Novel and diverse mycoviruses co-infecting a single strain of the phytopathogenic fungus

*Alternaria dianthicola*

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*Alternaria dianthicola* is a pathogenic fungus that causes serious leaf or flower blight on some medicinal plants worldwide. In this study, multiple dsRNA bands in the range of 1.2-10 kbp were found in a *Alternaria dianthus* strain HNSZ-1, and eleven full-length cDNA sequences of these dsRNA were obtained by high-throughput sequencing, RT-PCR detection and conventional Sanger sequencing. Homology search and phylogenetic analyses indicated that the strain HNSZ-1 was infected by at least nine mycoviruses. Among the nine, five viruses were confirmed to represent novel viruses in the families *Hypoviridae*, *Totiviridae*, *Mymonaviridae* and a provisional family *Ambiguiviridae*. Virus elimination and horizontal transmission indicated that the (-) ssRNA virus, AdNSRV1, might be associated with the slow growth and irregular colony phenotype of the host fungus. As far as we know, this is the first report for virome characterization of *A. dianthus*, which might provide important insights for screening of mycovirus for biological control and for studying of the interactions between viruses or viruses and their host.

KEYWORDS  
mycoviruses, *Alternaria dianthicola*, virome, virus diversity, biocontrol

Highlights

1. This research reported the characterization of the virome of an *Alternaria dianthicola* strain HNSZ-1.
2. Among the nine co-infecting viruses, five were novel viruses belonging to families *Hypoviridae*, *Totiviridae*, *Mymonaviridae* and a provisional family *Ambiguiviridae*.  

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3. Virus elimination, horizontal transmission and biological comparison revealed that the (+) ssRNA virus, AdNSRV1, might be related with the phenotypic change of the host fungus.

### Introduction

Mycoviruses (fungal viruses) are commonly infect all major groups of fungi, including plant-pathogenic fungi (Ghabrial and Suzuki, 2009; Pearson et al., 2009). In general, mycovirus infections are cryptic, while some other viral fungi were associated with hypovirulence which can alter several physiological and biochemical properties of their fungal hosts (Nuss, 2005; Pearson et al., 2009; Yu et al., 2010; Yang et al., 2018). Hypovirulence-associated mycoviruses have the potential to be used as a biocontrol material to control fungal diseases. The successful use of Cryphonectria hypovirus 1 (CHV1) to control chestnut blight in Europe has pioneered the use of mycoviruses for biocontrol (Rigling and Prospero, 2018). Recently, the DNA mycovirus Sclerotinia sclerotiorum hypovirulence associated DNA virus 1 (SsHADV-1) has been proven to be a usefully biocontrol agent since it could be transmitted extracellularly and convert the pathogenic host fungus into a beneficial, non-pathogenic endophyte (Rigling and Prospero, 2018; Zhang et al., 2020). Therefore, hunting of mycoviruses conferring hypovirulence has become a pathway for development of potential biological control strategies for fungal diseases (Xie and Jiang, 2014; Ghabrial et al., 2015; Garcia-Pedrajas et al., 2019).

Recently, with the development of high throughput sequencing, more and more studies revealed mixed infection of different mycoviruses or dsRNA elements in a single strain, such as the fungal strains Rosellinia necatrix, Botrytis cinerea, Rhizoctonia solani, Sclerotinia sclerotiorum, Sclerotium rolfsii (Xie and Ghabrial, 2012; Kondo et al., 2013; Bartholomáus et al., 2016; Hao et al., 2018; Zhu et al., 2018). For example, 17 different mycoviruses have been reported to co-infect a Rhizoctonia solani strain DC17 (Bartholomáus et al., 2016), and 21 mycoviruses have been found in a hypovirulent Sclerotium rolfsii strain BLH-1 (Zhu et al., 2018). Mu et al. (2021) have also reported 9 mycoviruses that were assigned into eight potential families and exhibited new evolutionary modes in a single S. sclerotiunum strain SX276. Different mycoviruses co-infected in a single fungal strain might provide some possibilities for virus recombination or horizontal gene transfer, thus promoting the evolution of viruses (Visaykrishna et al., 2015). In addition, co-infections by different mycoviruses might also provide theories for virus classification and interaction. It is known that some synergistic or antagonistic interactions might exist between co-infected mycoviruses, that might enhance or decrease their disease symptoms (Hillman et al., 2018; Thapa and Roossinck, 2019). Recently, there are examples of virus interactions between capsidless (+) ssRNA and dsRNA virus, where the former which belong mainly to Yadokarviridae can hijack the latter’s particles, such as the viruses in the order Ghabrivirales or genus Botyvirnavirus, for packaging (Zhang et al., 2016; Das et al., 2021; Jia et al., 2022).

