v) and visualized by Coomassie-Brilliant Blue staining. M, PageRuler Prestained Protein Ladder (Thermo Fisher Scientific). Two distinct bands are visible at around 125 kDa and 80 kDa corresponding to P1 and P3 of FsAV1. **C**: VLPs with an approximate size of 31 nm in diameter examined by transmission electron microscopy (LEO 906E, Zeiss, Germany) with 2% (w/v) uranyl acetate contrasting.

identical aa) with P1 of the Stemphylium lycopersici mycovirus (SIV, accession ID: YP_009551660.1). Alike other alternaviruses, within the conserved RdRP motif VI (GDD), the G residue is replaced with an A residue (Supplementary Figure 3). Additionally, to proof that the complete genome consists on dsRNA, the complementary poly(U) was confirmed by RT and PCR (Supplementary Figure 4).

Based on the amino acid sequence of the RdRP of FsAV1 and of RdRPs of different alternaviruses and chrysoviruses, a Maximum-Likelihood Tree was constructed to analyze the phylogenetic position of FsAV1. As displayed in Fig. 2B, FsAV1 clusters with high bootstrap support with AsV341, AfV-F, the Aspergillus heteromorphus alternavirus 1 (AheAV1) and the Cordyceps chanhua alternavirus 1 (CcAV1).

Segment 2 (dsRNA 2) is 2633 bp in length, its ORF spans from nucleotide position 54 to 2549 and encodes a protein (P2) containing 831 aa with a calculated molecular weight of 90.70 kDa. BLASTp search showed the highest similarity (E-value: 0.0) to the hypothetical protein P2 of CcAV1 (47.77% identical aa; accession ID: UPH33985.1).

The complete sequence of dsRNA 3 (segment 3) is 2403 bp in length. Its ORF ranges from nucleotide position 53 to 2239 and encodes a protein (P3) containing 728 aa with a calculated molecular weight of 78.47 kDa. BLASTp search showed similarities (E-value: 0.0) to three mycoviruses of the Alternaviridae family. With P3 of CcAV1 (accession ID: UPH33986.1) 55.56% and with AfV-F (accession ID: YP_007353983.1), 50.96% identical aa were shared; P3 of AheAV1 (accession ID: AZT88577.1) showed 50.00% identical aa.

Segment 4 (dsRNA 4) is 1721 bp in length. Its ORF is initiated at nucleotide position 187, terminated at position 1410 and encodes a protein (P4) containing 407 aa with a calculated molecular weight of 43.93 kDa. Compared to the other three dsRNAs, it harbors large UTRs (186 nts at the 5′ UTR and 311 nts at the 3′ UTR). BLASTp search exclusively showed distant similarities (E-value: 1e-04) to the hypothetical protein encoded on segment 4 of FoAV1 (36.59% identical aa, accession ID: QYY49565.1). Even though a fourth segment is also present in AaV1, AfV-F, AsV341 and SlV, no similarity was detected by BLASTp in relation to these. Wen et al. (2021) discussed segment 4 of FoAV1 to have a satellite RNA-like function, since it was spontaneously lost after subculturing. We speculate that other alternaviruses initially contained a satellite-like RNA, which was lost due to propagation in axenic cultures as it was shown for FoAV1.

According to the 9th report on subviral agents by the ICTV (2012), satellite-like nucleic acids are distinct from that of the helper virus and are coding for a non-structural protein or no protein. They may have functions in different steps during the viral replication-cycle of the helper virus. With the large UTRs, segment 4 of FsAV1 complies with the criterion being distinct from its helper virus. Since we did not find its encoded protein in purified VLPs and BLASTp did not show similarities to structural proteins, we assume that the ORF encodes for a non-structural protein. However, we cannot exclude that the protocol for VLP enrichment might be not suitable for the enrichment of P4 encapsidated particles. In contrast to segment 4 of FoVA1, the respective segment of FsAV1 is maintained during subculture (data not shown). Whether the fourth segment of FsAV1 is a viral segment or represents a satellite RNA cannot be deciphered with our data on hand.

