Novel Mycoviruses Discovered from a Metatranscriptomics Survey of the Phytopathogenic *Alternaria* Fungus

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**Abstract:** *Alternaria* fungus can cause notable diseases in cereals, ornamental plants, vegetables, and fruits around the world. To date, an increasing number of mycoviruses have been accurately and successfully identified in this fungus. In this study, we discovered mycoviruses from 78 strains in 6 species of the genus *Alternaria*, which were collected from 10 pear production areas using high-throughput sequencing technology. Using the total RNA-seq, we detected the RNA-dependent RNA polymerase of 19 potential viruses and the coat protein of two potential viruses. We successfully confirmed these viruses using reverse transcription polymerase chain reaction with RNA as the template. We identified 12 mycoviruses that were positive-sense single-stranded RNA (+ssRNA) viruses, 5 double-strand RNA (dsRNA) viruses, and 4 negative single-stranded RNA (−ssRNA) viruses. In these viruses, five +ssRNA and four −ssRNA viruses were novel mycoviruses classified into diverse the families Botourmiaviridae, Deltaflexivirus, Mymonaviridea, and Discoviridae. We identified a novel −ssRNA mycovirus isolated from an *A. tenuissima* strain HB-15 as *Alternaria* tenuissima negative-stranded RNA virus 2 (AtNSRV2). Additionally, we characterized a novel +ssRNA mycovirus isolated from an *A. tenuissima* strain SC-8 as *Alternaria* tenuissima deltaflexivirus 1 (AtDFV1). According to phylogenetic and sequence analyses, we determined that AtNSRV2 was related to the viruses of the genus *Sclerotimonavirus* in the family Mymonaviridae. We also found that AtDFV1 was related to the virus family Deltaflexiviridae. This study is the first to use total RNA sequencing to characterize viruses in *Alternaria* spp. These results expand the number of *Alternaria* viruses and demonstrate the diversity of these mycoviruses.

**Keywords:** mycovirus; *Alternaria*; metatranscriptomics; genome; positive- and negative single-stranded RNA viruses

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1. **Introduction**

Mycoviruses are wide distributions in the major taxonomic groups of filamentous fungi, yeasts, and oomycetes [1–7]. Most of the mycoviruses genomes are linear positive-sense single-stranded RNA (+ssRNA) or double-stranded RNA (dsRNA) [1,2,5,7]. Of these mycovirus genomes, a few have been found to include circular single-stranded DNA (ssDNA) or linear negative-sense single-stranded RNA (−ssRNA) [8–16].

*Alternaria* spp. can cause essential diseases in cereals, ornamental plants, vegetables, and fruits, and more than 95% of the species reported globally can become facultative parasites on different plants [17–20]. A recent study revealed that six *Alternaria* species are the causal agents of pear black spot diseases, resulting in massive economic losses in China [20].
To date, members of the genus *Alternaria* have been found to harbor several mycoviruses. Some *A. alternata* strains have been found to contain many uncharacterized virus-like dsRNAs [21,22]. Mycoviruses discovered in the strains of different species of the genus *Alternaria* have been classified into twelve families. Among these families, the family *Chrysoviridae* includes *Alternaria alternata* chrysovirus 1 (AaCV1) and *Alternaria solani* chrysovirus 1 (AsCV1) [23–26]. The family *Partitiviridae* includes *Alternaria alternata* partitivirus 1 (AaPV1) and *Alternaria tenuissima* partitivirus 1 (AtPV1) [27,28]. The family *Totiviridae* includes *Alternaria* arborescens victorivirus 1 (AaV1) and *Alternaria alternata* victorivirus 1 (AalVV1) [29,30]. The genus *Botybirnavirus* includes *Alternaria* botybirnavirus 1 (ABRV1) and *Alternaria alternata* botybirnavirus 1 (AaBRV1) [31–33]. The family *Mitoviridae* includes two mitovirus called *Alternaria* arborescens mitovirus 1 (AaMV1) and *Alternaria* solani mitovirus 1 (AsMV1) [34,35]. The family *Endornaviridae* includes *Alternaria* brassicicola endornavirus 1 (AbEV1) [36]. *Alternaria alternata* virus 1 (AaV1), which was identified in the proposed family *Alternaviridae*, was isolated from an *A. alternata* strain [37]. Importantly, AaV1 is the first dsRNA virus to be identified with both 5′ cap and 3′poly(A) structures on its genomic segments [38]. Moreover, strains of the genus *Alternaria* have been found to harbor *Alternaria* dianthicola dsRNA virus 1 (AdRV1), *Alternaria* longipes dsRNA virus 1 (AlRV1), and *Alternaria* alternata virus 1 (AaV1) which are unclassified dsRNA mycoviruses [37–40]. The member in the family *Hypoviridae* was found to include *Alternaria* alternata hypovirus 1 (AaHV1) [41]. In addition, *Alternaria* brassicicola fusarivirus 1 (AbFV1) and *Alternaria* solani fusarivirus 1 (AsFV1), which are positive ssRNA mycoviruses, are isolated in *A. brassicicola* and *A. solani*, respectively. AbFV1 and AsFV1 have been grouped with members of the recently proposed family *Fusariviridae* [42,43]. The other +ssRNA mycoviruses, *Alternaria* alternata magoulivirus 1 (AaMOV1) has been found to belong to the genus *Magoulavirus* in the family *Botourmiaviridae* [24]. Recently, full-length cDNA sequences of nine viruses were obtained by high-throughput sequencing in an *Alternaria dianthus* strain HNSZ-1 [44]. Among the nine viruses, five were confirmed to novel members in the families *Hypoviridae*, *Totiviridae*, *Mymonaviridae*, and a provisional family *Ambiguiviridae* [44]. Two −ssRNA, named *Alternaria* tenuissima negative-stranded RNA virus 1 (AtNSRV1) and *Alternaria* dianthus negative-stranded RNA virus 1 (AdNSRV1), belonged to the genus *Sclerotimonavirus* in the family *Mymonaviridae* [44,45]

In most cases, the host does not experience any phenotypic changes as a mycovirus infection [1,2,5,7]. Some mycoviruses, however, can cause debilitating symptoms in their hosts, including morphological changes, toxin production, and hypovirulence [4–7]. Among these hypovirulence-associated mycoviruses, some virus-infected strains can be used as biocontrol agents to prevent and treat fungal diseases in plants [3,7,45–49]. In some cases, phenotypic changes are result of mycoviruses harbored in the *Alternaria* fungi. For instance, AaCV1 not only restricted the growth of the host fungus but also rendered the host hypervirulent to the plant [26]. AaCV1-AT1 could reduce the growth rate and sporulation ability of the *A. tenuissima* strain [25]. AaV1 can cause impaired growth and unusual pigmentation in the host [38]. AaHV1 also can confer hypovirulence in other plant pathogenic fungi [41]. AdNSRV1 might be related to the phenotypic change of the host fungus [44].

In recent years, metatranscriptomics has been widely used in virus discovery. Many novel viruses have been discovered in fungi, which has significantly promoted the progress of viromics research and has sped up the discovery and understanding of unknown viruses [13,44,50–59]. In addition, transcriptomes data of fungi provided evidence of the existence of negative-sense RNA viruses in fungi before the first negative-sense RNA mycovirus was isolated [59].

In this study, we followed a metatranscriptomics approach to identify the mycovirus communities of six species of the genus *Alternaria*, which are associated with pear spot disease in China. We also identified near-full-length sequences of putative mycoviruses. We isolated a novel −ssRNA mycovirus from an *A. tenuissima* strain HB-15, which we
designated as Alternaria tenuissima negative-stranded RNA virus 2 (AtNSRV2). We also characterized a novel +ssRNA mycovirus from an *A. tenuissima* strain SC-8, which we designated as Alternaria tenuissima deltaflexivirus 1 (AtDFV1). Through phylogenetic and sequence analyses, we found AtNSRV2 to be related to the viruses of the genus *Sclerotimonavirus* in the family *Mymonaviridae*. We also found that AtDFV1 is related to the family *Deltaflexivirus*. In this study, we identified viruses in *Alternaria* spp., for the first time, using total RNA sequencing. These results significantly expanded the number of *Alternaria* viruses and revealed the abundant diversity of the mycoviruses.

2. Materials and Methods

2.1. Fungal Isolates and Culture Conditions

We selected 78 strains of *Alternaria* spp. from among isolated strains of pear black spot samples. We collected these samples from the primary among the 10 provinces that produce pears in China [20]. We used potato dextrose agar (PDA) plates to culture the strains in the dark at 28 °C. We used the sterile 25% glycerol solution to store the 5 mm mycelial agar discs at −80 °C. We used the partial region sequences of six loci to identify the strains: partial rDNA-ITS region, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), translation elongation factor 1-alpha (TEF 1α), endo polygalacturonase (endoPG), Alternaria major allergen gene (Alt al), and histone 3 (His 3) [20]. Table 1 and Supplementary Table S1 list the species of the strains used in this study and include the specific sources.

Table 1. Origin of the strains of *Alternaria* species used for high-throughput sequencing in this study.

| Area   | Source | Numbers of Strains | Species and Their Strains Numbers |
|--------|--------|--------------------|-----------------------------------|
|        |        |                    | A. tenuissima | A. alternata | A. gossypina | A. arborescens | A. gaisen | A. longipes |
| Anhui  | 8      | 5                  | 2            | 0           | 0            | 1            | 0         |
| Xinjiang | 5     | 2                  | 1            | 0           | 0            | 2            | 0         |
| Shandong | 6     | 5                  | 1            | 0           | 0            | 0            | 0         |
| Chongqing | 8     | 6                  | 1            | 0           | 0            | 1            | 0         |
| Sichuan | 9      | 5                  | 1            | 1           | 1            | 1            | 0         |
| Gansu  | 7      | 5                  | 2            | 0           | 0            | 0            | 0         |
| Yunnan | 3      | 1                  | 2            | 0           | 0            | 0            | 0         |
| Jilin  | 2      | 2                  | 0            | 0           | 0            | 0            | 0         |
| Guizhou | 2     | 2                  | 0            | 0           | 0            | 0            | 0         |
| Hubei  | 28     | 25                 | 3            | 0           | 0            | 0            | 0         |
| Total  | 78     | 58                 | 13           | 1           | 3            | 2            | 1         |

2.2. RNA Extraction and Sample Preparation for High-Throughput Sequencing

We prepared total RNA for high-throughput sRNA sequencing. We cultured 78 strains on cellophane membranes that overrode PDA plates at 28 °C for 7 days. By mixing the mycelia in equal proportion, we ground the samples using liquid nitrogen. We used a TRIzol RNA extraction kit (Thermo Fisher, Waltham, MA, USA) to extract the total RNA, which we then clarified with chloroform in 2 mL tubes. We used ethanol precipitation to obtain the total nucleic acid fractions. We used 75% alcohol to wash the sample two times, and then dissolved them in water treated with diethylpyrocarbonate (DEPC). The total RNA was detected by agarose gel electrophoresis with 1.2% (w/v). Then, we determined the concentration and selected qualified samples for high-throughput sequencing.