Alternaria sp. are notorious pathogens which are widely distributed in the natural environment and can cause disease in a wide variety of organisms including plants, humans and animals. Some of the phytopathogenic species can infect a wide range of plant species causing major losses for a large number of crops (Woudenberg et al., 2013). Moreover, toxins produced by some Alternaria species accumulated in agricultural products would also pose a threat to food safety (Ambra et al., 2013). Alternaria dianthcola is a plant pathogen that can cause flower blight on carnation in China and leaf blight on Withania somnifera in India, that brought great damage to such medical and ornamental plants (Maiti et al., 2007; Duan et al., 2015). Up to now, many mycoviruses have been reported in Alternaria spp., and some of them are related with the change of host phenotype. For example, Alternaria alternata hypovirus 1 (AaHV1) that was isolated from Alternaria alternata in an apple orchard has been confirmed to conserve hypovirulence in its host and other plant phytogenic fungi (Li et al., 2019). Alternaria alternata chrysovirus 1 (AaCV1) has been described to reduce growth rate of host fungus, but enhances the pathogenicity (Okada et al., 2018). In addition, AaCV1-AT1 has also been reported to reduce colony growth rate and conidial production ability on its host fungus (Ma et al., 2020). These studies indicated that there are many mycoviruses that might possess biocontrol potential in the fungal strains of Alternaria sp.

In this study, we molecularly characterized a variety of different mycoviruses from a single A. dianthcola strain HNSZ-1, by high-throughput sequencing, RT-PCR detection and conventional Sanger sequencing. In addition, effects of these viruses on their host were also been discussed.

### Materials and methods

#### Fungal isolates and growth conditions

*A. dianthcola* strain HNSZ-1 was collected from diseased leaf of *Dianthus chinensis* in Hunan province of China. All the strains were cultured on potato dextrose agar (PDA) at 27°C. The long-term preservation of fungal isolates was conducted by picking fungus plugs, growing on PDA for 7-10 days, in glycerin at -80°C. For dsRNA extraction, mycelial plugs were cultured on PD, with reciprocal shaking of 180 rpm, for 5 days at 27°C. Mycelia were harvested by filtration with sterile gauze.

#### DsRNA extraction and high-throughput sequencing

Extraction of dsRNA from mycelia was conducted by cellulose chromatography using the method as described by...
Morrison and Dodds (Morrison and Dodds, 1979). The dsRNA extractions were treated by DNase I or S1 nuclease (TaKaRa, Dalian, China) to eliminate possible contaminated DNA and ssRNA. The dsRNAs were then analyzed by 1% agarose gel electrophoresis.

The cDNA library construction and high-throughput sequencing was prepared using the previously described protocol (Bartholomäus et al., 2016). The cDNA was synthesized with the universal Primer-dN6 (GCCGGAGCTCTGCAGAATTC NNNNN), and the dsDNA was amplified using the Primer (GCCGGAGCTCTGCAGAATTC). All the PCR products were purified, sheared, and ligated with adaptors for Illumina sequencing. Sequencing was performed on the Illumina MiSeq 2000/2500 system by a pair-end sequencing run.

Bioinformatics analyses

Deep sequencing data was analyzed by CLC Genomic Workbench software package (CLC Bio-Qiagen, Boston, MA). After removing the low-quality sequence at the end and the joint sequence, the Clean Data was obtained and de novo assembled using the Trinity software (Grabherr et al., 2011). All generated contigs were compared to the National Center for Biotechnology Information (NCBI) database using BLASTx or tBLASTx with standard settings. The viral sequences were picked out and subjected for further confirmation.

