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Original article

Low mycovirus presence in soil saprotrophic fungi in Dutch sandy soils

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ABSTRACT

Saprotrophic fungi play a fundamental role in soil ecosystems as primary decomposers, driving nutrient and carbon turnover on a global scale. Mycoviruses are considered widespread and can affect saprotrophic fungi by altering their properties, such as growth rate, stress resistance, and metabolite production. To advance the understanding of the prevalence and diversity of mycoviruses of soil saprotrophic fungi, we conducted parallel mycovirus screening in 28 saprotrophic fungi representing major groups of soil fungi and concomitantly explored soil metatranscriptomic data. *De novo* assembly of RNA-sequencing data uncovered two viruses from two fungal strains. One of these viruses, a ssRNA virus from *Mucor* sp., appears to represent a putative novel species. Analysis of eukaryotic RNA viruses in soil metatranscriptomes indicated that even though fungal and oomycete hosts made up 39.6 % of the classified eukaryotic viruses, there was no saprotrophic host assignment. Both methods showed that there were few mycoviruses in the Dutch sandy soils with different land uses. This led to the conclusion that mycoviruses affecting saprotrophic fungi may either be rare in these types of soils or more difficult to detect compared to other fungal groups. These results offer new insights into the ecological dynamics and viral associations of soil saprotrophic fungi, highlighting the need for broader sampling and improved approaches to assess mycovirus diversity and its potential ecological significance.

1. Introduction

Viruses play a crucial role across environments in modulating microbial community composition and its metabolic functions. Soil, the most diverse habitat on earth [1], is a major reservoir of viruses [2,3]. Studies on the diversity of soil viral community through metaomics (e.g., metatranscriptomics and metagenomics) approaches have uncovered a remarkable diversity of viruses infecting all domains of life [4–6]. Despite this, the number of known classified viruses is just the tip of the iceberg, as it is estimated that the unknown virosphere can be up to 99.995 % of all virosphere [7]. It is thus highly likely that soils harbor many previously unknown viruses, given the vast diversity of their potential hosts. Previous research has shown that DNA viruses predominantly infect prokaryotes, while RNA viruses mainly target eukaryotic hosts, including unicellular protists, plants, animals, algae and fungi [8].

Recent studies recovering soil RNA have started to reveal significant diversity of RNA-viruses in soils [5,9–11]. Several researchers have also characterized the potential contribution of RNA viruses to soil carbon cycling. For instance, Starr et al. [9] found that there is a dynamic linkage among carbon input forms, host abundance patterns, and viral RNA communities, and that fungi were found to be the main hosts for viruses in their study system. RNA viral sequences from thawing permafrost were found to contain auxiliary metabolic genes (e.g., polygalacturonase and glycoside hydrolases), which play key roles in carbon degradation and acquisition [6,12]. Still, a lot is unknown about soil RNA viruses, partly due to their intracellular life strategy and hence their dependence on host cells, which makes it challenging to study them in soil ecosystems [13]. An improved understanding of RNA viruses in soil is essential to uncover their role in the ecological functioning of soils [14].

Viruses infect nearly all living organisms in soils, but most terrestrial RNA virus research focuses on virus interactions with plants, e.g. in relation to their roles as harmful pathogens to crops or as beneficial agents that help plants adapt to environmental changes and resist pathogens [15,16]. However, given the important ecological functions that fungi perform in soils, mycoviruses (viruses that infect fungi)

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deserve more attention. Mycoviruses are prevalent and widespread across the fungal kingdom [17] and screening of phytopathogenic fungi for the presence of double-stranded (ds) RNA showed that the infection incidence can be as high as 80 % [18]. While mycovirus infections typically remain latent and rarely trigger symptoms, replication of mycoviruses can still have an impact on host physiology and gene expression, potentially preventing antiviral responses [19]. Alterations to host metabolism, cellular transportation, RNA processing and signaling pathways may lead to profound effects on host fitness and ecology [20,21]. Researchers previously reported changes in host virulence, morphology, pigmentation, sporulation, and radial growth caused by mycoviruses [22–24]. The fungal functions in soil ecosystems, such as decomposition and nutrient cycling, may be impacted by such changes affecting ultimately the whole soil ecosystem.

Despite their potential importance, the diversity and roles of RNA mycoviruses infecting soil fungi remain poorly characterized. We know especially little about viruses affecting saprotrophic fungi, one of the most abundant fungal groups in soil [25]. Saprotrophic fungi affect carbon and nutrient cycling in the soils through their ability to produce diverse extracellular enzymes [26,27]. Furthermore, saprotrophic fungi modulate soil structure by promoting soil aggregation through the hyphal enmeshment effect [28,29], by increasing soil water retention, and via mitigation of soil erosion. Within the soil food web, saprotrophic fungi redistribute nutrients across microsites and can decouple local mineralization from plant uptake [30]. Viral infections of saprotrophic fungi could therefore influence a broader soil food web and plant functioning by altering decomposition and nutrient transfer pathways. Several researchers have reported the presence of mycoviruses in different fungi. Myers et al. [31] found that 21.6 % of the 333 earlier-diverging fungal isolates harbored RNA mycoviruses. Besides, 21 mycoviruses were identified from 166 strains of the tree parasitic fungal genus *Armillaria*, of which 14 were putative novel virus species [32]. Nevertheless, further identification of mycoviruses from a broader range of fungal groups, especially from saprotrophic fungi, is needed to improve existing viral databases, to provide an increased classification accuracy and robust evaluation of host associations.