2.3. High-Throughput Sequencing and Data Analysis

We sent the total RNA of the mixed strains to the Beijing Biomarker Technologies Company (Beijing, China), where it was constructed and sequenced for cDNA library. First, rRNA was removed, and then double-stranded cDNAs were synthesized using random hexamers (N6). The cDNA ends were repaired, and the A-tail was added and sequenced. The final library was obtained using the polymerase chain reaction (PCR). Then, the quality of the library was tested and sequenced using an Illumina HiSeq XTen platform.
A certain proportion of low-quality data is inevitable when obtaining raw data through sequencing. To ensure the reliability and accuracy of the analysis results, we had to preprocess the raw data, which were strictly controlled, and filtered as follows: we first removed the reads using adaptors, and then removed the low-quality reads for clean data. We analyzed the potential sequences of mycoviruses according to the splicing and assembly of clean data.

2.4. Validation of Virus-like Contigs by RT-PCR and Viral Sequencing Amplification

We isolated the total RNA of 78 strains and used reverse transcription polymerase chain reaction (RT-PCR) to investigate viruses in the fungal strains using specific primers, which were designed based on the assembled contigs (Supplementary Table S2). For cDNA synthesis, we added 1 µL random hexamers (N6), 2 µL DEPC-treated water, and 7 µL total RNA sample extracted from each tested strain to a 500 µL tube (RNase-free). After 8 min in boiling water bath, the sample was placed on ice for 3–5 min, the following system was added: 4 µL of DEPC-treated water, 4 µL of 5× M-MLV reverse transcription buffer, 1 µL of 2.5 mM dNTP, 0.5 µL of RNase inhibitor (TaKaRa, Dalian, China), 0.5 µL of M-MLV reverse transcriptase (PROMEGA, Madison, WI, USA). We mixed and centrifuged the sample, and then reverse transcribed the mixture for 1–2 h at 37 °C in a 20 µL reaction mixture. After reaction, obtained products were used for PCR or stored at −20 °C. The PCR products were electrophoresed in a 1.2% agarose gel, and stained with ethidium bromide (EB, 0.1 µg/mL) for visualization on a UV trans-illuminator. We used a Pmd18-T vector (TaKaRa, Dalian, China) to ligate the purified PCR product, which was transformed into competent cells of Escherichia coli DH5α. We conduct the sequencing at Sangon Biotech Co. (Shanghai, China).

We used RT-PCR to obtain full-length sequences of the viruses. We determined virus sequences using primers designed from the data obtained from the assembled contigs (Supplementary Table S3). We also amplified the cDNA ends sequence of virus using a SMARTer RACE 5'/3' kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. We ligated the purified PCR product into a pMD18-T vector, which was transformed into E. coli DH5α. The sequencing was completed at Sangon Biotech Co. We determined three or more independent clones for each product in both orientations.

2.5. Sequences Alignment and Phylogenetic Analysis

We calculated sequence similarities using BLAST program on the NCBI database (https://www.ncbi.nlm.nih.gov (accessed on 22 October 2022)). According to the alignment information, the viruses related information (e.g., nucleic acid type, virus species, and the number of open reading frames [ORFs]) was preliminarily confirmed. We used DNAMAN (Lynnon Corporation, Pointe-Claire, Quebec, Canada) to conduct the sequence assembly. We used the ORF Finder website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html (accessed on 22 October 2022)) to predict ORFs. We searched the CDD database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb. cgi (accessed on 22 October 2022)) and the Pfam database (http://pfam.xfam.org/ (accessed on 22 October 2022)) to predict the conserved domains in sequences. We used MEGA 10 and applied the maximum likelihood method to construct the phylogenetic trees, which we tested with 1000 bootstrap replicates. We used MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft/ (accessed on 22 October 2022)) to conduct multiple sequence alignments of proteins encoded by the contigs and the reference mycovirus.

3. Results

3.1. Diversity of Alternaria Viruses

Reportedly, several phytopathogenic fungi have fungal viruses. We identified the presence of mycoviruses in the fungi of Alternaria and generated an RNA sequencing library with 78 strains (Table 1). The library was sequenced and assembled at a considerable depth. After removing rRNA from the assembled data, we obtained 67,011,714 reads by fragment reverse transcription, and screened out 66,709,434 clean reads by quality control data.
Overall, a total of 131,203 contigs with a total length of 82,578,727 bp were yielded by transcriptional splicing, database alignment, and coding region prediction. The N50 and N90 values were 812 and 291, respectively. The results showed that the quality of the spliced sequences was good. We obtained 26 contigs that were derived from mycoviruses from this sequence (Table 2).

Table 2. Best BLASTx matches of contigs obtained in this study.

| No. | Contig Number | Contig Length (nt/bp) | Best Match | Host Strain | Name of Putative Virus | Protein | Cover % | aa Ident % | Taxon |
|-----|---------------|----------------------|------------|-------------|------------------------|---------|---------|-----------|-------|
| +ssRNA Virus | | | | | | | | | |
| 1 | contig 5 | 14,170 | Alternaria tenuissima hypovirus 1 | A. tenuissima: G-9, JL-7 | Alternaria tenuissima hypovirus 1 (AtHV1) | polyprotein | 90 | 97.89 | Hypocoviridae Hypovirus |
| 2 | contig 5012 | 2170 | Alternaria arborescens mitovirus 1 | A. tenuissima: G-9, G-21-2 | Alternaria tenuissima mitovirus 1 (AtMV1) | Polyprotein | 94 | 91.04 | Mitocoviridae Mitovirus |
| 3 | contig 5919 | 2004 | Neofusicoccum parvum narnavirus 2 | A. tenuissima: AH-29 | Alternaria tenuissima narnavirus 1 (AtNV1) | RdRp | 91 | 98.04 | Narnaviridae Narnavirus |
| 4 | contig 2423 | 2972 | Cladosporium cladosporioides ourmia-like virus 2 | A. tenuissima: G-5, G-41, GS-8, AH-25, A. gossypina: SC-16, A. arborescens: KEL-4-4 | Alternaria tenuissima ourmia-like virus 1 (AOLV1) | RdRp | 60 | 96.32 | Botourmiaviridae Magoulivirus |
| 5 | contig 2672 | 2845 | Alternaria alternata magoulivirus 1 | A. tenuissima: GZ-1 | Alternaria tenuissima ourmia-like virus 2 (AOLV2) | RdRp | 76 | 98.90 | |
| 6 | contig 4360 | 2324 | Plasmopara viticola associated ourmia-like virus 37 | A. tenuissima: SC-12 | Alternaria tenuissima ourmia-like virus 3 (AOLV3) | RdRp | 81 | 94.75 | Botourmiaviridae Betascleroulivirus |
| 7 | contig 5365 | 2102 | Plasmopara viticola associated ourmia-like virus 64 | A. arborescens: KEL-4-4 | Alternaria arborescens ourmia-like virus 1 (AarOLV1) | RdRp | 87 | 50.00 | Botourmiaviridae Deltascleroulivirus |
| 8 | contig 2521 | 2918 | Penicillium sumatrense ourmia-like virus 1 | A. alternata: GS-17, SC-32 | Alternaria alternata ourmia-like virus 1 (AalOLV1) | RdRp | 79 | 86.38 | Botourmiaviridae Botoulivirus |
| 9 | contig 6218 | 1950 | Plasmopara viticola associated ourmia-like virus 52 | A. tenuissima: G-21-2 | Alternaria tenuissima ourmia-like virus 4 (AOLV4) | RdRp | 96 | 64.17 | Botourmiaviridae Betabotoulivirus |
| 10 | contig 3454 | 2568 | Plasmopara viticola associated ourmia-like virus 65 | A. tenuissima: AH-25, A. gaisen: AH-20 | Alternaria tenuissima ourmia-like virus 5 (AOLV5) | RdRp | 83 | 94.85 | Botourmiaviridae Ournaviridae |
| 11 | contig 19,628 | 946 | Colletotrichum fructicola ourmia-like virus 2 | A. tenuissima: SC-12, HB-15 | Alternaria tenuissima ourmia-like virus 6 (AOLV6) | RdRp | 95 | 89.04 | Botourmiaviridae unclassified |
| 12 | contig 73 | 8352 | Agrostis stolonifera deltaflexiviruses 1 | A. tenuissima: SC-8 | Alternaria tenuissima deltaflexiviruses 1 (AtDFV1) | RdRp | 73 | 98.83 | Deltaflexiviridae Deltaflexiviruses |
Table 2. Cont.

| No. | Contig Number | Contig Length (nt/bp) | Best Match | Host Strain | Name of Putative Virus | Protein | Cover % | aa Ident % | Taxon |
|-----|---------------|-----------------------|------------|-------------|------------------------|---------|---------|------------|------|
|     |               |                       |            |             | dsRNA virus            |         |         |            |       |
| 13  | contig 10,828 | 1417                  | Alternaria longipes dsRNA virus 1 | *A. arborescens* | *A. arborescens* dsRNA virus 1 (AaRV1) | hypothetical protein | 51      | 97.96      | unclassified |
|     | contig 13,903 | 1210                  |            |             |                        |         |         |            |       |
| 14  | contig 1410   | 3617                  | Alternaria alternata chrysovirus 1 | *A. tenuissima* | *A. tenuissima* chrysovirus 1 (AtCV1) | RdRp | 91      | 97.84      |       |
|     | contig 2756   | 2814                  |            |             |                        |         |         |            |       |
|     | contig 2896   | 2759                  |            |             |                        |         |         |            |       |
|     | contig 7495   | 1762                  |            |             |                        |         |         |            |       |
| 15  | contig 11,000 | 1393                  | Alternaria dianthicola partitivirus 1 | *A. tenuissima* | *A. tenuissima* partitivirus 1 (AtPV2) | coat protein | 90      | 99.05      | Partitiviridae |
| 16  | contig 7155   | 1808                  | Alternaria tenuissima partitivirus 1 | *A. alternata* | *A. alternata* partitivirus 1 (AaPV1) | RdRp | 85      | 94.56      |       |
|     | contig 206    | 6162                  | Botryosphaeria dothidea botybirnavirus 1 | *A. tenuissima* | *A. tenuissima* botybirnavirus 1 (AtBBV1) | cap-pol fusion protein | 93      | 97.92      | Botybirnavirus |
|     | contig 253    | 5808                  |            |             |                        |         |         |            |       |
| 18  | contig 52     | 8970                  | Cryptonectria parasitica sclerotimonavirus 1 | *A. tenuissima* | *A. tenuissima* negative-stranded RNA virus 2 (AtNSRV2) | RdRp | 64      | 56.36      |       |
| 19  | contig 15,899 | 1079                  | Plasmopara viticola lesion associated mymonavirus 1 | *A. tenuissima* | *A. tenuissima* negative-stranded RNA virus 3 (AtNSRV3) | nucleocapsid | 59      | 43.12      | Discoviridae Sclerotimonavirus |
| 20  | contig 35     | 10,267                | Botrytis cinerea negative stranded RNA virus 10 | *A. tenuissima* | *A. tenuissima* negative-stranded RNA virus 2 (AtNSRV4) | RdRp | 49      | 30.71      |       |
| 21  | contig 192    | 6528                  | Coniothyrium diploidiella negative-stranded RNA virus 1 | *A. arborescens* | *A. arborescens* negative-stranded RNA virus 1 (AaNSRV1) | RdRp | 97      | 68.76      | Discoviridae unclassified |

We subjected the contigs to BLAST analysis and assigned the contigs to 21 putative novel mycoviruses. These mycoviruses were characterized by eight different viral families, including Botourmiaviridae, Botybirnaviridae, Chrysoviridae, Deltaflexiviridae, Hypoviridae, Partitiviridae, Mybuviridea, Mymonaviridae, and Narnaviridae. Among them, 12 viruses belonged to +ssRNA viruses, 5 viruses belonged to dsRNA viruses, and 4 viruses belonged to −ssRNA viruses (Table 2).
3.2. Detection and Validation of Alternaria Viruses by RT-PCR

According to the sequence of contigs obtained by high-throughput sequencing, specific primers were designed for RT-PCR detection. The results showed that 21 viruses could be detected in 22 strains of *Alternaria* spp. (Figure 1), including 16 strains of *A. tenuissima*, 3 strains of *A. alternata*, 1 strain each of *A. arborescens*, *A. gossypina*, and *A. gaisen*. These results indicated that the putative viral sequences were reliable. Strain G-21-2 of *A. tenuissima* and KEL-4-4 of *A. arborescens* were infected by four viruses (Figure 1). Strain HB-15 was infected by three viruses. Strains G-9 and AH-25 of *A. tenuissima* were infected by two viruses (Figure 1). Others harbored only one virus.