Interestingly, an over five viral genera conserved region including two highly conserved motifs (5′- GCTGCCCCC(A/G)GC-3′ and 5′-ATTGATCCGGC-3′) was detected. While this region is present at the 3′-terminus of segment 4 of FsAV1, it is mostly located at the 5′-terminus of other viruses (Supplementary Figure 5). This stretch is found in some viruses of the of *Megabirnaviridae, Totiviridae* or *Polycipiviridae* families and viruses of the genus *Botybirnavirus*. Among others, the highest

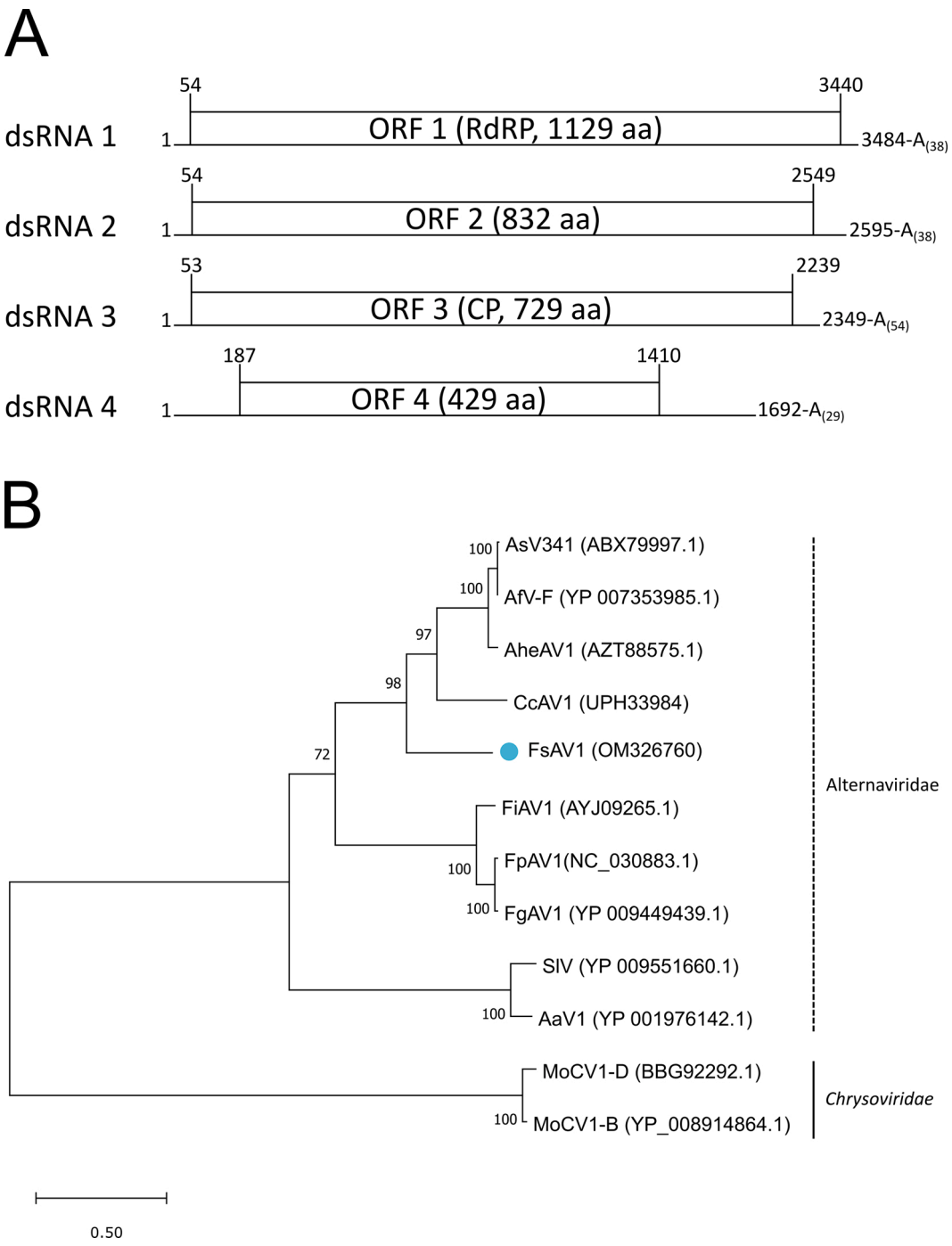

Fig. 2. **A**: Genome organization of FsAV1. The dsRNA segments are displayed as horizontal lines with their respective UTRs at each terminus. Poly(A) tails at the 3′-termini are indicated by A(n). ORFs are represented as boxes with start and stop codon positions indicated above the boxes. Note that the figures is not drawn to scale. **B**: Maximum-likelihood tree of FsAV1 and selected viruses with 1000 bootstrap replicates. Bootstrap values are displayed at the nodes. The scale bar (0.50) corresponds to the genetic distance. The dot indicates the new virus FsAV1. The abbreviated names of viruses and dsRNA elements are as follows: AsV341, Aspergillus mycovirus 341; AfV-F, Aspergillus foetidus virus–fast; AheAV1, Aspergillus heteromorphus alternavirus 1; CcAV1, Cordyceps chanhua alternavirus 1; FsAV1, Fusarium solani alternavirus 1; FiAV1, Fusarium incarnatum alternavirus 1; FpAV1, Fusarium poae alternavirus 1; FgAV1, Fusarium graminearum alternavirus 1; SlV, Stemphylium lycopersici mycovirus; AaV1, Alternaria alternata virus 1; MoCV1-D/B, Magnaporthe oryzae chrysovirus 1 D/B.

degree of similarity was shared with the 5′-termini of segment 1 and 2, respectively, of the Rosellinia necatrix megabirnavirus 1/W779 (RnMBV1) (Supplementary Table 2).

We conjecture that a former co-infection with a megabirna-like virus resulted in a horizontal gene transfer (HGT), and due to regulatory advantages, this fragment was stably maintained. Wang et al. (2015) found a papain-like protease domain on dsRNA 2 of Sclerotinia sclerotiorum megabirnavirus 1 (SsMBV1) which is phylogenetically related to the protease p29 of the ORFA-encoded protein of Cryphonectria hypovirus 1 (CHV1) and speculated also about HGT. Additionally, it also may be possible that this specific region preserved after different viruses emerged from the same ancestor. Overall, due to the high rate of conservation found in viruses of different families, a biological function seems likely.