Viral genome sequences validation and their full-length determination

To verify the presence of putative viruses in strain HNSZ-1, RT-PCR detections were conducted using specific primers designed according to the viral sequences obtained from the Illumina sequencing. Total RNA was isolated from collected mycelial mass using the RNeasy mini kit (Qiagen, Valencia, CA). The cDNA sequences were generated by reverse transcription reaction using RevertAid First Strand cDNA Synthesis Kit and hexdeoxyribonucleotide mixture random primers (Takara Dalian, China). PCR amplifications were carried out using the cDNA as template and the amplicons were analyzed by agarose gel electrophoresis and sequenced.

In order to complete the obtained viral genome sequences, the sequence gaps were filled by RT-PCR amplification with specific primers, and the 5’-and 3’-terminal sequences of all viral sequences infecting strain HNSZ-1 were obtained by ligase-mediated terminal amplification method as described previously (Xiang et al., 2017). The expected PCR amplicons were purified and cloned into pMD18-T vector (TaKaRa) for sequencing, with each base being sequenced in at least three independent clones.

Results and discussion

Multiple dsRNA species in strain HNSZ-1

When extracted by cellulose chromatography and digested by DNase I and S1 nuclease, diverse dsRNAs fragments were detected from strain HNSZ-1, as illustrated using agarose gel electrophoresis, which indicated the presence of multiple mycoviruses infection. At least eight dsRNA fragments, ranging from 1.2 kbp to 10 kbp in size, were observed (Figure 7A).
To investigate the mycoviruses infecting strain HNSZ-1, high-throughput sequencing library was constructed and sequenced on Illumina MiSeq 2500 platform. A total of 34,478,052 raw reads were obtained. After filtering of the low-quality reads, 34,315,594 high-quality sequence reads were de novo assembled into 5,924 contigs. Through preliminary BLASTx against to the NCBI NR database, 11 assembled contigs similar to viruses were obtained. Among these contigs, 9 contigs were significantly similar to the amino acid sequences of RdRp domains of 9 different viral sequences, suggesting that these contigs represent 9 distinct nearly complete mycovirus-like genome sequences. Analysis of the putative viral sequences indicated that they were belonged to +ssRNA mycoviruses in the families Narnaviridae, Hypoviridae, Ambiguiaviridae, -ssRNA mycovirus in the family Mymonaviridae, and dsRNA mycoviruses in the families Totiviridae, Partitiviridae and an unclassified taxon. To identify the really presence of these mycoviruses in strain HNSZ-1, RT-PCR was performed using the total RNA extracted from strain HNSZ-1 and specific primers designed based on the viral contigs. Results showed that all the viral contigs were probable viral origin.

The terminal genome sequences of the mycoviruses were determined by using ligase mediated terminal amplification technique. In combination with the high-throughput sequencing and conventional Sanger sequencing, the genome sequences of the 9 different mycoviruses were completed. Detailed information of these mycoviruses including their best matches deduced by BLASTx searches were listed in Table 1. According to the subsequent sequence analysis, those mycoviruses were temporarily designed as Alternaria dianthicola hypovirus 1 (AdHV1), Alternaria dianthicola narnavirus 1 (AdNV1), Alternaria dianthicola narnavirus 2 (AdNV2), Alternaria dianthicola umbra-like virus 1 and 2 (AdULV1), Alternaria dianthicola umbra-like virus 2 (AdULV2), Alternaria dianthicola victorivirus 1 (AdVV1), Alternaria dianthicola partitivirus 1 (AdPV1), Alternaria dianthicola negative-stranded RNA virus 1 (AdNSRV1), Alternaria dianthicola dsRNA virus 1 (AdRV1). Among the identified viruses, AdRV1 was reported previously in our lab (Hu et al., 2020), thus, we focused on other eight viruses in the following analysis.