![Figure 1](image-url)

**Figure 1.** Detection of the 21 putative mycoviruses in different strains by RT-PCR. We used the assembled contigs to design the specific primers (see Supplementary Table S2). Only 22 strains of *Alternaria* species used for high-throughput sequencing were detected viruses. Lane Marker, DNA Marker II (TianGEN, Beijing, China). For abbreviations of virus names used in detection of the viruses, see Table 2.

3.3. Positive-Sense Single-Stranded RNA Virus

We used the obtained contigs and identified 12 positive-sense single-stranded RNA viruses. These sequences could be classified into four families, including *Hypoviridae*, *Narnaviridae*, *Deltaflexiviridae*, and *Botourmiaviridae* (Table 2).

The family *Hypoviridae* contains the genus *Hypovirus*. Contig 5 (14,170 nt) had a large ORF (484–13,302 nt), which encoded a 4272 aa protein. We found that this predicted amino acid sequence best resembled the RdRp of *Alternaria* alternata hypovirus 1 (AaHV1, GenBank: QFR36339) and had a 97.89% homology (Table 2). Thus, this virus should be a new strain of AaHV1. This hypovirus was detected in *A. tenuissima* strain JL-7 and G-9 (Figure 1). Therefore, the virus might be called *Alternaria* tenuissima hypovirus 1 (AtHV1).

The family *Mitoviridae* had only one genome and encoded one ORF, including genera *Duamitovirus*, *Koaramitovirus*, *Triamitovirus*, and *Unuamitovirus*. The contig 5012 (2170 nt) had a large ORF (72–2048 nt) that encoded a 658 aa protein. We found that a predicted amino acid sequence of the protein best resembled the polyprotein of *Alternaria* arborescens mitovirus 1 (AaMV1, GenBank: YP_009270635), which had a 91.04% homology. Therefore,
this virus was a strain of AaMV1 that belonged to the genus Duamitovirus in the family Mitoviridae (Table 2). We detected this virus in A. tenuissima strains G-9 and G-21-2 (Figure 1) and called it Alternaria tenuissima mitovirus 1 (AtMV1).

The family Narnaviridae had only one genome and encoded one ORF, including one genus Narnavirus. The contig 5919 (2004 nt) had a 98.04% homology with the RdRp of Neofusicoccum parvum narnavirus 2 (NpNV2, GenBank: QDB74995), which meant this virus was a strain of NpNV2 and belonged to the genus Narnavirus. The results of RT-PCR revealed that A. tenuissima strain AH-29 harbored this virus and was called Alternaria tenuissima narnavirus 1 (AtNV1) (Figure 1).

The twelve genera Botoulivirus, Betabotoulivirus, Magoulivirus, Penoulivirus, Ourmiavirus, Rhizoulivirus, Betarhizoulivirus, Scleroulivirus, Betascleroulivirus, Deltascleroulivirus, Gammascleroulivirus, and Epsilonscleroulivirus are in the family Botourmiaviridae. In this study, eight of the obtained contigs were homologous with numbers of this family, including two magouliviruses, one ourmiavirus, one botoulivirus, one betabotoulivirus, one betascleroulivirus, one deltascleroulivirus, and one unclassified (Table 2). According to the BLASTx result, contig 2423 (2972 nt) best resembled the RdRp of Alternaria alternata magoulivirus 1 (AaMOV1, GenBank: A_13793PavOLV64, GenBank: QGY72594) at 50.00% similarity (Table 2). We determined that this virus was a strain of AaMOV1. We detected the virus in A. tenuissima strains G-5, G-41, GS-8, and AH-25 (Figure 1). We called it Alternaria tenuissima ourmia-like virus 1 (AtOLV1). AtOLV1 was also detected in A. arborescens strain KEL-4-4 and A. gossypina strain SC-16 (Figure 1). We found the greatest similarity with contig 2672 (2845 nt), which best resembled the RdRp of Alternaria alternata magoulivirus 1 (AaMOV1, GenBank: UOV22670) at 98.90% homology and Plasmopara viticola associated ourmia-like virus 2 (CcOLV2, GenBank: QDB75008) at 96.32% similarity (Table 2).

Therefore, the virus was a strain of CcOLV2, which was detected in A. arborescens strain KEL-4-4 and Plasmopara viticola associated ourmia-like virus 32 (DMG-F_40507PavOLV32, GenBank: QGY72562) at 98.07% homology (Table 2). Therefore, we determined that this virus was a strain of CcOLV2 and called it Alternaria tenuissima ourmia-like virus 2 (CcOLV2). According to the BLASTx result, contig 4360 (2324 nt) best resembled the RdRp of Plasmopara viticola associated ourmia-like virus 64 (DMG-B_Contig4PavOLV37, GenBank: QGY72567) at 94.75% similarity (Table 2). We determined that this virus was a strain of DMG-B_Contig4PavOLV37. We detected this virus in A. tenuissima strain SC-12 (Figure 1) and called it Alternaria tenuissima ourmia-like virus 3 (AtOLV3). According to the BLASTx result, contig 5365 (2102 nt) best resembled the RdRp of Plasmopara viticola associated ourmia-like virus 64 (DMG-A_13793PavOLV64, GenBank: QGY72594) at 50.00% similarity (Table 2). We detected this virus in A. arborescens strain KEL-4-4 and called it Alternaria arborescens ourmia-like virus 1 (AaORV1). According to the BLASTx result, contig 2521 (2918 nt) best resembled the RdRp of Alternaria alternata magoulivirus 1 (AaMOV1, GenBank: A_13793PavOLV64, GenBank: QGY72594) at 50.00% similarity (Table 2). We detected this virus in A. tenuissima strain GZ-1 and called it Alternaria tenuissima ourmia-like virus 3 (AtOLV3). Therefore, we determined that this virus was a strain of DMG-B_Contig4PavOLV37. We detected this virus in A. tenuissima strain GZ-1 and called it Alternaria tenuissima ourmia-like virus 3 (AtOLV3). According to the BLASTx results, contig 6218 (946 nt) best resembled the RdRp of Plasmopara viticola associated ourmia-like virus 52 (DMG-E_27866PavOLV52, GenBank: QGY72582) at 64.17% similarity (Table 2). We detected this virus in A. arborescens strain GZ-1 and called it Alternaria arborescens ourmia-like virus 4 (AtOLV4). Therefore, AtOLV4 was a novel ourmia-like virus. According to the BLASTx result, contig 3454 (2568 nt) best resembled the RdRp of Plasmopara viticola associated ourmia-like virus 65 (DMG-A_388322PavOLV65, GenBank: QGY72595) at 94.85% similarity (Table 2). Therefore, the virus was a strain of DMG-A_388322PavOLV65. We detected this virus in A. tenuissima strain AH-25 (Figure 1) and called it Alternaria tenuissima ourmia-like virus 5 (AtOLV5). AtOLV5 was also detected in A. gossypina strain AH-20 (Figure 1). According to the BLASTx results, contig 19,628 (946 nt) and contig 49,207 (482 nt) best resembled the RdRp of Colletotrichum fructicola ourmia-like virus 2 (CIOLV2, GenBank: UOV22974) at 89.04% and 86.44% similarity, respectively. We detected this virus in A. tenuissima strains SC-12 and HB-15 (Figure 1). Therefore, we called it Alternaria tenuissima ourmia-like virus 6 (AtOLV6) and AtOLV6 might be a novel virus. Unfortunately, not constructing phylogenetic tree for lacking the GDD motif in the amino acid sequence of RdRp of AtOLV6 encoded by contig 19,628.

The family Magouliviridae had the RdRp of Plasmopara viticola associated ourmia-like virus 2 (CcOLV2, GenBank: QDB75008) at 96.32% similarity (Table 2). We detected this virus in A. tenuissima strains G-9 and G-21-2 (Figure 1) and called it Alternaria tenuissima ourmia-like virus 3 (AtOLV3). According to the BLASTx result, contig 3454 (2568 nt) best resembled the RdRp of Alternaria alternata magoulivirus 1 (AaMOV1, GenBank: A_13793PavOLV64, GenBank: QGY72594) at 50.00% similarity (Table 2). We detected this virus in A. tenuissima strain GZ-1 and called it Alternaria tenuissima ourmia-like virus 3 (AtOLV3). Therefore, we determined that this virus was a strain of DMG-B_Contig4PavOLV37. We detected this virus in A. tenuissima strain SC-12 (Figure 1) and called it Alternaria tenuissima ourmia-like virus 3 (AtOLV3). According to the BLASTx result, contig 5365 (2102 nt) best resembled the RdRp of Plasmopara viticola associated ourmia-like virus 64 (DMG-A_13793PavOLV64, GenBank: QGY72594) at 50.00% similarity (Table 2). We detected this virus in A. arborescens strain KEL-4-4 and called it Alternaria arborescens ourmia-like virus 1 (AaORV1). According to the BLASTx result, contig 2521 (2918 nt) best resembled the RdRp of Alternaria alternata magoulivirus 1 (AaMOV1, GenBank: A_13793PavOLV64, GenBank: QGY72594) at 50.00% similarity (Table 2). We detected this virus in A. tenuissima strain GZ-1 and called it Alternaria tenuissima ourmia-like virus 3 (AtOLV3). Therefore, we determined that this virus was a strain of DMG-B_Contig4PavOLV37. We detected this virus in A. tenuissima strain SC-12 (Figure 1) and called it Alternaria tenuissima ourmia-like virus 3 (AtOLV3). According to the BLASTx results, contig 6218 (946 nt) best resembled the RdRp of Plasmopara viticola associated ourmia-like virus 52 (DMG-E_27866PavOLV52, GenBank: QGY72582) at 64.17% similarity (Table 2). We detected this virus in A. arborescens strain GZ-1 and called it Alternaria arborescens ourmia-like virus 4 (AtOLV4). Therefore, AtOLV4 was a novel ourmia-like virus. According to the BLASTx result, contig 3454 (2568 nt) best resembled the RdRp of Plasmopara viticola associated ourmia-like virus 65 (DMG-A_388322PavOLV65, GenBank: QGY72595) at 94.85% similarity (Table 2). Therefore, the virus was a strain of DMG-A_388322PavOLV65. We detected this virus in A. tenuissima strain AH-25 (Figure 1) and called it Alternaria tenuissima ourmia-like virus 5 (AtOLV5). AtOLV5 was also detected in A. gossypina strain AH-20 (Figure 1). According to the BLASTx results, contig 19,628 (946 nt) and contig 49,207 (482 nt) best resembled the RdRp of Colletotrichum fructicola ourmia-like virus 2 (CIOLV2, GenBank: UOV22974) at 89.04% and 86.44% similarity, respectively. We detected this virus in A. tenuissima strains SC-12 and HB-15 (Figure 1). Therefore, we called it Alternaria tenuissima ourmia-like virus 6 (AtOLV6) and AtOLV6 might be a novel virus. Unfortunately, not constructing phylogenetic tree for lacking the GDD motif in the amino acid sequence of RdRp of AtOLV6 encoded by contig 19,628.
We analyzed the relationships among three novel viruses and other mycoviruses in the family Botourmiaviridae. Then, we constructed a phylogenetic tree based on the RdRp amino acid sequence of AarOLV1, AalOLV1, AtOLV4, and other related viral sequences, such as ourmiaviruses, sclerouliviruses, magouliviruses, botoliviruses, and mitoviruses. However, this was not used in phylogenetic analysis due to the lack of GDD motif in the amino acid sequence of RdRp of AtOLV6 encoded by contig 19,628. In the obtained phylogenetic tree, AalOLV1 was grouped with some botoliviruses (Figure 2). AtOLV4 and some betabotoliviruses were grouped in a branch (Figure 2). AarOLV1 was clustered with some deltascerouliviruses in a group (Figure 2). As a result, we identified AalOLV1 and AtOLV4 as the new members of the recent genus Botolivirus and Betabotolivirus, and AarOLV1 as a new member of the genus Deltasclerouliviruses within the family Botourmiaviridae.