Recently developed techniques such as viromics, metagenomics and metatranscriptomics have been widely applied for advancing our understanding of the soil virosphere. With extra enrichment steps, virome sequencing has provided a deeper perspective on the viral community, accelerating the exploration of soil viral diversity [33,34]. Yet mycoviruses are, in large part, not captured by viromes because of the absence of an extracellular phase in their life cycle. Metatranscriptomics is an effective tool for RNA virus discovery for a wide range of sample types, and this has led to a rapid expansion of the number of available viral sequences in public databases. Despite these improvements, unfortunately, current metatranscriptomic approaches often miss the specific associations of viruses with their respective fungal species [35]. We propose that integrating metatranscriptomes from environmental samples to transcriptomes of cultivated fungi can offer a more comprehensive understanding of the diversity of mycoviruses of saprotrophic fungi and their interactions with host organisms.

In this study, we aimed to expand the understanding of the prevalence and diversity of mycoviruses in soil saprotrophic fungi by using Dutch sandy soils as a case study. In total, 28 saprotrophic fungal isolates from sandy soils (previously characterized in Ref. [29]) were examined for the presence of RNA viruses, and metatranscriptomic data from soil samples of a similar soil type were used to estimate the relative abundance of mycoviruses in these soil ecosystems. This combined approach allowed us to match hosts to their specific viruses. We hypothesized that mycoviruses would be prevalent in both isolated saprotrophic fungi and the soil metatranscriptome, and we further expected to discover novel viral species due to the limited prior research targeting mycoviruses in soil saprotrophic fungi. Moreover, we hypothesized that metatranscriptomic analyses would reveal a broader diversity of mycoviruses, whereas culture-based isolation would complement this by providing direct host-virus associations.

2. Materials and methods

2.1. Fungal isolation

Fungi were isolated using sites and methods as described previously in Ref. [29]. In brief, soils were collected from four sites within the Netherlands in April 2020. Soil 1 was collected from a pasture used for conventional farming (North-Brabant, N51.6423, E5.1998), Soil 2 was collected from a farm managed as an organic pasture (North-Brabant, N51.6460, E5.2172), Soil 3 was collected at the experimental farm (Limburg, N51.3219, E5.5105) and Soil 4 was collected from a grassland (Gelderland, N52.0830, E6.0639) abandoned from intensive agricultural management 2 years prior to the sampling. All samples were characterized as sandy soil. Fungal isolation from collected soil samples was performed using a serial dilution method with NaCl buffer. The dilutions were inoculated on Rose-Bengal agar (RBA), malt extract agar (MEA), Czapek's agar (CZA) and yeast extract peptone dextrose agar (YEPD), water agar (WA), potato dextrose agar (PDA), PDA with supplemented benomyl (0.5 ml/L agar) and 1:10 PDA plates. To inhibit bacterial growth, media were supplemented with antibiotics (15 mg/L ampicillin, 15 mg/L streptomycin, and 30 mg/L chlortetracycline). Plates were incubated at 20 °C for 2 weeks, followed by constant isolation until pure cultures were obtained. One set of the isolates was kept at 10 °C for short-term preservation and a second set was stored at −80 °C for long-term preservation for further analyses. In total, ten strains from Soil 1, seven strains from Soil 2, eight strains from Soil 3, and three strains from Soil 4 were used in the current study.

2.2. Total RNA extraction from fungal strains

We selected 28 cultivable fungi (Table S1) from three major saprotrophic phyla present in soils: *Ascomycota, Basidiomycota* and *Mucoromycota*, all of which are well-established saprotrophic fungal groups involved in soil organic matter decomposition and commonly found in soils [36]. Preliminary molecular identification of the strains was reported by Hannula et al. [29] based on their nuclear ribosomal internal transcribed spacer (ITS) sequences. These ITS sequences were combined with the sequences of the type species of the related genera to determine taxonomical positions of the strains. All sequences were aligned with MAFFT [37] and edited on BioEdit [38]. The ModelFinder [39] was used to determine the best-fitting model for phylogenetic analysis.

The selected fungi were incubated on PDA plates covered with porous cellophane membranes (Thistle Scientific, UK) at 20 °C in dark for 5–7 days. Mycelia were collected and immediately grounded into fine powder in liquid nitrogen. Total RNA was extracted from approximately 100 mg of mycelia using TRIzol reagent (Sigma-Aldrich, US), following the manufacturer's instructions, and treated with TURBO DNA-free Kit (Invitrogen, US) to remove residual DNA. RNA quantity and quality were checked by Tape Station 4200 (Agilent, US). Total RNA was also treated with S1 nuclease which specifically degrades ssRNA to assist viral RNA gel screening. This step helps distinguish potential dsRNA viral genomes, as many mycoviruses are dsRNA viruses. Both total RNA and S1-treated RNA were then screened for virus-specific RNA bands using 1 % agarose gel electrophoresis. All detected extra bands were sized using comparison to a ladder. Based on literature, RNA mycovirus genome segments often range in size from 2.5 to 23 kb [40].

2.3. Transcriptomic library preparation and sequencing

rRNA was depleted from 500 ng of total RNA isolated from fungal cultures using QIAseq FastSelect Kit for Yeast (QIAGEN, Germany). Sequencing libraries were established from rRNA-depleted total RNA samples using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB#E7760 S/L). Briefly, rRNA-depleted RNA was

fragmented and converted to cDNA, which was then ligated to Illumina sequencing adapters, followed by PCR amplification. The quality and yield after sample preparation were measured with the Fragment Analyzer 5300 (Agilent). Paired-end sequencing was conducted on an Illumina NovaSeq 6000 platform at GenomeScan (Leiden, Netherlands) according to the manufacturer's protocols. Libraries were clustered and sequenced at a final concentration of 1.1 nM. Data processing, including image analysis, base calling, and quality assessment, was performed using the Illumina data analysis pipeline RTA v3.4.4 and BclConvert v3.10.5.