FsAV1 possesses isometric VLPs with an approximate size of 31 nm (standard error of the mean: 0.35) in diameter (Fig. 1C), which is in

accordance to the size Aoki et al. (2009) determined for AaV1 (33 nm). When examining VLPs by SDS-PAGE and Coomassie staining, two bands were distinctly visible at around 125 kDa and 80 kDa (Fig. 1B) corresponding to P1 (126.34 kDa) and P3 (78.47 kDa), which was further verified by protein sequencing (Supplementary Figure 6 and 7). Therefore, the hypothetical proteins P2 and P4 of FsAV1 may be non-structural and their function remain unclear. This result is contrarily to the assumption that the coat protein of AheAV1, which is also within the Alternaviridae family, is built of protein encoded on segment 2 (Gilbert et al., 2019) but in line with the results of Wu et al. (2021), who experimentally showed the major structural protein to be encoded on segment 3 of AaV1.

Two isogenic virus free strains (NW-FVA 2572-C3 and NW-FVA 2572-C4) were obtained after growing NW-FVA 2572 from 10 single conidia. Virus absence was confirmed by RT-PCR and virus specific primers (Supplementary Figure 8). The virus-infected isolate exhibited extended radial growth of its mycelium with the formation of airy, cotton-like structures as compared to the virus-free isolate. Whereas, no significant alteration in the pigmentation of the colonies was observed (Supplementary Figure 9). Even though only 10 cultures of single conidia of FsAV1 were screened, the results indicate a high rate of vertical transmission, which was also observed for hypoviruses by Peever et al. (2000) and the Alternaria alternata partitivirus 1 by Da Xavier et al. (2018).

In conclusion, we reported an alternavirus with four segments isolated from *F. solani*. Due to a motif found on the 3′-UTR of segment 4, HGT with a megabirna-like virus was discussed.

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CRediT authorship contribution statement

Tobias Lutz: Investigation, Conceptualization, Writing – original draft. **Elma Japić:** Investigation. **Steffen Bien:** Investigation. **Gitta Jutta Langer:** Conceptualization, Writing – original draft. **Cornelia Heinze:** Conceptualization, Writing – original draft, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2022.198817.

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