One novel virus characterized in the family Hypoviridae

Viruses in the family Hypoviridae are +ssRNA viruses whose genomes generally ranged from 9 to 13 kbp containing one or two ORFs (Ghabrial et al., 2015; Suzuki et al., 2018). In the past, Hypoviridae contained only one genus, Hypovirus, including the mycoviruses isolated from Cryphonectria parasitica. At present, the members of this family are divided into three genera, including Alphahypovirus, Betahypovirus and Gammahypovirus. Viruses belong to Alphahypovirus were represented by Cryphonectria hypovirus 1 (CHV1) and Cryphonectria hypovirus 2 (CHV2), which contained two ORFs and in a larger size (12.5-13 kbp) than those in betahypovirus (9.1-10.4 kbp). Genus Betahypovirus was represented by CHV3 and CHV4 encoding only one polyprotein and four conserved protein domains. Viruses in the Genus Gamahypovirus contained the largest genome represented by Sclerotinia sclerotiorum hypovirus 2 (Mahmoud and Michael, 2014). While Setosphaeria turcica hypovirus 1 (StHV1) from Setosphaeria turcica, Valsa ceratospersa hypovirus 1 (VcHV1) from Valsa ceratospersa (Hajime et al., 2012), CHV3 and CHV4 from Cryphonectria parasitica are supposed to be part of the Betahypovirus genus, encoding only one polyprotein and four conserved protein domains. Viruses in the Gammahypovirus genus were larger than other viruses and only contained a single ORF.

In this study, a 9,443 bp long RNA fragment with a G+C content of 48.77% was identified, which showed similarity to viruses in the Hypoviridae family and be designated as AdHV1. AdHV1 contains a large ORF, encoding a 2,745 amino acid polyprotein with a calculated molecular mass of 310.17 kDa (Figure 1A). Three conserved domains of UDP-glucosyltransferase, RdRp and helicase-associated domain were found. It is well known that many polyproteins encoded by hypoviruses, such as CHV1 and CHV4, were processed by papain-like cysteine protease that was located in the N-terminal regions (Koonin et al., 1991; Aulia et al., 2021). Unexpectedly, the papain-like cysteine protease motif was not found in the AdHV1 polyprotein by conserved domain search in NCBI. However, alignment of the N-terminal region of the AdHV1 (186-307 aa region) with the papain-like cysteine protease regions of other hypoviruses revealed the presence of conserved three cysteine protease core residues, including cysteine, histidine and glycine (Figure 1B). This indicated that the AdHV1 encoded polyprotein might also be processed by cysteine protease. BLASTp homology search revealed that the polyprotein of AdHV1 had 58.63% to 67.61% aa sequence identity to proteins encoded by viruses in genus Betahypovirus, including Setosphaeria turcica hypovirus 1 (StHV1), Fusarium concentricum hypovirus 1 (FcHV1) and CHV4 (Daniela et al., 2005; Gilbert et al., 2019; Mizutani et al., 2021). Phylogenetic tree of AdHV1 and other related hypoviruses were generated using the conserved regions of polyprotein, it revealed that AdHV1 is a novel member of the family Hypoviridae (Figure 1C).

Two viruses in the family Narnaviridae

Viruses in the Narnaviridae family contained the simplest genomes, ranging from 2.3 to 3.6 kbp, and included a single ORF encoding RdRp (Hillman and Cai, 2013). Two contigs showed similarity to members of the family Narnaviridae. We designated the two viruses as AdNV1 and AdNV2. AdNV1
and AdNV2 were in lengths of 3159 and 2192 nucleotides (nts), respectively (Figure 2A). Like most narnaviruses, both the AdNV1 and AdNV2 contained a single ORF, encoding proteins that showed 96.74% and 97.09% identities to the RdRps of Erysiphe necator associated narnavirus 48 and Plasmodora viticola lesion associated narnavirus 1, respectively (Chiapello et al., 2020). Phylogenetic analysis indicated that AdNV1 and AdNV2 were grouped in the clade of Narnavirus genus, in the family Narnaviridae (Figure 2B).

### Two novel viruses in the family Ambiguiviridae

Two viral contigs were similar to viruses in a new proposed family Ambiguiviridae. The family Ambiguiviridae contained viruses closely related to members of the genus Umbravirus in the family Tombusviridae, as well as the (+) ssRNA viruses from insects (Gilbert et al., 2019). All the two viruses, with sizes of 4568 bp and 3380 bp, respectively, contained two ORFs, with the second ORF encoded an RdRp. They were named as AdULV1 and AdULV2, respectively (Figure 3A). BLASTp searches showed that the RdRp of AdULV1 shared 54.49% to 70.16% identities with the RdRps of some unclassified viruses, like Plasmodora viticola lesion associated ambiguivirus 1 (Chiapello et al., 2020) and Erysiphe necator associated ambiguivirus 3. AdULV2 showed the maximal 48.09% identity with Macrophomina phaseolina umbra-like virus 1 (Wang et al., 2021), followed by Erysiphe necator umbra-like virus 2, with 45.24% identity. Phylogenetic analysis based on the RdRps domain of AdULV1 and AdULV2 and other selected viruses showed that AdULV1 and AdULV2 were clustered with members of the proposed family Ambiguiviridae (Figure 3B).