2.4. Soil total RNA extraction and metatranscriptomic sequencing

Soil sample was collected at Meijendel, a sandy coastal dune area in the north of the Hague, the Netherlands (52 °11 N, 4° 31 'E) as previously described by Zhang et al. [41]. Topsoil was collected to a depth of 15 cm, sieved through a 5 mm mesh and used to grow native plant species in the soil collection area, namely *Jacobaea vulgaris* (common ragwort), *Cirsium vulgare* (bull thistle), *Trifolium repens* (white clover) and *Daucus carota* (wild carrot). A total of 27 soil samples from these pots were used in the present study for RNA extraction but as we were not interested in plant effects, we combined these samples in downstream bioinformatic analyses. Total RNA was extracted using the RNeasy PowerSoil Total RNA Kit (QIAGEN). The concentration and quality of the RNA were assessed by loading 1 μl of the extracted raw RNA onto Tape Station 4200 (Agilent). Subsequently, contaminants such as DNA, salts, and residual reagents were removed using the RNeasy MinElute Cleanup Kit (QIAGEN). Following this step, ribodepletion was performed with the Ribo-Zero Magnetic Kit for Bacteria (Illumina, US). RNA Clean & Concentrator Kit (Zymo Research, US) was used to eliminate any remaining buffers and proteins from the rRNA-depleted RNA. All extraction and purification steps were performed according to the manufacturers' protocols. Double-stranded cDNA was synthesized from the purified and concentrated RNA obtained in the final step. Library preparation was carried out using the Illumina Nextera XT DNA library protocol and paired-end sequencing was performed by FG Technologies (Leiden, The Netherlands).

2.5. Identification of RNA viral sequences

The raw reads were analyzed using a pipeline designed in workflow engine Snakemake v8.16.0 [42]. Trimming and quality control of initial reads were conducted with fastp using the default parameters. The trimmed reads were *de novo* assembled using rnaviralSPAdes v3.15 [43], a version that allows identification of RNA viruses. We used HMM-guided assembly for all k-mers using the default settings of the rnaviralSPAdes. The resulting assembled contigs were visualized using Bandage v0.8.1 [44] and subjected to further identification via DIAMOND BLASTx v2.1.9 [45] against both the NCBI non-redundant reference database containing all organisms (downloaded in July 2024) and a curated viral RNA-dependent RNA polymerase (RdRp)-scan database (downloaded in July 2024) [46]. This RdRp-specific database contains all RNA virus-encoded RdRps from the NCBI non-redundant database as well as the RdRp sequences from recent metagenomic studies [46]. Contigs without any matches in the NCBI non-redundant or RdRp-scan database were translated and compared to the RdRp Hidden Markov Models (HMMs) profile database using HMMer3 v3.4 with default settings in order to further detect RdRp sequences with low identity levels to known viruses [47]. The exact identities of putatively viral contigs identified using DIAMOND BLASTx, were further queried using NCBI BLASTn and NCBI VIRUS databases to determine the nucleotide-level percentage identity to the closest BLAST hit and to confirm their homology and taxonomy. Viral classification was determined using the International Committee on Taxonomy of Viruses (ICTV) database. Putative host or viral source assignment for each viral entry was initially determined using the RdRp-scan database [46]. Sequences were manually searched against the Virus-Host DB [48] and the NCBI Virus database (online version, accessed in September 2024) to support and confirm these assignments.

2.6. Mycovirus characterization

The open reading frames (ORFs) of viral contigs were predicted using the NCBI ORF finder program (https://www.ncbi.nlm.nih.gov/orffinder/) with the standard genetic code. Predicted ORF amino acid (aa) sequences were screened against the Conserved Domain Database (CDD v3.21 - 62456 PSSMs; https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to identify conserved motifs. Significant matches were retained using an E-value cutoff of $\leq 1 \times 10^{-5}$, with all detected hits exhibiting E-values $<1 \times 10^{-10}$. Genome organization diagrams were generated with IBS 2.0 [49] to highlight the positions of ORFs and functional regions. Sequence similarity searches were performed using BLASTx and BLASTp against the NCBI non-redundant (nr) protein database (accessed in July 2024), with an E-value cutoff of $\leq 1 \times$ 10-5 to confirm viral identities and to establish taxonomic affiliations.

All the RdRp sequences of identified viral contigs were aligned with MUSCLE [50] on MEGAX [51] and trimmed using ClipKIT [52] in default mode (smart-gap). The phylogenetic trees were constructed using the maximum likelihood method implemented on the W-IQ-TREE web server [53–55]. The best-fit models were defined with ModelFinder [39] according to the Bayesian Information Criterion (BIC). Branch support values were assessed through 1000 bootstrap replicates, and the resulting phylogenetic trees were visualized using online iToL server v6 [56]. The accession numbers of the proteins and the associated virus names are shown in Tables S1 and S2.

3. Results

3.1. Taxonomic distribution of the fungal strains and preliminary mycovirus screening

Fungal strains used in this study were identified based on their ITS sequences. The alignment of the sequences consisted of 37 sequences with 702 columns, 556 distinct patterns and 419 parsimony-informative sites. The phylogenetic tree of the strains is shown in Fig. 1 together with their corresponding colony morphologies.

To identify potential mycoviruses with a culture-based approach and to narrow down candidates for transcriptome sequencing, we first assessed the presence of viral RNA by visualizing the total RNA and double-stranded (ds) RNA for each fungal strain. The detection of abnormal RNA bands was used for a rough indication of the presence of mycoviruses (Fig. S1). Six strains, including *Pseudogymnoascus* sp. (3V; originating from soil 3), *Oidiodendron truncatum* (3AB; soil 3), *Mucor* sp. (2F; soil 2), *Cladosporium* sp. (1C; soil 1), *Apiotrichum lignicola* (3E; soil 3), and *Penicillium* sp. (C; soil 1), showed abnormal band patterns and were selected for further sequencing. No other fungi showed abnormal bands indicating they would not contain mycoviruses.