**Figure 2.** Phylogenetic analysis of AarOLV1, AalOLV1, AtOLV4. The novel viruses obtained by high throughput sequencing were indicated by a red circle (●). The data coverage percentages were shown by the numbers on the left of branches. We constructed a phylogenetic tree using the maximum likelihood method. We based the 1000 bootstrap replications on the best-fit protein evolution (LG+G+I+F) model. We set the gamma value at 2.

The BLASTx results showed that contig 73 (8352 nt) was similar to RdRps of Agrostis stolonifera deltaflexivirus 1 (AsDFV1, 98.83% identity, GenBank: QQG34628), Alternaria alternata deltaflexivirus 1 (AaDFV1, 98.34% identity, GenBank: QTZ98076), Erysiphe necator associated deltaflexivirus 1 (EnDFV1, 95.61% identity, GenBank: QKN22722), and Triticum polonicum deltaflexivirus 1 (TpDFV1, 96.84% identity, GenBank: QQG34637). Contig 73 was identified from A. tenuissima strain SC-8 by RT-PCR amplification. We designated this virus to be Alternaria tenuissima deltaflexivirus 1 (AtDFV1).

3.3.1. Characterization of the Virus AtDFV1 Genome

We obtained the cDNA sequence of the AtDFV1 segment by combining the contig 49207 (946 nt) and contig 19628 (946 nt) best resembled the RdRp of Colletotrichum fructicola ourmia (CRfOLV2, GenBank: UOV22974) at 89.04% similarity. The amino acid sequence of RdRp of AtDFV1 was clustered with some botoliviruses such as ourmiaviruses, sclerouliviruses, magouliviruses, botoliviruses, and mitoviruses. Unfortunately, not constructing phylogenetic tree for lacking the GDD motif in the amino acid sequence of AtDFV1.

We obtained the cDNA sequence of the AtDFV1 segment by combining the contig 49207 (946 nt) and contig 19628 (946 nt) best resembled the RdRp of Colletotrichum fructicola ourmia (CRfOLV2, GenBank: UOV22974) at 89.04% similarity. The amino acid sequence of RdRp of AtDFV1 was clustered with some botoliviruses such as ourmiaviruses, sclerouliviruses, magouliviruses, botoliviruses, and mitoviruses. Unfortunately, not constructing phylogenetic tree for lacking the GDD motif in the amino acid sequence of AtDFV1.
excluding the ploy(A), which had a GC content of 51.4%. According to the BLASTn, this sequence best resembled segment Agrostis stolonifera deltaflexivirus 1, and had a 93.47% homology (GenBank: MW328744, E-value = 0.0, coverage 99%). We found that the AtDFV1 5′- and 3′-untranslated regions (UTR) were 19 nt and 146 nt long, respectively. The AtDFV1 contains four ORFs (I-IV). ORFs I and II were arranged in a line on the genome, and ORFs III and IV had 55 nt overlaps (Figure 3A).

![Figure 3. AtDFV1 genomic organization. (A) Organization and genome size with two conserved domains in the RdRp protein. Boxes indicate the position and size of each ORF and are labeled with Roman numerals, except for ORF I which encodes RdRp protein. (B–D) Amino acid sequences of the viral methyltransferase, viral RNA helicase, and RNA-dependent RNA polymerase, respectively, of AtDFV1 with members of the family of Deltaflexivirus. Black indicates the conserved sequences, shading indicates the conserved sequence level, and the darkest color indicates the most conserved sequence. For abbreviations of virus names and viral protein accession numbers used in alignment analysis, see Supplementary Table S4.]

We determined that ORF I (20–6241 nt) encoded a protein (P1) that had 2073 amino acid (aa) residues and a mass of 232 kDa. According to BLASTp, the P1 protein had 98.74% homology with the RdRp of Agrostis stolonifera deltaflexivirus 1 (AsDFV1) (GenBank: QQG34628, E-value = 0.0, coverage 99%). The P1 protein also had high homology with the RdRp of some deltaflexivirus (see Supplementary Table S5). In addition, four motifs domains were found from the P1 protein, including viral methyltransferase (Mtr, Pfam01660, position 191 to 511 aa, E-value = 1.1 × 10^{-31}), viral RNA helicase (Hel, Pfam01443, position 1212 to 1478 aa, E-value = 0.082), RNA-dependent RNA polymerase (RdRp, Pfam00978, position 1781 to 1934, E-value = 2.9 × 10^{-5}), and protein of unknown function (DUF3581, Pfam12119, position 807 to 838, E-value = 0.5). Therefore, ORF I encoded the viral RdRp with a methyltransferase and viral RNA helicase (Figure 3A). We observed that the homol-
ogous domains from selected other deltaflexivirus aligned with conserved domains (Mtr, Hel, and RdRp) of the putative P1 of AtDFV1. We used a pairwise comparison to identify the amino acid sequence identity (Figure 3B–D). ORF II (6622–6978 nt) encoded a protein (P2) with 118 aa residues and a mass of 13 kDa. According to BLASTp, the P2 protein was 98.31% similar to a hypothetical protein (P2) of AsDFV1 (GenBank: QQQ34629, E-value = 0.0, coverage 100%). We found a motif that was homologous with viral RNase III in mycoviruses (Mycovirus_RNase, Pfam20614, position 28 to 99, E-value = 7.0 × 10^{-10}). We also found that ORF III (7290–7823 nt) encoded a protein (P3) that had 177 aa residues and a mass of 18 kDa. According to BLASTp, the P3 protein was exactly the same as hypothetical protein 3 (HP3) of AsDFV1 (accession: QQQ34630, E-value = 1.0 × 10^{-112}, coverage 100%). We did not find any motifs in the P3 amino acid sequence. We observed that ORF IV (7780–8256 nt) encoded a protein (P4) with 158 aa residues and a mass of 16 kDa. According to BLASTp, the P4 protein was 97.47% similar to a hypothetical protein 4 (HP4) of AsDFV1 (accession: QQQ34631, E-value = 6.0 × 10^{-112}, coverage 100%). We did not find any motifs in the P4 amino acid sequence. We deposited the corresponding sequences in GenBank (accession: ON263576).

3.3.2. Phylogenetic Analysis of AtDFV1

We constructed a maximum-likelihood phylogenetic tree using the entire replicase of AtDFV1 and members of Alphaflexiviridae, Deltaflexiviridae, and Gammaflexiviridae. We verified the existence of motifs I-VI in AtDFV1 and members of the deltaflexivirus family due to the amino acid alignment with the predicted RdRp (Figure 3D). According to this phylogenetic analysis, AtDFV1, AaDFV1, EnDFV1, and TpDFV1 were related to viruses in the family Deltaflexiviridae (Figure 4).
3.4. Double Strand RNA Virus

In this study, five mycoviruses belonged to dsRNA viruses, of which four viruses could be classified into the families Chrysoviridae, Partitiviridae, and the genus Botybirnavirus, and one had no classification status.

According to the BLASTx results, four contigs were homologous at 96.90–100.00% with the corresponding proteins encoded by Alternaria alternata chrysovirus 1 (AaCV1), which was a betachrysovirus in the family Chrysoviridae (Table 2). The BLASTx search also revealed that contig 1410 (3617 bp) had a 99.37% identity with the RdRp of AaCV1 (GenBank: QJW39304). Contig 2756 (2814 bp) shared a 100.00% similarity with the CP of AaCV1. Therefore, these contigs might belong to a new strain of the AaCV1. As shown in Figure 1, we detected the virus using the special primers based on contig 2756 in A. tenuissima strain SC-8 and called it as Alternaria tenuissima chrysovirus 1 (AtCV1).

At present, the family Partitiviridae contains five genera, namely, Alphapartitivirus, Betapartitivirus, Cryspovirus, Deltapartitivirus, and Gammapartitivirus. Contig 11,000 (1393 bp) had 99.05% homology with a coat protein of Alternaria dianthicola partitivirus 1 (AdPV1, GenBank: UYZ32457) (Table 2). Therefore, the virus corresponding to this sequence was a new strain of AdPV1. The RT-PCR results showed that this virus was detected in A. tenuissima strains G-21-2 and G-24-2. Therefore, we called it Alternaria tenuissima partitivirus 2 (AtPV2). Contig 7155 (1808 bp) shared 89.38% similarity with a dsRNA1 segment of Alternaria tenuissima partitivirus 1 (AtPV1, GenBank: MT648466). According to the BLASTx result, contig 7155 best resembled the RdRp of AtPV1 with 94.59% similarity (Table 2). Therefore, the virus corresponding to this sequence might be a new strain of AtPV1. Because we detected this contig in the A. alternata strain KEL-9-7, we temporarily called it as Alternaria alternata partitivirus 1 (AaPV1).

The genus Botybirnavirus presently includes 21 members. BLASTn searches revealed that two contigs were notable for regions with very strong similarity to viral cap-pol fusion protein gene and hypothetical protein gene of Botryosphaeria dothidea botybirnavirus 1 (BdBRV1). BLASTx searches showed that the contig 206 (6162 bp) sequence shared high similarity with the cap-pol fusion protein gene of BdBRV1 (97.92% identity, GenBank: AXP19719), Bipolaris maydis botybirnavirus 1 (BmBRV1; 97.87% identity, GenBank: YP_009551519), and Sclerotinia sclerotiorum botybirnavirus 4 (SsBRV4; 98.32% identity, GenBank: QUE49104). The BLASTx searches showed that contig 253 (5808 bp) best resembled the hypothetical protein of BdBRV1 (98.81% identity, GenBank: AXP19720), BmBRV1 (98.42% identity, GenBank: YP_009551518), and SsBRV4 (98.47% identity, GenBank: QUE49105). Thus, these contigs might represent a virus that was a new strain of BmBRV1. According to the RT-PCR results, this virus was detected in A. tenuissima strains G-21-2 and GZ-2. Therefore, we called it Alternaria tenuissima botybirnavirus 1 (AtBRV1).