### A novel virus in the family Totiviridae

The family Totiviridae had five genera, namely Victorivirus, Giardiaivirus, Leishmanaviirus, Trichomonasivirus and Totivirus (Ghabrial et al., 2015). Viruses in this family are characterized by linear dsRNA genomes of 4.6-7.0 kbp, which contain two ORFs encoding CP and RdRp or RdRp domain of a fusion protein (Wickner, 2012). One viral sequence, in the length of 5318 bp, was similar to members of the family Totiviridae, and was named AdVV1. AdVV1 had two separate ORFs, encoding CP and RdRp of 791 aa and 835 aa, respectively. The 5’ and 3’ untranslated regions (UTRs) of AdVV1 were 333 bp and 70 bp, respectively (Figure 4A). Both the CP and RdRp of AdVV1 showed the maximal identities of 63.79% and 58.50% to the corresponding proteins of Fusarium asiaticum victorivirus 2 (FaVV2). It is worth noting that the ORF2 and ORF1 were

| Virus name                      | Sequence length (bp) | Closest relative virus                           | Maximum Identity (%) | Genome type | Tentative virus classification | Accession numbers |
|--------------------------------|----------------------|--------------------------------------------------|----------------------|-------------|--------------------------------|-------------------|
| Alternaria dianthicola hypovirus 1 | 9443                 | Fusarium concentricum hypovirus 1                | 58.82                | +ssRNA      | Hypoviridae                     | ON843755          |
| Alternaria dianthicola narnavirus 1 | 3159                 | Erysiphe necator associated narnavirus 48        | 96.74                | +ssRNA      | Narnaviridae                    | ON843756          |
| Alternaria dianthicola narnavirus 2 | 2192                 | Plasmodora viticola lesion associated narnavirus 1 | 97.09                | +ssRNA      | Narnaviridae                    | ON843757          |
| Alternaria dianthicola umbra-like virus 1 | 4568                 | Plasmodora viticola lesion associated ambiguivirus 1 | 54.49                | +ssRNA      | Ambiguiviridae                  | ON843758          |
| Alternaria dianthicola umbra-like virus 2 | 3380                 | Macrophomina phaseolina umbra-like virus 1       | 48.09                | +ssRNA      | Ambiguiviridae                  | ON843759          |
| Alternaria dianthicola victorivirus 1 | 5318                 | Fusarium asiaticum victorivirus 2                | 58.50                | dsRNA       | Totiviridae                     | ON843760          |
| Alternaria dianthicola partitivirus 1 | S1:1770 S2:1553 S3:1297 | Alternaria tenuissima partitivirus 1              | 94.26                | dsRNA       | Partitiviridae                  | ON843761          |
| Alternaria dianthicola negative-stranded RNA virus 1 | 9013                 | Sclerotinia sclerotiorum negative-stranded RNA virus 3-U | 56.03                | -ssRNA      | Mymonaviridae                   | ON843763          |
| Alternaria dianthicola dsRNA virus 1 | 3014                 | Trichoderma harzianum mycovirus 1                | 68.89                | dsRNA       | Unclassified                    | MT241326 (Hu et al., 2020) |
not overlapped in AdVV1. It is known that the ORFs of viruses in the genus *Victorivirus* were commonly overlapped by the terranucleotide AUGA or pentanucleotide UAAUG, the typical characteristic of the coupled termination-reinitiation strategy for translation of downstream ORF in victoriviruses (Li et al., 2011; Zisis et al., 2013). Multiple aa sequences alignment showed that the CP of AdVV1 has an Alanine-Glycine-Proline-rich region on its C-terminal region as reported for victoriviruses, and the RdRp contained eight motifs conserved in the genus *Victorivirus* (Figure S1). We performed phylogenetic analysis based on the RdRp domains of AdVV1 and other selected viruses and revealed that AdVV1 was a new virus, which aggregated with members of the genus *Victorivirus* in the family *Totiviridae* (Figure 4B).