We further applied a combination of hyphal tipping and single-spore isolation as described by Cao et al. [57] and Noman et al. [58] to reduce viral titer. These methods have been reported to help "cure" fungal strains and produce isogenic lines [59]. By comparing colony phenotypes and growth rates before and after the treatment, we identified changes, such as shifts in pigmentation, growth rate, or sporulation, that could indicate a viral infection. Based on these observations, another six fungi - *Penicillium* sp. (2X), *Bionectriaceae* sp. (1X), *Mucor* sp. (1A), *Mucor* sp. (2D), *Penicillium* sp. (1E) and *Entomortierella* sp. (2B) were further selected for transcriptomic analyses. In the end, a total of 12 fungal strains were used for transcriptome sequencing.

3.2. Identification of mycoviruses in fungal strains

Transcriptome sequencing of 12 fungal strains provided 221,510

Fig. 1. Overview of the taxonomic and morphological diversity of fungal strains used in the study (shown in bold). The unrooted tree has been generated with ITS sequences (Table S1) and using maximum likelihood method. T type strain. *Central strain according to MycoBank database (www.mycobank.org). The best-fitting model for the phylogenetic tree was SYM + G4 according to the BIC on ModelFinder [39]. An additional three strains (isolate C, 2B, and 3K) were identified based on their transcriptome data. A detailed version of this tree is provided in Fig. S2.

contigs and >99 % of these contigs mapped to the genome of the fungal host itself. Results of the BLASTx search against both the NCBI non-redundant reference database (un-targeted approach) and a curated viral RdRp-scan database (targeted approach [46]) revealed that two different fungi, *Mucor* sp. (2F) and *Penicillium* sp. (C), were hosts of two distinct mycoviruses. No viral contigs were detected in the transcriptomes of the other ten fungal strains. A few fungal transcriptomes contained RNA mapping to bacteria, indicating potential endosymbionts, but due to the scope of the study, these were not investigated further.

The amino acid sequence of the ssRNA-RT virus detected from *Penicillium* sp. was identical to mycovirus *Penicillium camemberti virus* - GP1 (KT833324.1) belonging to the family *Pseudoviridae*. The maximum likelihood phylogenetic tree for this virus was generated using the best model WAG + I and it consisted of eight sequences (716 aa) (Fig. S3). The 8338 aa contig, assembled from *Mucor* sp. (2F) Transcriptomic data, was identified as a putative novel mycovirus and tentatively named as Mucor sp. Negative-stranded RNA virus 1 (MuNSRV1) with accession number PV357561. Its closest match with 52.42 % identity was found to be the Combu negative-strand RNA mycovirus (MN082151.1) [60] previously reported from *Mucor irregularis* (Table 1). However, the classification of the Combu negative-strand RNA mycovirus for the order and family ranks was not available. Therefore, preliminary phylogenetic tree was generated by combining the RdRp sequences of Combu negative-strand RNA mycovirus and MuNSRV1 together with the available RdRp sequences of the representatives of each viral family listed in the "Species lists" of the ICTV and NCBI databases (Table S2). The best model for this preliminary tree was VT + F + G4 and it contained 301 sequences (3581 aa). Based on this tree (data is not shown), a second phylogenetic tree was generated with only the sequences from the members of the *Bunyaviricetes* and *Monjiviricetes*, the closest viruses to the Combu negative-strand RNA mycovirus and MuNSRV1 (Fig. 2c). The best model for this final tree was VT + F + G4 and it consisted of 53 sequences (2566 aa). The descriptions of the two viral contigs belonging to Combu negative-strand RNA mycovirus and MuNSRV1 are listed in Table 1.

Table 1
Mycoviruses identified in fungal strains *Mucor* sp. (2F) and *Penicillium* sp. (C), using fungal transcriptome sequencing.

Fungal species	Viral taxon	Virus name	Genome polarity	Conserved domain	Length (bp)	BlastX FirstHIT (nr NCBI)	Identity (%)	Accession (nucleotide)	Accession (protein)	Host fungi (NCBI)
Mucor sp.	unclassified ssRNA(−) virus	*Mucor* sp. Negative-stranded RNA virus 1 (MuNSRV1)	ssRNA(−)	RdRp	8338	Combu negative-strand RNA mycovirus	52.42	MH990635.1	QAB47441.1	*Mucor irregularis*
Penicillium sp.	*Pseudoviridae*	*Penicillium camemberti* virus - GP1 (PcVGP1)	ssRNA-RT	gag-pol polyprotein	6378	*Penicillium camemberti* virus - GP1	100.00	KT833324.1	AOW69231.1	*Penicillium camemberti*