The BLASTn results revealed that the contig 10,828 (1417 bp) and contig 13,903 (1210 bp) were notable for regions that had a very strong similarity to the sequences of Alternaria longipes dsRNA virus 1 (AlRV1; GenBank: KJ817371, 91; 88% and 90.58% identity). According to BLASTx, contig 10,828 best resembled the hypothetical protein of AlRV1 with 97.96% homology and contig 13,903 best resembled the RdRp of AlRV1 with 97.84% homology (Table 2). Therefore, the virus was a strain of AlRV1. This virus was detected in A. arborescens strain KEL-4-4 and called it Alternaria arborescens dsRNA virus 1 (AaRV1).

Therefore, we determined that dsRNA viruses in the pooled RNA-Seq sample were not new mycoviruses.

3.5. Negative-Sense Single-Stranded RNA Viruses

Based on their RdRp amino acid sequences, we identified four negative-stranded RNA viral sequences in our samples. We assigned these viruses to two taxonomical groups of negative-strand viruses: two were assigned to the family Mymonaviridea, and two were assigned to the family Discoviridae (Table 2).

The BLASTx searches revealed that contig 52 (8970 nt) shared 56.36% homology with RdRp of Cryphonectria parasitica sclerotimonavirus 1 (CpSMV1, GenBank: QMP84020).
We used RT-PCR with specific primers to investigate the tested strains and found that strain HB-15 harbored contig 52 virus (AtNSRV2). The complete genome of AtNSRV2 was obtained using RT-PCR and RACE. According to BLASTx, contig 15,899 (1079 nt) best resembled the putative nucleocapsid of Plasmopara viticola lesion associated mymonavirus 1 (PvLMVV1; GenBank: QHD64781, 43.12% identity) and ORF3 of Botrytis cinerea mymonavirus 1 (BcMMVV1; GenBank: AXS76908, 29.13% identity). Thus, we determined this virus was a novel mymonavirus. As shown in Figure 1, we detected this virus in A. tenuissima strain G-21-1 and called it Alternaria tenuissima negative-stranded RNA virus 3 (AtNSRV3).

We found that contig 35 (10,267 nt) had one large ORF (<1–10,230 nt) that encoded a 3410 aa protein. According to the motif scan results, this protein contained a conservative Bunyavirus RNA-dependent RNA polymerase domain from 1886 aa to 2499 aa (pfam04196; E-value = 4.8 × 10^{-11}). Furthermore, we detected a type II toxin-antitoxin system motif from amino acids 2812 to 2902 (E-value = 0.23) and a L protein N-terminus motif from 2812 aa to 2902 aa (E-value = 0.076) in the hypothetical protein. According to BLASTp, the protein had low homology with the RdRp of Botrytis cinerea negative stranded RNA virus 10 (BcNSRV10) with only 30.71% similarity, and Macrophomina phaseolina negative-stranded RNA virus 1 (MpNSRV1) had only 32.24% similarity. As shown in Figure 1, we detected this virus in A. tenuissima strain HB-15 and called this novel virus Alternaria tenuissima negative-stranded RNA virus 4 (AtNSRV4).

### 3.5.1. Characterization of the Virus AtNSRV2 Genome

We determined the complete cDNA sequence of AtNSRV2 using sequence verification and terminal cloning. The full-length sequence of AtNSRV2 was 9067 nt. The GC content of the whole genome was 53.3%, the 5′ and 3′-untranslated regions (UTRs) were 300 nt and 85 nt long, respectively. We predicted that the full length of the AtNSRV2 had five major ORFs (ORFs I-V). These non-overlapping ORFs were arranged in a line along the viral genome (Figure 5A). We also identified the conserved noncoding sequences (3′-AUUU/AAAUAAAACUUAGGA-5′), which were downstream the ORFs (Figure 5B). Because the gene-junction sequences were ubiquitous in the viral genome, we determined that they were a characteristic feature of the mononegaviruses. We deposited the nucleotide sequence of AtNSRV2 in GenBank (accession number: OP566533).

We found that ORF I encoded a protein with 253 amino acid residues and had a mass of 29 kDa. According to BLASTp, the protein had 33.62% homology with a hypothetical protein of Botrytis cinerea negative-stranded RNA virus 4 (BcNSRV4) (GenBank: QJW39407). We also obtained three motifs from the hypothetical protein encoded ORF I, including Family of unknown function (DUF5798; position 118 to 179 aa; pfam19111, E-value = 0.0032), LRRC37A/B like protein 1 C-terminal domain (LRRC37AB_C; position 198 to 251 aa; pfam14914, E-value = 0.073), and LMBR1-like membrane protein (LMBR1; position 100 to 230 aa; pfam04791, E-value = 0.098). We found that ORF II encoded a protein of 402 amino acid residues and had a mass of 44 kDa. According to BLASTp, it was 47.46% similar to the N protein (nucleoprotein) of the SsNSRV-1 (GenBank: YP_009094314). We discovered that ORF III encoded a protein of 52 amino acid residues and had a mass of 6 kDa. We did not find any significant similarity protein sequences in the BLASTp search. We found that ORF IV encoded the largest protein of 1931 amino acids in length and had a molecular mass...
of 220 kDa. According to BLASTp, the L protein was 56.48% similar to the RdRp of CpSMV1 (GenBank: QMP84020). The P4 protein of AtNSRV2 also had high homology with the RdRp of other −ssRNA mycoviruses (see Supplementary Table S6). The conserved domain predicted that the protein contained a mononegavirales mRNA-capping region V domain (Mononeg_mRNAcap; position 1127 to 1272 aa; pfam14318, E-value = 1 × 10⁻⁶) and a mononegavirales RdRp domain (Mononeg_RNA_pol; position 21 to 1041 aa; pfam00946, E-value = 3.3 × 10⁻¹ⁱ). Based on the multiple alignment of the sequences of the RdRp amino acid of AtNSRV2 and other related viruses from Sclerotimonavirus, we identified four conserved motifs (I–IV) (Figure 5C). We found that ORF V encoded a protein of 186 amino acid residues and had a mass of 20 kDa. According to BLASTp, the protein best resembled ORF4 of Plasmopara viticola lesion associated mononega virus 2 (PvLAMV2), with a 41.67% similarity (GenBank: QHD64788). The conserved domain predicted that the protein contained a Mannosidase Ig/CBM-like domain (Mannosidase_ig; position 61 to 102 aa; pfam17786, E-value = 0.16).

**Figure 5.** AtNSRV2 genomic organization. (A) Organization and genome size, and a conserved domain in the L protein. Boxes on the genome indicate the position and size of each ORF, which are labeled with Roman numerals, except for two ORFs, N and L, which encode the nucleoprotein (N) and RNA dependent RNA polymerase (RdRp) protein (L). (B) Putative gene junction regions among the ORFs in AtNSRV2. Alignment of the putative gene junction sequences is shown in a 3′-to-5′ orientation. (C) Amino acid sequence alignment of core RdRp motifs of AtNSRV2 and selected viruses from the genus Sclerotimonavirus. Conserved sequences are highlighted in black. Black highlights indicate the conserved sequences, shading indicates the conserved sequence levels, and the darkest color indicates the most conserved sequence. For abbreviations of virus names and viral protein accession numbers used in alignment analysis, see Supplementary Table S4.
3.5.2. Phylogenetic Analysis of the Novel −ssRNA Viruses

We constructed a phylogenetic tree based on the RdRp aa sequences of AtNSRV2, AaNSRV1, and AtNSRV4 as well as members of the families *Mymonoviridae* and *Discoviridae*. We found that AtNSRV2 formed a supported clade with the following viruses: Soybean leaf-associated negative-stranded RNA virus 1 (SlaNSRV-1), Fusarium gramineartm negative-stranded RNA virus 1 (FgNSRV1), AtNRV1, BcNSRV3, SlaNSRV2, and CpSMV1 (Figure 6). They were close to the clade and included members of the genus *Sclerotimonavirus* but were separate from other genera in the family *Mymonoviridae* (Figure 6). Therefore, according to genomic characteristics and phylogenetic analysis, we proposed that AtNSRV2 should be a new member of the genus *Sclerotimonavirus* in the family *Mymonoviridae*. We also identified novel −ssRNA mycoviruses that should be grouped with members in the family *Discoviridae*. We included AaNSRV1 in a group with CdNSRV1, BcNSRV2, and Fusarium poae negative-stranded virus 2 (FpNSRV2), which were classed a genus *Orthodiscovirus* in the family *Discoviridae*. We also included AtNSRV4 in a clade with the members in the family *Discoviridae*, but we considered it to be part of different groups. Therefore, AaNSRV1 and AtNSRV4 are in two highly supported groups inside the family *Discoviridae* (Figure 6).

![Figure 6. Phylogenetic analyses of the RdRp sequences including the novel −ssRNA mycoviruses and other selected viruses. We used the maximum likelihood method to conduct 1000 bootstrap replications. The data coverage percentage was shown by the numbers on the left of branches. The position of viruses AtNSRV2, AtNSRV4, and AaNSRV1 were shown by the red circle (•). We constructed the phylogenetic tree based on the best-fit protein evolution (LG+G+F) model and set the gamma value was 2.](image-url)

The genomic −ssRNA of a novel mycovirus called AtNSRV2 was from strain HB-15. It was completely sequenced, and we found the virus to have a similar genome.
structure and high sequence similarity with members of the genus *Sclerotimonavirus* in the family *Mymonaviridae*.

4. Discussion

To search for novel mycoviruses, we used fungal strains from different regions to apply viral metagenomics to a mixed pool. This method allowed us to identify a great variety of new mycoviruses with different classes of genomes. In this study, we collected a total of 78 strains, including 58 *A. tenuissima*, 13 *A. alternata*, 3 *A. arborescens*, 2 *A. gaisen*, 1 *A. gossypina*, and 1 *A. longipes*, from pear spot disease from different regions of China, and mixed a fungal pool used to search for novel mycoviruses. As a result, we identified 21 putative viral sequences, most of which were nearly the full-length genome. We found 12 +ssRNA viruses, 5 dsRNA, and 4 −ssRNA viruses. We also identified sequences that were near full-length sequences in putative mycoviral genomes. Notably, the following eight distinct lineages were similar to these viral families: *Botourmiaviridae*, *Chrysoviridae*, *Deltaflexiviridae*, *Discoviridae*, *Hypoviridae*, *Partitiviridae*, *Mitoviridae*, *Mymonaviridae*, and *Narnaviridae*. In these putative viruses, nine were new mycoviruses. Through our analysis, we found that most of these viruses were dsRNA and ssRNA, and we did not find any DNA virus in the 78 strains. We used RT-PCR with specific primers, which had been designed based on the assembled contigs, and confirmed this variety of viruses. Two viruses from strains of *A. tenuissima* were provided a complete genome sequence using RT-PCR and RACE in our study.