**A novel virus in the family Partitiviridae**

Viruses in family *Partitiviridae* are characterized by small and isometric viruses with bisegmented dsRNA genomes, ranging from 1.4 to 2.4 kbp in size (Liang et al., 2021). The two dsRNA segments separately encode an RdRp and a CP, respectively. However, in addition to RdRp and CP coding sequences, genome segments encoding unknown proteins, such as satellites or defective RNAs, also exist in *partitiviridae*. Up to now, there are seven known or proposed genera in this family, namely *Alphapartitivirus*, *Betapartitivirus*, *Gammapartitivirus*, *Deltapartitivirus*, *Cryspovirus*, *Epsilonpartitivirus* and *Zetapartitivirus* (Vainio et al., 2018; Gilbert et al., 2019).
In this study, virome analysis of strain HNSZ-1 revealed the presence of three viral sequences, in the lengths of 1770 bp, 1553 bp and 1297 bp, which might represent the genome of a novel virus, designated AdPV1 (Figure 5A). Each of the segments consist of a single ORF encoding a 57.78 kDa, 46.16 kDa and 35.11 kDa protein, respectively. BLASTp homology search revealed that the 57.78 kDa protein was similar to the RdRp of Alternaria tenussima partitivirus 1 with 94.26% identity (AttPV1) (Liang et al., 2021). The 46.16 kDa protein was 96.30% and 85% identical to the CP of Plasmopara viticola lesion associated partitivirus 10 (PVaPV10) (Chiapello et al., 2020) and AttPV1, respectively. The 35.11 kDa protein was similar to the hypothetical protein of PVaPV10 with 78.90% identity. A multiple nucleotide sequence alignment of the three viral segments revealed the presence of conserved sequences "CGAAAUU" at their 5' UTRs, and high sequence identity at their 3' UTRs (Figure 5B). Therefore, it is speculated that these three RNA fragments might be the same virus genome, according to the sequence homology search and non-coding regions alignment. Phylogenetic analysis based on the RdRp domain suggested that AdPV1 was most likely to be a novel virus related to Gammapartitivirus in the family Partitiviridae (Figure 5C).

**A novel virus in the family Mymonaviridae**

Viruses in *Mymonaviridae* typically have a linear (-) ssRNA genome, about 10 kbp in size, and enveloped by filamentous virions. Sclerotinia sclerotiorum negative-stranded RNA virus 1 (SsNSRV-1) that was isolated from the devastating plant pathogen fungus *Sclerotinia sclerotiorum* is the first reported (-) ssRNA mycovirus and subsequently be affiliated with the *Mymonaviridae* family (Liu et al., 2014; Amarasinghe et al., 2017; Jiang et al., 2019; Vainio and Suzuki, 2020).

One viral sequence, designated AdNSRV1, was identified in this study. The full length of the AdNSRV1 genome was 9013 nts, with five nonoverlapping ORFs encoding proteins of 235, 395, 57, 1933 and 184 aa, respectively (Figure 6A). The 5' and 3' UTRs were 79 nt and 269 nt long, respectively. Multiple alignment of nucleotide sequence between these ORFs found a semi-conserved sequence, "UAAAACUUAGGAA", occurred downstream of each ORFs and upstream of ORF1, which was a common feature of Mononegaviruses (Figure 6B). These regions might most likely be the gene-junction regions in AdNSRV1 genome and were broadly similar to sequences found in other mononegaviruses (Pringle and Easton, 1997; Wang et al., 2018).
BLAST analysis of AdNSRV1 revealed that, among the five ORFs, the largest ORF4 contained conserved regions corresponding to motif pfam00946 (Mononegavirales RNA dependent RNA polymerase; aa sites 238-1040; e-value 2.92e-75) and was 56.03% identical to the RdRp of Sclerotinia sclerotiorum negative-stranded RNA virus 3-U; The ORF2 showed sequence identity with the putative nucleocapsid protein of Plasmopara viticola lesion associated ambigivirus 2 (PvLamV2) (Chiapello et al., 2020) and Cryphonectria parasitica sclerotimonavirus 1. ORF1 and ORF5 were predicted to encode hypothetical proteins showing a low level of sequence identity to other members of Mymonaviridae. The ORF3 showed no similarity with any other viruses in the NCBI database.