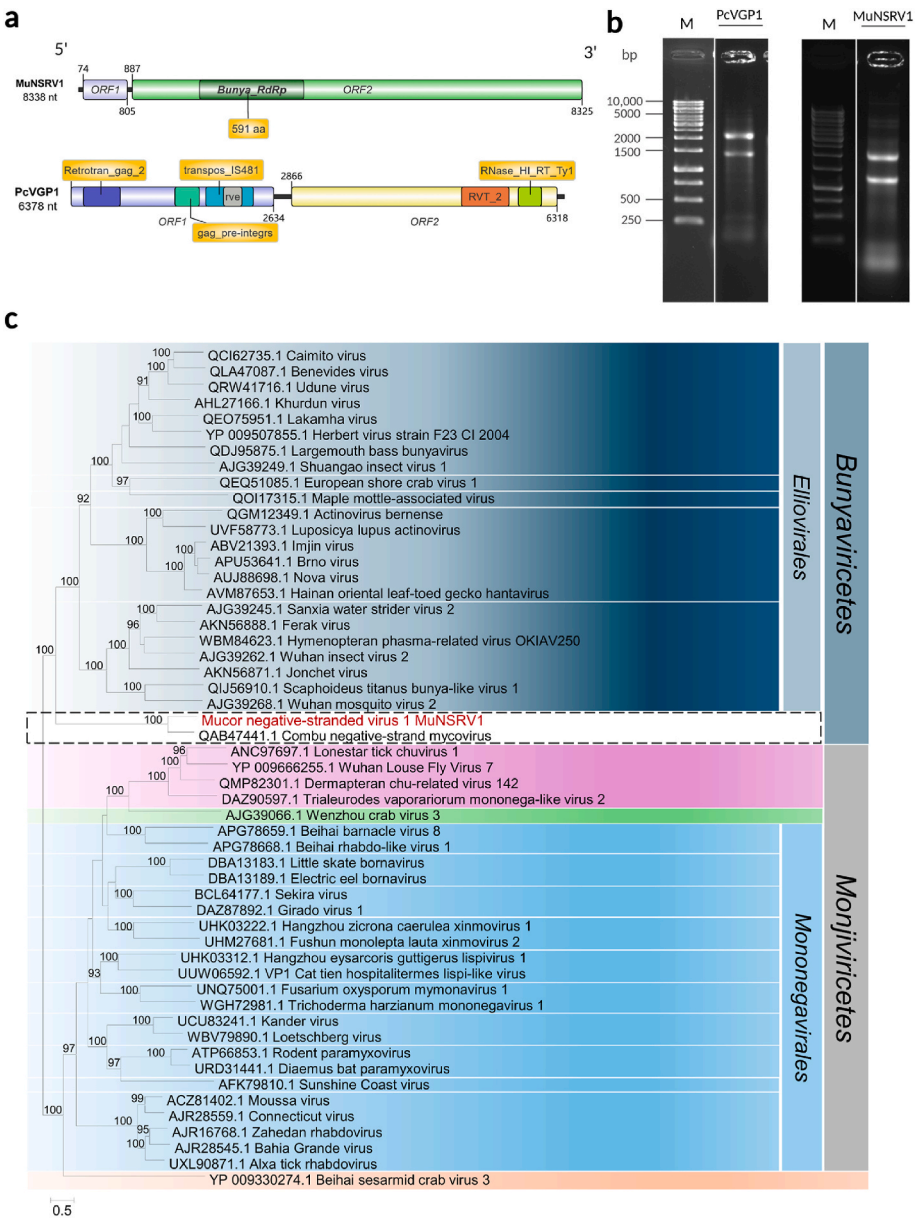

Fig. 2. Genome characterization and phylogenetic analysis of two mycoviruses from *Mucor* sp. and *Penicillium* sp. Strains, respectively (a) Schematic representation of genome structures. The functional protein domains are shown in color shade upon the ORFs (b) 1 % Agarose-gel electrophoresis profiles of the total RNA preparations from fungal strains *Mucor* sp. and *Penicillium* sp. (c) Midpoint rooted-phylogenetic tree generated with the maximum likelihood method and the best fit model VT + F + G4 using RdRp sequences of the putative novel virus MuNSRV1 and other selected viruses classified in *Riboviridae*. Values of ≥90 % for maximum likelihood are shown on the branches. The MuNSRV1 is shown in red. Beihai sesarmid crab virus 3 is used as an outgroup. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. Genome characterization and phylogenetic analysis of the putative novel virus infecting Mucor sp

The MuNSRV1 genome encodes ORF1 with 243 aa and ORF2 with 2478 aa. The conserved domain of RdRp (Bunya RdRp super family, cl20265) was located at position 766–1356 aa of ORF2 (Fig. 2a). Typical bunya-like conserved motifs [61] were identified in the RdRp of MuNSRV1. The alignment of the RdRp sequence of MuNSRV1 with selected members of *Bunyaviricetes* (Fig. S4) indicated that this mycoviral sequence contains six conserved motifs (A–E, and H). Motif C (SDD), motif D (KK), motif E (EXXS) and motif H (K) were entirely conserved in MuNSRV1, whereas a change of lysine (K) by arginine (R) in motif A, and a change of Glutamine (Q) by Methionine (M) in motif B were observed.

3.4. RNA viral occurrence in soil metatranscriptomes

Sequencing depth ranged from 25 to 52 million reads per sample. A total of 8,959,487 RNA contigs were assembled, and among them, only 232 potential RNA virus contigs were identified. Duplicate virus-associated sequences were merged across samples by combining contigs that matched the same viral reference in the database with >90 % sequence similarity, resulting in total of 101 potential RNA virus contigs (Table S3). All these contigs had amino acid identity values between 24.2 and 70.6 %, indicating a high degree of potentially novel species.

Taxonomic host assignment of the detected RNA virus-associated contigs was achieved at different levels using the RdRp-scan reference database [46]. A total of 81.2 % RNA viral contigs could be classified at the phylum level, with *Lenarviricota* accounting for 58.4 % of the abundance. At the family level, 57.4 % of RNA viral contigs were

classified, with *Fiersviridae* and *Mitoviridae* together accounting for 38.6 % (Fig. 3a). However, only 50.5 % of the RNA viral contigs could be assigned to a putative host (Fig. 3b). Prokaryotic RNA viruses were all assigned to bacteriophages belonging to the family *Fiersviridae*. The eukaryotic RNA viruses, on the other hand, were assigned a wide variety of eukaryotes, including algae, fungi, insects, mammals, mollusks, oomycetes and plants (Fig. 3b). Eukaryotic RNA viruses accounted for 47.5 % of the total detected RNA viruses and 94.1 % of the viruses with an assigned host (Fig. 3b).

Insects were identified as the most abundant hosts assigned to 37.5 % of eukaryotic RNA viruses and 17.8 % of all RNA viral contigs. Fungal and oomycete viruses together accounted for 39.6 % of the eukaryotic viruses, and 89.4 % of them were classified in the phylum *Lenarviricota*. A total of 57.9 % of these fungal and oomycete viruses were assigned to the family *Mitoviridae*, while the rest were classified in *Botourmiaviridae*, *Fiersviridae*, *Narnaviridae* and *Togaviridae* (Fig. 3c). The ecological functions of fungal and oomycete hosts included insect pathogens, arbuscular mycorrhizal fungi and plant pathogens (Fig. 3b; Fig. 4). Despite the availability of several records from common saprophytes, such as *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium* (https://www.genome.jp/virushostdb/index/host/all), no saprotrophic host was assigned to any viruses detected in our study.