Viruses are commonly present in *Alternaria* spp. Recently, 14 mycoviruses in 7 families have been associated with fungi from *Alternaria*. Of these fungi, we also identified AaMV1, AaHV1, AaCV1, and AIV1 in our study. These four viruses were found from the different isolates of *A. arborescens*, *A. alternata*, and *A. longipes* [24–26,34,39,40]. In our study, 10 viruses were discerned initially from other hosts with more than 90% similarity were detected in *Alternaria* isolates. A larger body of research has found that mycovirus occurs in two fungi hosts that are taxonomically. Some dsRNA and ssRNA mycoviruses were previously identified as infecting other fungal species or fungal genera. For example, *Bipolaris maydis* botybirnavirus 1 (BmBBV1) was found in *Bipolaris maydis* and *Botryosphaeria dolichoides* [60]. Ophiostoma novo-ulmi mitovirus 3a-Ld (OnuMV3a-Ld) was found in *Sclerotinia homoeocarpa* and *Ophiostoma novo-ulmi* [61]. Previously, *Helminthosporium victoriae* virus 190S was identified in *Helminthosporium victoriae* and *Bipolaris maydis* [62,63], and the mitovirus *Hymenoscyphus fraxineus* mitovirus 1 (HfMV1) was found in the *Hymenoscyphus fraxineus* and *H. albidus* [64,65]. Another study identified a mycovirus that infected different fungal strains through metatranscriptomics. Two viruses, Macrophomina phaseolina mitovirus 4 and Rhizoctonia solani mitovirus 10, were different strains of the same virus infected different hosts [59] and Botrytis fuckeliana totivirus 1 was found in *Botrytis cinerea* and *B. fuckeliana* [14]. Additionally, *Sclerotinia sclerotiorum* hypovirus 1-A, *Sclerotinia sclerotiorum* hypovirus 2, *Sclerotinia sclerotiorum* negativestranded RNA virus 5, *Sclerotinia sclerotiorum* partitivirus 2 were found in *B. cinerea* and *S. sclerotiorum* [14]. Both *S. sclerotiorum* and *B. cinerea* are necrotrophic fungi. They have wide hosts ranges, and their genomes show high sequence identity as well as a similar arrangement of genes [66]. Given that these mycoviruses can be found in coinfections in common plant hosts, these mycoviruses may transfer horizontally between coinfected fungi. An essential resource may be transmitted between different fungi hosts. For example, *Sclerotinia sclerotiorum* hypovirulence associated DNA virus 1 (SsHADV-1) could infect a mycophagous insect, *Lycoriella ingenua*, which could act as a transmission vector [67]. Cryphonectria hypovirus 1 (CHV1) could replicate and spread in *Nicotiana tabacum*, which is a model plant [68]. Plant-fungal-mediated routes may disseminate the same viruses between different fungi in nature.

We used RT-PCR and confirmed a variety of viruses in each strain. Our results identified 22 strains that were infected by various viruses. Interestingly, the *A. tenuissima* strain G-21-2 contained four viruses that belonged to four distinct lineages. Moreover,
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five strains showed coinfection by various viruses. Plant pathogenic fungal species often experience coinfection with multiple mycoviruses [69]. For example, an avirulent R. solani isolate DC-17 harbored 17 different mycovirus species that were assigned to eight or more families [70]. In addition, a single Fusarium mangiferae strain SP1 was coinfected by 11 mycoviruses that belonged to three families [71]. Sahin et al. detected eight new fungal viruses co-infesting a single isolate of the hypogeous ectomycorrhizal fungus Picoa juniperi using high-throughput sequencing [72]. Similar to isolate DC-17 and SP1, many plant pathogenic fungal species co-infected by different mycovirus, such as B. dothidea [73,74], B. cinerea [75], Magnaporthe oryzae [76], R. necatrix [77], Rhizoctonia solani [78], Sphaeropsis sapinea [79], S. sclerotiorum [80–82], and S. nivalis [83]. Therefore, a strain harbored different viruses might be for horizontal transmission of viruses.

Recently, deep sequencing has been used to identify the diversity of fungal viruses within fungal species from diverse geographic regions [52,55,70,71,82,84–90]. Unlike other dsRNA extraction and cloning methods, this approach could obtain viral information for different classified viruses regardless of their genome types [13,14,56,86]. Deep sequencing also has been used to identify mycoviruses from diverse fungal strains in an experimental project. For instance, using a high-throughput sequencing-based metatranscriptomic approach, 66 previously undescribed mycoviruses were obtained from five fungal species, including Colletotrichum truncatum, Diaporthe longicolla, Macrophomina phaseolina, R. solani, and S. sclerotiorum [13]. Multiple mycoviruses, which were coinfected in a fungal strain, were often efficiently identified by deep sequencing. For example, using deep sequencing, 17 different mycovirus that were assigned to different families were found in an R. solani isolate DC-17 [70]. Eleven mycoviruses were identified as being part of 3 families in a single F. mangiferae strain SP1[71]. Eight new fungal viruses that co-infected a single isolate of P. juniperi were found using high-throughput sequencing [72]. In addition, high-throughput sequencing has been used to detect mycoviruses on the phyllosphere and arbuscular mycorrhizal fungi in the roots [54,85]. For example, 22 putative mycovirus genomes have been organized into 10 taxonomic groups and assembled from soybean leaf metatranscriptomes [54]. The diversity, evolution, and annual variation of mycovirus in S. sclerotiorum within a single field for three years were investigated using the metatranscriptomic approach [86].

Alternaria spp. have been reported to have several RNA viruses, including dsRNA viruses, positive-sense ssRNA viruses, and other unidentified viruses [21–45]. The virus SsNSRV1 was the first member of the family Mymonaviridae [12]. Recently, two sclerotimonaviruses in this family have been found in A. tenuissima and A. dianthicola, respectively [44,45]. In this study, four negative-stranded RNA viruses have been identified. AtNSRV2 and AtNSRV3 were members of the genus Sclerotimonavirus in the family Mymonaviridae. The genome of the virus AtNSRV2 also was obtained. According to BLASTp, the L protein of AtNSRV2 was similar to the RdRp of SlaNRV2 with 55.85% identity. The virus SlaNRV2 was assembled from soybean leaf metatranscriptomes [54]. AaNSRV1 and AtNSRV4 were new members of the family Discoviridae. More research is needed to address these differences and to confirm their molecular and biological characterization. A lot of −ssRNA mycoviruses were classed into the order Discoviridae [3,4]. A recently reported BcNSRV1, RsNSRV4, and Macrophomina phaseolina negative-stranded RNA virus 1 (Mp-NSRV1) are new members of the order [8,13]. This is the first time that a mycovirus with a negative-stranded ssRNA genome has been reported to have infected an Alternaria strain.

5. Conclusions

In conclusion, diversity analysis of mycoviruses from 78 strains was executed by using high-throughput sequencing technology. The used stains were collected from 10 pear production areas and belonged to six species of the genus Alternaria. We excavated at least 21 different mycoviruses. The +ssRNA viruses belonged to the families Mitoviridae, Nar- naviridae, Deltaflexiviridae, Hypoviridae, and Botourmiaviridae. The dsRNA viruses belonged to the families Chrysoviridae, Partitiviridae, and a genus Botybironavirus, and one unclassified
The −ssRNA viruses belonged to the families Mymonaviridae of order Mononegavirales, and Discoviridae of order Bunyavirales. We also identified near-full-length sequences of mycoviral genomes that are putative. We isolated a novel −ssRNA mycovirus from an A. tenuissima strain HB-15, which we designated as AtNSRV2. We also characterized a novel +ssRNA mycovirus from an A. tenuissima strain SC-8, which we designated as AtDFV1. Through phylogenetic and sequence analyses, we found AtNSRV2 to be related to the viruses of the genus Sclerotimonavirus in the family Mymonaviridae. We also found that AtDFV1 is related to the family Deltaflexiviruses. The results of this study significantly enhanced the number of Alternaria viruses and identified their abundant diversity.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14112552/s1, Table S1: Origin of the strains of Alternaria species used in this study; Table S2: RT-PCR primers used for detection viruses in this study; Table S3: RT-PCR primers used for obtaining full-length sequences of the viruses in this study; Table S4: Abbreviations of virus names and viral protein accession numbers used in alignment analysis in this study; Table S5: Best BLASTp matches of P1 of Alternaria tentissima deltaflexivirs 1; Table S6: Best BLASTp matches of P4 of Alternaria tentissima negative-stranded RNA virus 1.

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References
1. Ghabrial, S.A.; Caston, J.R.; Jiang, D.; Nibert, M.L.; Suzuki, N. 50-plus years of fungal viruses. Virology 2015, 479, 356–368. [CrossRef] [PubMed]
2. Ghabrial, S.A.; Suzuki, N. Viruses of plant pathogenic fungi. Annu. Rev. Phytopathol. 2009, 47, 353–384. [CrossRef] [PubMed]
3. Kondo, H.; Botella, L.; Suzuki, N. Mycovirus diversity and evolution revealed/inferred from recent studies. Annu. Rev. Phytopathol. 2022, 60, 307–336. [CrossRef]
4. Kotta-Loizou, I. Mycoviruses and their role in fungal pathogenesis. Curr. Opin. Microbiol. 2021, 63, 10–18. [CrossRef] [PubMed]
5. Pearson, M.N.; Beever, R.E.; Boine, B.; Arthur, K. Mycoviruses of filamentous fungi and their relevance to plant pathology. Mol. Plant Pathol. 2009, 10, 115–128. [CrossRef]
6. Myers, J.M.; James, T.Y. Mycoviruses. Curr. Biol. 2022, 32, R150–R155. [CrossRef]
7. Xie, J.T.; Jiang, D.H. New insights into mycoviruses and exploration for the biological control of crop fungal diseases. Annu. Rev. Phytopathol. 2014, 52, 45–68. [CrossRef]
8. Donaire, L.; Pagán, I.; Ayllón, M.A. Characterization of Botrytis cinerea negative-stranded RNA virus 1, a new mycovirus related to plant viruses, and a reconstruction of host pattern evolution in negative-sense ssRNA viruses. Virology 2016, 499, 212–218. [CrossRef]
9. Hao, F.; Wu, M.; Li, G. Characterization of a novel genomovirus in the phytopathogenic fungus Botrytis cinerea. Virology 2021, 553, 111–116. [CrossRef]
10. Khalifa, M.E.; MacDiarmid, R.M. A mechanically transmitted DNA mycovirus is targeted by the defence machinery of its host, Botrytis cinerea. Viruses 2021, 13, 1315. [CrossRef]
Viruses 2022, 14, 2552

11. Li, P.; Wang, S.; Zhang, L.; Qiu, D.; Zhou, X.; Guo, L. A tripartite ssDNA mycovirus from a plant pathogenic fungus is infectious as cloned DNA and purified virions. *Sci. Adv.* 2020, 6, eaay9634. [CrossRef]

12. Liu, L.J.; Xie, J.T.; Cheng, J.S.; Fu, Y.P.; Li, G.Q.; Yi, X.H.; Jiang, D.H. Fungal negative-stranded RNA virus that is related to bornaviruses and nyaviruses. *Proc. Natl. Acad. Sci. USA* 2014, 111, 12205–12210. [CrossRef] [PubMed]

13. Marzano, S.Y.L.; Nelson, B.D.; Ajayi-Oyetunde, O.; Bradley, C.A.; Hughes, T.J.; Hartman, G.L.; Eastburn, D.M.; Domier, L.L. Identification of diverse mycoviruses through metatranscriptomics characterization of the viromes of five major fungal plant pathogens. *J. Virol.* 2016, 90, 6846–6863. [CrossRef] [PubMed]

14. Ruiz-Padilla, A.; Rodriguez-Romero, J.; Gómez-Cid, I.; Pacifico, D.; Ayllón, M.A. Novel mycoviruses discovered in the mycovirome of a necrotrophic fungus. *mBio* 2021, 12, e03705-20. [CrossRef] [PubMed]

15. Wang, L.; He, H.; Wang, S.C.; Chen, X.G.; Qiu, D.W.; Kondo, H.; Guo, L.H. Evidence for a novel negative-stranded RNA mycovirus isolated from the plant pathogenic fungus *Fusarium graminearum*. *Virology* 2018, 518, 232. [CrossRef]

16. Yu, X.; Li, B.; Fu, Y.P.; Jiang, D.H.; Ghabrial, S.A.; Li, G.Q.; Peng, Y.L.; Xie, J.T.; Cheng, J.S.; Huang, J.B.; et al. A geminivirus-related DNA mycovirus that confers hypovirulence to a plant pathogenic fungus. *Proc. Natl. Acad. Sci. USA* 2010, 107, 8387–8392. [CrossRef] [PubMed]

17. Bajwa, R.; Mukhtar, I.; Mushtaq, S. New report of *Alternaria alternata* causing leaf spot of *Aloe vera* in Pakistan. *Can. J. Plant Pathol.* 2010, 32, 490–492. [CrossRef]

18. Lamondia, J.A. Outbreak of brown spot of tobacco caused by *Alternaria alternata* in Connecticut and Massachusetts. *Plant Dis.* 2001, 85, 230. [CrossRef]

19. Peever, T.L.; Su, G.; Carpenter-Boggs, L.; Timmer, L.W. Molecular systematics of citrus-associated *Alternaria* species. *Mycolgia* 2004, 96, 119–134. [CrossRef]

20. Wang, W.Q.; Li, Y.; Xiang, J.; Hong, N.; Wang, G.P. Identification and pathogenicity of *Alternaria* species causing black spot in pear producing regions in China. *J. Fruit Sci.* 2020, 37, 1922–1933.