Phylogenetic analysis was performed using neighbor joining (NJ) model. Phylogenetic tree was constructed based on multiple amino acid sequence alignment of the RdRps of AdNSRV1 and other selected mononegaviruses, including species from each genus within the Bornaviridae, Mymonaviridae, Nyamiviridae, Rhabdoviridae, Lispiviridae families, and an out group from Ophioviridae. The result indicated that AdNSRV1 was clustered with the Mymonaviridae family, thus it might be predicted as a novel member in this family (Figure 6C).
Virus elimination, horizontal transmission and effects of viruses infected on HNSZ-1

In order to check the possible effects of the viruses on the host fungus, we used protoplast regeneration method for virus curing. After dsRNA extraction and RT-PCR detection, two single-protoplast regeneration derivatives, HNSZ1-P4 and HNSZ1-P13, were obtained, and confirmed to lack the AdNSRV1 (Figure 7A, C). In addition, in order to reintroduce AdNSRV1 to the AdNSRV1-curing strain, virus horizontal transmission experiments were carried out using HNSZ1 and HNSZ1-P4 as the donor and recipient of AdNSRV1, respectively. The HNSZ1-P4 derivative strain HNSZ1-P4-T1 re-infected by AdNSRV1 was obtained.

Although no virus-free and other strains carrying different virus combination was obtained. The growth rate and morphology were compared between the original strain HNSZ-1, the two derivative subcultures HNSZ1-P4 and HNSZ1-P13, and the re-infected HNSZ1-P4-T1. On PDA, strains HNSZ1-P4 and HNSZ1-P13 showed regular colony margin as compared to the original strain HNSZ-1 and the re-infected strain HNSZ1-P4-T1 (Figure 7B). The average growth rate of strain HNSZ-1 and HNSZ1-P4-T1 were significantly lower than that of HNSZ1-P4 and HNSZ1-P13, but no significant difference was exhibited between the HNSZ1-P4 and HNSZ1-P13 (Figure 7D). These results indicated that AdNSRV1 in strain HNSZ-1 might be involved in the phenotypic alteration of the host fungus. However, the effects of each virus on the host fungus or any interactions between these co-infected viruses remain warrant further exploration.

Conclusions

In this study, we characterized the virome of an A. dianthicola strain HNSZ-1, that was isolated from the diseased leaves of D. chinensis. After dsRNA extraction, high-throughput sequencing, RT-PCR detection and conventional Sanger sequencing, the complete genome of nine viruses co-infected in strain HNSZ-1 were obtained. Genome organization, homology search and phylogenetic analysis revealed that, among the nine viruses, a dsRNA virus named AdRV1 has been identified to be an unclassified virus in our previously study (Hu et al., 2020), and five viruses named AdHV1, AdULV1, AdULV2, AdNSRV1 and AdVV1 were predicted to represent new mycovirus species belonging to the families Hypoviridae, Ambiguiviridae, Mymonaviridae and Totiviridae, respectively. Meanwhile, viruses AdNV1, AdNV2 and AdPV1 have more than 95% similarity to the viral sequences in other fungal species in the NCBI database. Thus, they might be new strains of these previously recorded mycoviruses, which were assigned to the
families Narnaviridae and Partitiviridae, respectively. Thus far, the identification of co-infection of mycoviruses in this study enriched the diversity of virus constitution in Alternaria.

The application of high-throughput sequencing technology for virus hunting has greatly promoted the development of virology in recent years that accelerated the discovery of virus diversity, origin and evolution from various materials including animals, plants, fungi and environment (Adams et al., 2009; Roossinck et al., 2015; Marzano et al., 2016; Shi et al., 2016; Chiapello et al., 2020; Myers et al., 2020; Wang et al., 2021; Ruiz-Padilla et al., 2021; Kondo et al., 2022; Dominguez-Huerta et al., 2022). For mycoviruses, many studies have reported that the co-