3.5. Phylogenetic diversity of the fungal and oomycete RNA viruses in soil

In total, 19 classified eukaryotic RNA viral contigs were putatively assigned to fungal or oomycete hosts. Because RNA viruses often have segmented genomes [61], clustering viral contigs directly may lead to an overestimation of their diversity. To address this, the viral contigs were instead grouped into clusters based on the similarity of their RdRp amino acid sequences. This phylogenetic analysis demonstrated the distribution of RNA viruses among fungi and oomycetes in soil, showing a distinct clustering pattern into four main clades including five viral families (Fig. 4). *Mitoviridae* was the dominant family with nine newly identified viruses positioned within its clades, known to harbor viruses that are infecting fungi, oomycetes, plants and insects (Fig. 4). Due to the >90 % similarity of amino acid sequences and identical RdRp organization, virus 2 and virus 4 were classified in *Fiersviridae* and considered to be variants of the same virus, which has around 45 % identity with an already known *Emesvirus* [62]. Two other soil viruses were classified in the families *Botourmiaviridae* and *Togaviridae* and their closest relatives were previously reported from soil and river water samples (Fig. 4; Table S4).

4. Discussion

In the present study, we investigated the presence of mycoviruses on saprotrophic fungi, a group that is often overlooked in (myco)viral ecology. Screening 28 fungal strains isolated from sandy soils resulted in the identification of two mycoviruses, including one putative novel ssRNA virus. In parallel, through analysis of soil metatranscriptomes, a diverse array of RNA viral sequences was uncovered with eukaryotic and

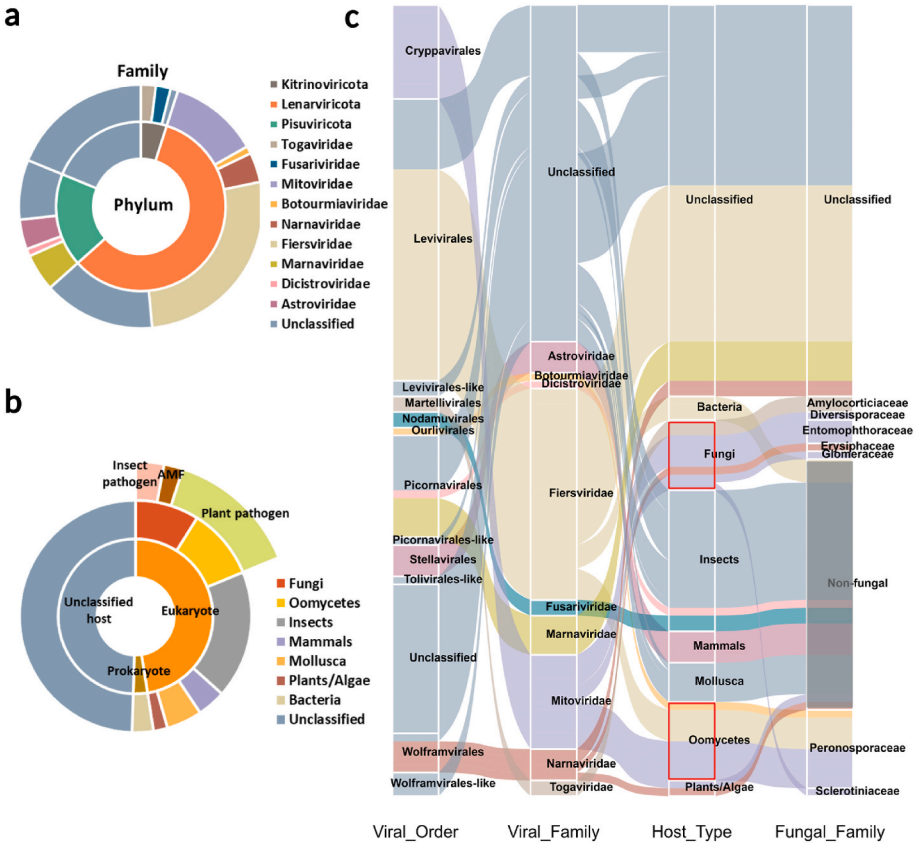

Fig. 3. Taxonomic classification and host range of RNA viruses identified from soil metatranscriptomes. (a) Taxonomic composition of the soil RNA viruses identified in this study. The double-layer donut chart demonstrates the distribution (%) of the viral contigs in the taxonomic rank of phylum and family. "Unclassified" indicates the contigs that could not be further classified at the family level. (b) Potential host assignments of corresponding RNA viruses. The inner layer shows the percentage of eukaryotic, prokaryotic and unclassified hosts. The middle and outer layers show the host lineage and ecological functional classification of fungi and oomycetes. (c) Connections of RNA viral groups and predicted host assignments. The Sankey diagram shows the viral proportions and host lineage correspondence. Viruses obtained from fungal and oomycete hosts are shown in red squares. The height of the bars in each stratum indicates the percentage of RNA viral contigs. Threads were colored by viral family classified consistent with (a). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