21. Shepherd, H.S. Virus like particles in tentoxin-producing strains of *Alternaria alternata*. *J. Virol.* 1988, 62, 3888–3891. [CrossRef]

22. Zabalgogeazcoa, I.; Petrunak, D.; Christ, B.J.; Gildow, F.E. Unencapsidated double-stranded RNA associated with membrane vesicles in isolates of *Alternaria solani*. *Mycol. Res.* 1997, 101, 604–608. [CrossRef]

23. Hu, C.; Li, S.; Wu, C.; Mi, Y.; Cai, Q.; Zhou, T.; Zhao, C.; Wu, X. Complete genome sequence of the first chrysovirus from the phytopathogenic fungus *Alternaria solani* on potato in China. *Arch. Virol.* 2021, 166, 3493–3497. [CrossRef] [PubMed]

24. Li, B.; Cao, Y.; Ji, Z.; Zhang, J.; Meng, X.; Dai, P.; Hu, T.; Wang, S.; Cao, K.; Wang, Y. Coinfection of two mycoviruses confers hypovirulence and reduces the production of mycotoxin alternariol in *Alternaria alternata* f. sp. mali. *Front. Microbiol.* 2022, 13, 910712. [CrossRef] [PubMed]

25. Ma, G.; Zhang, X.; Hua, H.; Zhou, T.; Wu, X. Molecular and biological characterization of a novel strain of the phytopathogenic fungus *Alternaria tenuissima* causing leaf blight of watermelon. *Virus Res.* 2020, 280, 197904. [CrossRef] [PubMed]

26. Okada, R.; Ichinose, S.; Takeshita, K.; Urayama, S.I.; Fukuhara, T.; Komatsu, K.; Arie, T.; Ishihara, A.; Egusa, M.; Kodama, M.; et al. Molecular characterization of a novel mycovirus in *Alternaria alternata* manifesting two-sided effects: Downregulation of host growth and upregulation of host pathogenicity. *Virology* 2018, 519, 23–32. [CrossRef]

27. Xavier, A.D.S.; Barros, A.P.O.; Godinho, M.T.; Zerbini, F.M.; Souza, F.O.; Bruckner, F.P.; Alfenas-Zerbini, P. A novel mycovirus associated to *Alternaria alternata* comprises a distinct lineage in *Partitiviridae*. *Virus Res.* 2018, 244, 21–26. [CrossRef]

28. Wang, X.; Ma, Z.; Li, R.; Zhou, T.; Zhao, C.; Wu, X. Complete genome sequence of a novel partitivirus infecting the phytopathogenic fungus *Alternaria tenuissima*. *Arch. Virol.* 2022, 167, 635–639. [CrossRef] [PubMed]

29. Komatsu, K.; Katayama, Y.; Omatsu, T.; Mizutani, T.; Fukuhara, T.; Kodama, M.; Arie, T.; Teraoka, T.; Moriyama, H. Genome sequence of a novel victorivirus identified in the phytopathogenic fungus *Alternaria arborescens*. *Arch. Virol.* 2016, 161, 1701–1704. [CrossRef]

30. Jamal, A.; Sato, Y.; Shahi, S.; Shamsi, W.; Kondo, H.; Suzuki, N. Novel victorivirus from a Pakistani isolate of *Alternaria alternata* lacking a typical translational stop/start sequence signature. *Viruses* 2019, 11, 577. [CrossRef]

31. Shamsi, W.; Sato, Y.; Jamal, A.; Shahi, S.; Kondo, H.; Suzuki, N.; Bhatti, M.F. Molecular and biological characterization of a novel botrytiviruses identified from a Pakistani isolate of *Alternaria alternata*. *Virus Res.* 2019, 263, 119–128. [CrossRef] [PubMed]

32. Ma, G.; Liang, Z.; Hua, H.; Zhou, T.; Wu, X. Complete genome sequence of a new botrytiviruses isolated from a phytopathogenic *Alternaria alternata* in China. *Arch. Virol.* 2019, 164, 1225–1228. [CrossRef] [PubMed]

33. Xiang, J.; Fu, M.; Hong, N.; Zhai, L.; Xiao, F.; Wang, G. Characterization of a novel botrytiviruses isolated from a phytopathogenic *Alternaria alternata*. *Arch. Virol.* 2017, 162, 3907–3911. [CrossRef] [PubMed]

34. Komatsu, K.; Katayama, Y.; Omatsu, T.; Mizutani, T.; Fukuhara, T.; Kodama, M.; Arie, T.; Teraoka, T.; Moriyama, H. Genome sequence of a novel mitovirus identified in the phytopathogenic fungus *Alternaria arborescens*. *Arch. Virol.* 2016, 161, 2627–2631. [CrossRef]

35. Chen, Y.; Shang, H.H.; Yang, H.Q.; Gao, B.D.; Zhong, J. A mitovirus isolated from the phytopathogenic fungus *Alternaria brassicicola*. *Arch. Virol.* 2017, 162, 2869–2874. [CrossRef]

36. Shang, H.H.; Zhong, J.; Zhang, R.J.; Chen, C.Y.; Gao, B.D.; Zhu, H.J. Genome sequence of a novel endornavirus from the phytopathogenic fungus *Alternaria brassicicola*. *Arch. Virol.* 2015, 160, 1827–1830. [CrossRef]
37. Aoki, N.; Moriyama, H.; Kodama, M.; Arie, T.; Teraoka, T.; Fukuhara, T. A novel mycovirus associated with four double-stranded RNAs affects host fungal growth in Alternaria alternata. *Virus Res.* 2009, 140, 179–187. [CrossRef]

38. Wu, C.F.; Aoki, N.; Takeshita, N.; Fukuhara, T.; Chiura, H.X.; Arie, T.; Kotta-Loizou, I.; Okada, R.; Komatsu, K.; Moriyama, H. Unique terminal regions and specific deletions of the segmented double-stranded RNA genome of Alternaria alternata virus 1, in the proposed family Alternaviridae. *Front. Microbiol.* 2021, 12, 77962. [CrossRef]

39. Hu, Z.; Guo, J.; Gao, B.D.; Zhong, J. A novel mycovirus isolated from the plant-pathogenic fungus *Alternaria dianthioca*. *Arch. Virol.* 2020, 165, 2015–2109. [CrossRef]

40. Lin, Y.; Zhang, H.; Zhao, C.; Liu, S.; Guo, L. The complete genome sequence of a novel mycovirus from *Alternaria longipes* strain HN28. *Arch. Virol.* 2013, 160, 577–580. [CrossRef]

41. Li, H.; Bian, R.; Liu, Q.; Yang, L.; Pang, T.; Salaipeth, L.; Andika, I.B.; Kondo, H.; Sun, L. Identification of a novel hypovirulence-inducing hypovirus from *Alternaria alternata*. *Front. Microbiol.* 2019, 10, 1076. [CrossRef] [PubMed]

42. Zhong, J.; Shang, H.H.; Zhu, C.X.; Zhu, J.Z.; Zhu, H.J.; Hu, Y.; Gao, B.D. Characterization of a novel single-stranded RNA virus, closely related to fusariviruses, infecting the plant pathogenic fungus *Alternaria brassicicola*. *Virus Res.* 2016, 217, 1–7. [CrossRef]

43. Gong, W.; Liu, H.; Zhu, X.; Zhao, S.; Cheng, J.; Zhu, H.; Zhong, J.; Zhou, Q. Molecular characterization of a novel fusarivirus infecting the plant-pathogenic fungus *Alternaria solani*. *Arch. Virol.* 2021, 166, 2063–2067. [CrossRef]

44. Zhong, J.; Li, P.; Gao, B.D.; Zhong, S.Y.; Li, X.G.; Gambino, G.; Chitarra, W. Isolation, molecular characterization and virome analysis of culturable wood fungal endophytes in esca symptomatic and asymptomatic grapevine plants. *Environ. Microbiol*. 2019, 21, 2886–2904. [CrossRef] [PubMed]

45. Anagnostakis, S.L. Biological control of chestnut blight. *Science* 1991, 255, 666–671. [PubMed] [CrossRef]

46. Bocos-Asenjo, I.T.; Niño-Sánchez, J.; Ginesy, M.; Diez, J.F. New insights on the integrated management of plant diseases by RNA strategies: Mycoviruses and RNA interference. *Int. J. Mol. Sci.* 2022, 23, 9236. [CrossRef]

47. MacDonald, W.L.; Fulbright, D.W. Biological control of chestnut blight: Use and limitations of transmissible hypoviroplasm. *Plant Dis.* 1991, 75, 656–661. [CrossRef]

48. Tian, B.; Xie, J.; Fu, Y.; Cheng, J.; Li, B.; Chen, T.; Zhao, Y.; Gao, Z.; Yang, P.; Barbetti, M.J.; et al. A cosmopolitan fungal pathogen of dicots adopts an endophytic lifestyle on cereal crops and protects them from major fungal diseases. *ISME J.* 2020, 12, 3120–3135. [CrossRef]

49. Yu, X.; Li, B.; Fu, Y.; Xie, J.; Cheng, J.; Ghabrial, S.A.; Li, G.; Yi, X.; Jiang, D. Extracellular transmission of a DNA mycovirus and its use as a natural fungicide. *Proc. Natl. Acad. Sci. USA* 2013, 110, 1452–1457. [CrossRef]

50. Konno, H.; Chiba, S.; Toyoda, K.; Suzuki, N. Evidence for negative-strand RNA virus infection in fungi. *Virology* 2013, 433, 201–209. [CrossRef] [PubMed]

51. Konno, H.; Kanematsu, S.; Suzuki, N. Viruses of the white root rot fungus, *Rosellinia necatrix*. *Adv. Virus Res.* 2013, 86, 177–214. [PubMed]

52. Linnakoski, R.; Sutela, S.; Coetzee, M.P.; Duong, T.A.; Pavlov, I.N.; Litovka, Y.A.; Hantula, J.; Wingfield, B.D.; Vainio, E.J. Armillaria root rot fungi host single-stranded RNA viruses. *Sci. Rep.* 2021, 11, 7336. [CrossRef] [PubMed]

53. Marzano, S.L.; Domier, L.L. Novel mycoviruses discovered from metatranscriptomics survey of soybean phyllosphere phyto-biomes. *Viruses* 2016, 21, 332–342. [CrossRef]