**FIGURE 5**

Genome organization and phylogenetic placement of Alternaria diantichola partitivirus 1 (AdPV1). (A) A schematic diagram of the genome organization of AdPV1. The genome of AdPV1 contained three dsRNA segments, with each one has a single ORF, encoding a RdRp, CP and a protein with unknown function, respectively. (B) Multiple alignments of 5' and 3'-terminal nucleotide sequences of the AdPV1 genomic segments. (C) NJ phylogenetic tree was constructed based on the RdRp aa sequences.
Characterization of the genome structure and phylogenetic analysis of Alternaria dianthicola negative-stranded RNA virus 1 (AdNSRV1). (A) Genome size and organization of AdNSRV1. Five ORFs were predicted in the AdNSRV1 genome, which were indicated by the rectangular boxes. Conserved sequences are highlighted in shade. (B) Comparison of putative gene junction regions between the ORFs in AdNSRV1 shown in 3'- to 5'-orientation. (C) Phylogenetic analysis of AdNSRV1. A phylogenetic tree based on aa alignments of L proteins was constructed, using the NJ method. The virus AdNSRV1 was represented in the phylogenetic tree.
infection of a large number of viruses in single fungal strains is a common phenomenon, which usually appeared in the necrotrophic fungi *R. solani* and *S. sclerotiorum* (Bartholomäus et al., 2016; Mu et al., 2021). Although a number of viruses have been reported in *Alternaria* species, no strain has been infected by comparable number of virus species as has been identified in this study. Therefore, we speculate that there are a large number of mycoviral resources in *Alternaria*, given that the *Alternaria* is a large taxonomic fungal group. Our study is a step forward the screening of mycoviruses infecting *Alternaria* species.

In this study, we obtained two protoplast regeneration derivatives, lacking the AdNSRV1 infection, and found that AdNSRV1 might be related to growth rate and morphology in the host *A. dianthicola*. This was confirmed by virus elimination and horizontal transmission experiments. Of course, the direct evidence that AdNSRV1 had explicit effects on its host remain limited. Further efforts including virions transfection of AdNSRV1 to the virus-free strains were also warranted. In addition, we also conducted the virus horizontal transmission experiments in order to transfer AdNSRV1 singly from HNSZ-1 to other virus-free strains with the same genetic background, but failed. It is possible that other viruses co-infecting with AdNSRV1 might have potential interactions with AdNSRV1, as have been inferred by previously reports (Li et al., 2022a; Li et al., 2022b). Since we have not obtained other derivative strains infected by each virus alone or by different virus combination, the possible effects of each virus on its host fungus, or whether there is any interaction between these co-infected viruses, remains to be elucidated. In conclusion, to our knowledge, this is the first report for virome characterization of *A. dianthus*. This study might provide an important insight for understanding the diversity and evolutionary of mycoviruses and screening

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**FIGURE 7**

Mycovirus content and biological features of strain HNSZ-1 and other derivative strains. (A) Detection of viruses in strain HNSZ-1, its protoplast regeneration derivatives HNSZ-1-P4 and HNSZ-1-P13, and the re-infected derivative strain HNSZ1-P4-T1 by dsRNA extraction. Strain HNSZ-1 and HNSZ1-P4-T1 were the original and re-infected strains, respectively, that were co-infected by all the nine viruses. Strains HNSZ-P4 and HNSZ-1-P13 were detected to lack the AdNSRV1. (B, C) Colony morphology (B) and growth rate ** means significant difference at P < 0.05. (C) Detection of viruses in strain HNSZ-1, its protoplast regeneration derivatives HNSZ-1-P4 and HNSZ-1-P13, and the re-infected strain HNSZ1-P4-T1. (D) Detection of viruses in strain HNSZ-1, the two protoplast regeneration derivatives HNSZ-1-P4 and HNSZ-1-P13, and the re-infected strain HNSZ1-P4-T1 by RT-PCR. Strain HNSZ-1 was the original strain co-infected by nine viruses, while strains HNSZ-P4 and HNSZ-1-P13 were detected to lack the AdNSRV1. Strain HNSZ1-P4-T1 was a derivative strain of HNSZ1-P4 obtained by virus horizontal transmission experiment, that was re-infected by nine viruses including AdNSRV1.
mycoviruses for biological control. To a certain extent, it was also conducive to study the interactions between viruses or viruses and their fungal host.

**Data availability statement**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

**Author contributions**

JZ, PL, and SZ: sampling, investigation, writing-original draft. JZZ and XL: investigation, results interpretation, reviewing and editing original draft. JZ and BG: conceptualization, supervision, results interpretation, writing-reviewing and editing- original draft. All authors contributed to the article and approved the submitted version.

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**Conflict of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Supplementary material**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.980970/full#supplementary-material

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