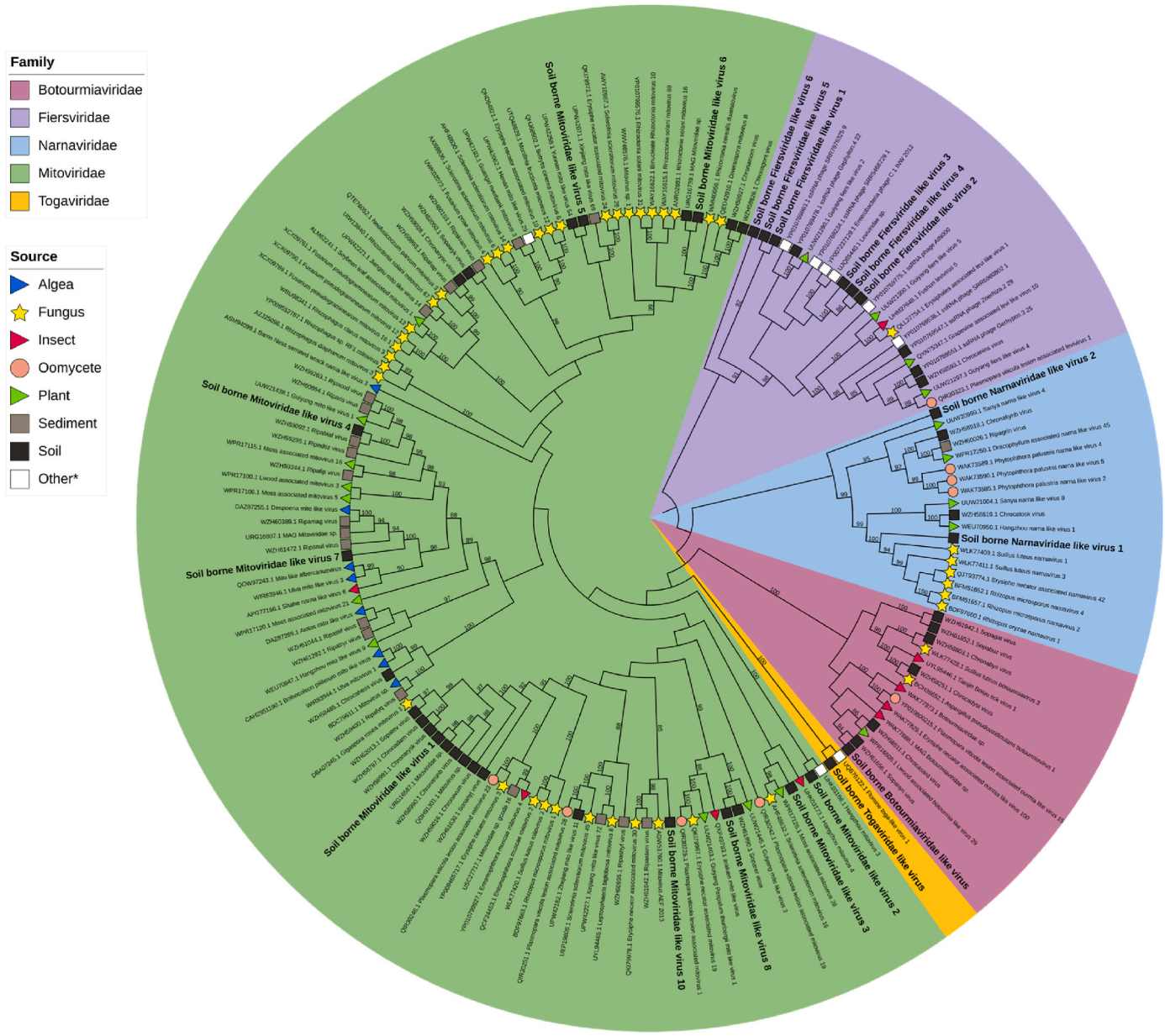

Fig. 4. The phylogenetic tree constructed using the maximum likelihood method showing taxonomic positions and host information of the soil viruses detected in the current study and their closest relatives based on RdRp aa sequences. The list of viruses is shown in Table S4. Values of ≥85 % for maximum likelihood are shown on the branches. The soil viruses detected in the study are shown in bold.

prokaryotic hosts; however, none of these viruses could be confidently assigned to have saprotrophic fungi as a host based on the RdRp-scan reference database.

The list of fungi screened for viruses evaluated in our study reflects the wide taxonomic range of typical soil saprotrophic fungi [29], including members from *Ascomycota, Basidiomycota* and *Mucoromycota*. In our study using a combination of RNA screening basing on electrophoresis and transcriptome sequencing, only two mycoviruses were found across the 28 saprotrophic fungal strains. This number is much lower than our expectation and contrary to our hypothesis. The results obtained by the culture-based approach were confirmed through metatranscriptomic soil analysis. Using metatrascriptomes we detected no viruses that could be confidently assigned to have saprotrophic fungal hosts, while viruses known to infect insect pathogenic, plant pathogenic and arbuscular mycorrhizal fungi were detected. However, 49.5 % of the virus contigs from the soil metatranscriptomes could not be assigned to any known host and hence we do not know if they potentially affect saprotrophic fungi. This large amount of viruses with unknown hosts reflects the uncertainty of the available data and potentially obscures findings. Given that a vast diversity of undescribed mycovirus species is thought to exist in nature [17,31,63], it is estimated that 30–80 % of fungal species are infected by mycoviruses [18]. Screening of mycoviruses from saprotrophic fungi has been limited in the past, despite their important roles in soil functioning [64]. Saprotrophic fungi may be underrepresented in the databases as a result of the previous focus on the identification of mycoviruses from pathogenic, mutualistic, and economically important fungi due to their explicit impact on agriculture, forestry, and industry. Therefore, it is important to screen mycoviruses from soil saprotrophic fungi and improve the databases for further studies.

While our results provide initial insight into the occurrence of mycoviruses in the analyzed Dutch sandy soils, it is not negligible that the lack of viral assignment to saprotrophic fungal hosts could also be partially due to limitations of the techniques used in the study. The

process of isolating fungi into pure cultures itself might reduce or eliminate viral load, yet, comparable studies have detected many mycoviruses with the same approach [31,32]. When the pure cultures are derived from spores, viral transmission can fail, or in the case of cultures derived from hyphal sectors, mycoviruses can be excluded from hyphal tips [59]. Moreover, the stability of viral infection further varies among taxa and viral groups [59,63]. Here we have tried to overcome these challenges by intentionally avoiding re-culturing steps and not using hyphal tips as source material. However, more studies using saprotrophic cultures should be performed in variable soils and methods need to be developed to standardize fungal culturing and isolation to detect specifically mycoviruses.