54. Mizutani, Y.; Uesaka, K.; Ota, A.; Calassanzio, M.; Ratti, C.; Suzuki, T.; Fujimori, F.; Chiba, S. De novo sequencing of novel mycoviruses from *Fusarium sambucinum*: An attempt on direct RNA sequencing of viral dsRNAs. *Front. Microbiol.* 2021, 12, 641484. [CrossRef] [PubMed]

55. Mu, F.; Xie, J.; Cheng, S.; You, M.; Barbetti, M.; Jia, J.; Wang, Q.; Cheng, J.; Fu, Y.; Chen, T.; et al. Virome characterization of a collection of *Sclerotinia sclerotiorum* from Australia. *Front. Microbiol.* 2018, 8, 2540. [CrossRef]

56. Myers, J.M.; Bonds, A.E.; Clemens, R.A.; Thapa, N.A.; Simmons, D.R.; Carter-House, D.; Ortizan, J.; Liu, P.; Miralles-Durán, A.; Desiró, A.; et al. Survey of early-diverging lineages of fungi reveals abundant and diverse mycoviruses. *mBio* 2020, 11, e02027-20. [CrossRef]

57. Pandey, B.; Naidu, R.A.; Grove, G.G. Detection and analysis of mycovirus-related RNA viruses from grape powdery mildew fungus *Erysiphe necator*. *Arch. Virol.* 2018, 163, 1019–1030. [CrossRef]

58. Wang, J.; Ni, Y.; Liu, X.; Zhao, Y.; Xiao, Y.; Xiao, X.; Li, S.; Liu, H. Divergent RNA viruses in *Macrophomina phaseolina* exhibit potential as virocontrol agents. *Plant Bio.* 2020, 7, vea095. [CrossRef]

59. Zhai, L.; Yang, M.; Zhang, M.; Hong, N.; Wang, G. Characterization of a botyribnavirus conferring hypovirulence in the phytopathogenic fungus *Botryosphaeria dothidea*. *Viruses* 2019, 11, 266. [CrossRef]

60. Feng, F.; Xu, R.; Boland, G. Hypovirulence-associated double-stranded RNA from *Sclerotinia homoeocarpa* is conspecific with *Ophiostoma novo-ulmi* mitovirus 3a-Ld. *Phytopathology* 2003, 93, 1407–1414. [CrossRef] [PubMed]

61. Wu, R.; Yang, Y.; Duan, X.; An, H.; Du, Z.; Zhang, S.; Zhang, X. Four distinct isolates of *Helminthosporium victoriae* virus 190S identified from *Bipolaris maydis*. *Virus Res.* 2020, 285, 197941. [CrossRef] [PubMed]

62. Huang, S.; Ghabrial, S.A. Organization and expression of the double-stranded RNA genome of *Helminthosporium victoriae* virus 190S virus, a totiviruses infecting a plant pathogenic filamentous fungus. *Proc. Natl. Acad. Sci. USA* 1996, 93, 12541–12546. [CrossRef] [PubMed]
64. Schoebel, C.N.; Prospero, S.; Gross, A.; Rigling, D. Detection of a conspecific mycovirus in two closely related native and introduced fungal hosts and evidence for interspecific virus transmission. *Viruses* **2018**, *10*, 628. [CrossRef]

65. Schoebel, C.N.; Zoller, S.; Rigling, D. Detection and genetic characterisation of a novel mycovirus in *Hymenoscyphus fraxineus*, the causal agent of ash dieback. * Infect. Genet. Evol.* **2014**, *28*, 78–86. [CrossRef]

66. Amselem, J.; Cuomo, C.A.; van Kan, J.A.; Viaud, M.; Benito, E.P.; Couloux, A.; Coutinho, P.M.; de Vries, R.P.; Dyer, P.S.; Fillinger, S.; et al. Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *PLoS Genet.* **2011**, *7*, e1002230. [CrossRef]

67. Liu, S.; Xie, J.; Cheng, J.; Li, B.; Chen, T.; Fu, Y.; Li, G.; Wang, M.; Jin, H.; Wan, H.; et al. Fungal DNA virus infects a mycophasgous insect and utilizes it as a transmission vector. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 12803–12808. [CrossRef]

68. Bian, R.; Andika, I.B.; Pang, T.; Lian, Z.; Wei, S.; Niu, E.; Wu, Y.; Kondo, H.; Liu, X.; Sun, L. Facilitative and synergistic interactions between fungal and plant viruses. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 3779–3788. [CrossRef]

69. Bartholomäus, A.; Wibberg, D.; Winkler, A.; Pühler, A.; Schlüter, A.; Varrelmann, M. Deep sequencing analysis reveals the mycoviral diversity of the virome of an avirulent isolate of *Rhizoctonia solani* AG-2-2 IV. *PLoS ONE*. **2016**, *11*, e0165965. [CrossRef]

70. Thapa, V.; Roossinck, M.J. Determinants of coinfection in the mycoviruses. *Front. Cell. Infect. Microbiol.* **2019**, *9*, 169. [CrossRef]

71. Khan, H.A.; Shamsi, W.; Jamal, A.; Javaied, M.; Sadiq, M.; Fatma, T.; Ahmed, M.; Waseem, M.; Babar, S.; et al. Assessment of mycoviral diversity in Pakistani fungal isolates revealed infection by 11 novel viruses of a single strain of *Fusarium mangiferae* isolate SPI. *J. Gen. Virol.* **2021**, *102*, 001690. [CrossRef]

72. Sahin, E.; Keskin, E.; Akata, I. Novel and diverse mycoviruses co-inhabiting the hypogeous ectomycorrhizal fungus *Picea juniperi*. *Virology* **2021**, *552*, 10–19. [CrossRef] [PubMed]

73. Wang, L.; Jiang, J.; Wang, Y.; Hong, N.; Zhang, F.; Xu, W.; Wang, G. Hypovirulence of the phytopathogenic fungus *Botryosphaeria dothidea*: Association with a co-existing chrysovirus and a partitivirus. *J. Virol.* **2014**, *88*, 7517–7527. [CrossRef] [PubMed]

74. Li, J.; Zhai, L.; Zhang, M.; Luo, G.; Wen, Y.; Cao, T.; Xie, H.; Zhang, J.; Liu, M. Molecular characterization of a novel virus isolate from *Botryosphaeria dothidea*, the causal agent of longan leaf spot disease. *Arch. Virol.* **2022**, *167*, 2417–2422. [CrossRef] [PubMed]

75. Hao, F.; Ding, T.; Wu, M.; Zhang, J.; Yang, L.; Chen, W.; Li, G. Two novel hypovirulence-associated mycoviruses in the phytopathogenic fungus *Botrytis cinerea*: Molecular characterization and suppression of infection cushion formation. *Viruses* **2018**, *10*, 254. [CrossRef]

76. Liu, Y.; Zhang, L.; Esmael, A.; Duan, J.; Bian, X.; Jia, J.; Xie, J.; Cheng, J.; Fu, Y.; Jiang, D.; et al. Four novel botourmiaviruses co-infecting an isolate of the rice blast fungus *Magnaporthe oryzae*. *Viruses* **2020**, *12*, 1383. [CrossRef]

77. Chun, J.; Kim, D.H. Co-infection of a novel fusagavirus and a partitivirus in a Korean isolate of *Rosellinia necatrix* KACC40168. *Viruses Gen.* **2021**, *57*, 121–126. [CrossRef]

78. Li, Y.; Li, S.; Zhao, Y.; Zhou, T.; Wu, X.; Zhao, C. Six novel mycoviruses containing positive single-stranded RNA and double-stranded RNA genomes co-Infect a single strain of the *Rhizoctonia solani* AG-3 PT. *Viruses* **2022**, *14*, 813. [CrossRef]

79. Preisig, O.; Wingfield, B.D.; Wingfield, M.J. Coinfection of a fungal pathogen by two distinct double-stranded RNA viruses. *Virology* **1998**, *252*, 399–406. [CrossRef]

80. Hai, D.; Li, J.; Lan, S.; Wu, T.; Li, Y.; Cheng, J.; Fu, Y.; Lin, Y.; Jiang, D.; Wang, M.; et al. Discovery and evolution of six positive-sense RNA viruses co-infecting hypovirulent strain SCH733 of *Sclerotinia sclerotiorum*. *Phytopathology* **2022**, *113*, 001690. [CrossRef] [PubMed]

81. Khalifa, M.E.; Pearson, M.N. Molecular characterization of three mitoviruses co-infecting a hypoviral isolate of *Sclerotinia sclerotiorum* fungus. *Virology* **2013**, *441*, 22–30. [CrossRef] [PubMed]

82. Mu, F.; Li, B.; Cheng, S.; Jia, J.; Jiang, D.; Fu, Y.; Cheng, J.; Lin, Y.; Chen, T.; Xie, J. Nine viruses from eight lineages exhibiting new evolutionary modes that co-infect a hypovirulent phytopathogenic fungus. *PLoS Pathog.* **2021**, *17*, e1009823. [CrossRef] [PubMed]

83. Wu, M.; Deng, Y.; Zhou, Z.; He, G.; Chen, W.; Li, G. Characterization of three mycoviruses co-infecting the plant pathogenic fungus *Sclerotinia nivialis*. *Virus Res.* **2016**, *223*, 28–38. [CrossRef] [PubMed]

84. Chen, F.; Pu, Z.; Ni, H.; Wang, Y.; Yan, B. Multiple mycoviruses identified in *Pestalotiopsis* spp. from Chinese bayberry. *Virology.* **2021**, *18*, 43. [CrossRef]

85. Ezawa, T.; Ikeda, Y.; Shimura, H.; Masuta, C. Detection and characterization of mycoviruses in arbuscular mycorrhizal fungi by deep-sequencing. *Methods Mol. Biol.* **2015**, *1236*, 171–180. [CrossRef]

86. Jia, J.; Fu, Y.; Jiang, D.; Mu, F.; Cheng, J.; Lin, Y.; Li, B.; Marzano, S.L.; Xie, J. Interspecific dynamics, diversity and evolution of the virome in *Sclerotinia sclerotiorum* from a single crop field. *Virus Evol.* **2021**, *7*, vea032. [CrossRef]

87. Kartali, T.; Nyilasi, I.; Kocsübő, S.; Patai, R.; Polgár, T.F.; Szindely, N.; Nagy, G.; Bodai, L.; Lipinszki, Z.; Vágvölgyi, C.; et al. Characterization of four novel dsRNA viruses isolated from *Mucor hiemalis* strains. *Viruses* **2021**, *13*, 2319. [CrossRef]

88. Li, Y.; Zhou, M.; Yang, Y.; Liu, Q.; Zhang, Z.; Han, C.; Wang, Y. Characterization of the mycoviruse from the plant-pathogenic fungus *Cercospora beticola*. *Viruses* **2021**, *13*, 1915. [CrossRef]

89. Marais, A.; Faure, C.; Comont, G.; Candresse, T.; Stempien, E.; Corio-Costet, M.F. Characterization of the mycoviruse of the phytopathogenic fungus, *Neofusosicoccum parvum*. *Viruses* **2021**, *13*, 375. [CrossRef]

90. Sutela, S.; Piri, T.; Vainio, E.J. Discovery and community dynamics of novel ssRNA mycoviruses in the conifer pathogen *Heterobasidion parviporum*. *Front. Microbiol.* **2021**, *12*, 770787. [CrossRef]