With an increasing number of viruses detected from environmental metagenomic and metatranscriptomic analyses [33–35], a proper classification of viruses becomes even more urgent. Increasing the data diversity in terms of substrates, hosts, or locations in databases is a crucial step for improving the classification but also understanding the ecology of the viruses. Here, we contributed to the database with putatively novel unclassified fungal viruses. However, further research is needed to understand the role of these mycoviruses in soil ecosystem functions. Even a rare infection of a mycovirus in saprotrophic fungi may still hold ecological significance, since it may potentially alter decomposition dynamics, nutrient turnover, and downstream ecosystem processes.

The prevalence and diversity of mycoviruses appears to differ significantly across different fungal lifestyles and environmental contexts. The number of detected viruses of saprotrophic fungi isolated from sandy soils in our study is much lower than previous reports of mycovirus presence in e.g. phytopathogenic fungi. For instance, coinfections involving more than ten mycoviruses within a single strain of *Fusarium poae* [65] and a diverse array of mycoviruses in *Sclerotinia sclerotiorum* have been reported [66]; both highlighting the high prevalence of mycoviruses in fungal pathogens [24,67]. This striking difference between plant-pathogenic and free-living saprotrophic fungi suggests distinct factors affecting virus presence in different fungal guilds. We propose that, besides the limitations in databases, the lower detection of viruses in saprotrophic fungi within the sandy soil environment, compared to those found in plant pathogenic fungi, could also be related to a naturally lower viral load associated with fungal lifestyle in heterogeneous soil conditions or to the immense fungal diversity in soil [25].

Unlike mutualists and pathogens, the independent lifestyle of soil saprotrophs potentially limits the opportunities for viral transmission [65,68,69]. Fungi living closely to plants or insects have established close coevolutionary relationships that can facilitate viral cross-kingdom transmission [70–72]. The widely observed cross-infection of plant viruses in fungi supports this possibility [73,74]. For example, for fungi that were isolated from plants with viral disease symptoms, around half of the fungal strains were detected with plant viruses [75]. Viral transmission among different fungal strains relies on compatibility and the intracellular transmission of mycoviruses is subjected by host mechanisms including cell division, hyphal anastomosis and the formation of sexual and asexual spores [76]. The systems controlling hyphal fusion, including vegetative and mating incompatibility, can regulate fungal cells connection, and in turn limit the spread of viruses across different fungal strains or species [77] making vegetative incompatibility a significant barrier to horizontal mycovirus transmission between fungal species or strains [78]. Moreover, in vertical propagation, viruses are usually eliminated in sexual spores during ascospore formation [79]. Soil ecosystems present unique challenges to fungal transmission that may explain why we detected so little mycoviruses affecting saprotrophic fungi. In soil environments, a three times higher fungal diversity was observed compared to fungi dwelling in leaves and roots [80]. This high diversity increases the likelihood of encountering incompatible fungal strains, making horizontal viral transmission less likely or even a rare phenomenon. Additionally, saprotrophic fungi residing in bulk soils are often dispersed and isolated within the soil matrix as compared to plant associated fungi resulting in a lower probability of physical contact with compatible species and fusion. Moreover, fluctuating soil conditions, such as temperature changes, nutrient scarcity, and desiccation, influence fungal reproduction, adaptation, and competition efficiency [81,82]. For example, some fungi simplify their reproduction to energy-efficient forms under harsh conditions [83,84]. These stress responses may create unfavorable intracellular environments for viral propagation, further leading to lower numbers of mycovirus in soil saprotrophic fungi.

Combined findings from fungal strains and complex soil viral communities suggest that mycoviruses in saprotrophic fungi remain difficult to detect and are largely uncharacterized. Additionally, soil type and detection techniques might affect the detection rate of these mycoviruses. Broader screening of saprophytic fungi, different soil types, and improved methodology, such as using both *in vitro* and *in silico* approaches for additional screening, are needed to capture the diversity of mycoviruses in soil saprotrophic fungi more accurately. Promising directions include targeted dsRNA or viral particle enrichment to improve assembly of complete viral genomes, as well as development of curated reference databases for divergent RNA virus detection. Culture-based approaches can be combined with curing assays that directly link viral presence to host phenotypes. Combining these methods will not only enhance the identification of virus-host associations and reveal virus spatial distributions in soils but also allow us to investigate how viral infection alters the ecological functions of fungi. Additionally, investigating viral transmission strategies in soils and the impact of environmental factors on viral prevalence can provide deeper insights into fungi-host interactions in soil ecosystems.

5. Conclusion

In this study we detected very low numbers of mycoviruses in saprotrophic fungi in sandy soils in the Netherlands. The low number of viral sequences recovered from saprotrophic fungal strains and soil metatranscriptomes could be due to limited opportunities for horizontal viral transmission, and the isolated nature of free-living saprotrophic fungi within soil matrices or be a special feature of these soils. We cannot furthermore rule out that biases in databases towards commercially important viruses infecting plant-pathogenic fungi could obscure our findings. Overall, our findings highlight the need for large-scale, multiphasic approaches with improved methodological sensitivity, to advance our understanding of the prevalence and ecological significance of mycoviruses.

CRediT authorship contribution statement

Ruiqi Wang: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hazal Kandemir:** Writing – review & editing, Visualization, Methodology. **Jing Zhang:** Investigation. **T. Martijn Bezemer:** Writing – review & editing. **Peter M. van Bodegom:** Writing – review & editing. **S. Emilia Hannula:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejsobi.2025.103787.

Data availability

Nucleotide sequences that were generated during the study were deposited into GeneBank nucleotide database with the accession numbers PV357561 and PV357562. We have preserved the fungal strains identified in this study as virus-infected, and we are willing to share them with researchers who are interested in further studies.

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