Discovery and exploration of widespread infection of mycoviruses in *Phomopsis vexans*, the causal agent of phomopsis blight of eggplant in China

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*Phomopsis vexans*, which causes Phomopsis blight of eggplant, has been reported worldwide. To study the biocontrol of this disease, 162 leaf and fruit samples of eggplant Phomopsis blight were collected from Hunan, Hubei, Jiangxi, Sichuan, Zhejiang, Fujian, Guangdong and Anhui Provinces from 2017 to 2019. Eighty-seven pathogenic fungus isolates were identified as *P. vexans*. The following studies were conducted: screening of sporulation medium, spore morphology analysis, mycovirus detection and identification of novel mycoviruses in these isolates. The results showed that eggplant tissue medium was the most suitable medium for rapid sporulation, and all isolates had mycoviruses consisting of mainly mixed infections. The genome of these mycoviruses varied from 1-15 kb. Five novel mycoviruses infecting *P. vexans* were obtained, including "*Phomopsis vexans* fusarivirus 1" (PvFV1), "*Phomopsis vexans* ourmia-like virus 1" (PvOLV1), "*Phomopsis vexans* endornavirus 2" (PvEV2), "*Phomopsis vexans* partitivirus 1" (PvPV1) and "*Phomopsis vexans* victorivirus L1" (PvVV1L). Thus, PvVV1L displays a unique genome structure, and this is the first report of a victorivirus consisting of two segments and of a deltapartitivirus infecting the fungus host.
1 Introduction

Mycoviruses are viruses that infect and replicate in fungal cells (Hollings, 1962; Son et al., 2015). Most mycoviruses infect their fungal hosts asymptptomatically (cryptic infections). However, several mycoviruses clearly can result in phenotypic alterations in the host, such as hypovirulence and debilitation. Therefore, mycoviruses have great potential to be exploited as biological agents against fungal diseases (Nuss, 2005; Jiang et al., 2013; Ghabrial et al., 2015; Li et al., 2019). Since Cryphonectria hypovirus 1 (CHV1) was successfully used to control chest blight disease in Europe (Nuss, 1992; Xie and Jiang, 2014; Ghabrial et al., 2015), an increasing number of hypoviruses have been detected in fungi, such as Sclerotinia sclerotiorum, Botrytis cinerea, and Botryosphaeria dothidea (Xiao et al., 2014; Hao et al., 2018; Azhar et al., 2019; Zhai et al., 2019). The mycovirus SSHADV-1 was used to transform the pathogenic fungus Sclerotinia sclerotiorum into an endophytic fungus that could coexist with, promote the growth and enhance the disease resistance of rape. The novel concept of a “plant vaccine” for the control of fungal diseases has been proposed, and it has also been pointed out that mycoviruses might be an important factor in the formation of endophytic fungi. Mycoviruses play a significant role in the interaction between plants and fungi (Zhang et al., 2020). The development and application of high-throughput next-generation sequencing (NGS) technologies and bioinformatics have greatly enhanced the discovery of new viruses in many organisms, including fungi (Marzano et al., 2016; Zhang et al., 2018).

Some mycoviruses with attenuated virulence may not affect the growth rate of the host after infection but show physiological alterations in the host, such as hypovirulence and debilitation. For example, Penicillium digitatum polymycovirus 1 (PdPMV1) and Penicillium digitatum Narna-like virus 1 (PdNLV1) have no significant effect on the growth morphology of the host when coinfected with Penicillium digitatum; however, they reduce the sensitivity of the host to fungicides (Niu et al., 2018). Torres-Trenas et al. used a GFP fluorescence labeling technique, demonstrated that mycoviruses in Fusarium oxysporum affect its fixed speed and spatial distribution in plant roots (Torres-Trenas et al., 2019).

Mycoviruses can also promote the virulence of their fungal hosts. Curvularia thermal tolerance virus (CThTV) in the endophytic fungus Curvularia protuberate can enhance the heat tolerance of its fungal host and the plants simultaneously (Marquez et al., 2007), which provides material for studying the tripartite interaction among viruses, fungi and plants. Kotta-Loizou and Coutts found that mycoviruses can improve the pathogenicity of Beauveria bassiana to Galleria mellonella (Kotta-Loizou and Coutts, 2017). Talaromyces marneffei partitivirus 1 (TmPV1) can enhance the pathogenicity and tolerance of host fungi and increase the lethality of the host to mice in Talaromyces marneffei (Lau et al., 2018).

Mycoviruses are divided into five categories according to the nucleic acid type: retroviruses, negative single-stranded RNA viruses, positive single-stranded RNA viruses, double-stranded RNA viruses and DNA viruses. Double-stranded RNA viruses are divided into 7 families: Totiviridae, Partitiviridae, Chysoviridae, Endornaviridae, Reoviridae, Quadrirhabdovidae and Megabirnaviridae. Negative single-stranded RNA viruses include the single-negative-stranded RNA virological family (Myxomaviridae); DNA viruses comprise the Geminivirus-like family (Genomoviridae); retroviruses include the transposable virofamily (Metavirusidae) and pseudoviridae (Pseudoviridae); positive single-stranded RNA viruses approved by the International Commission for the Classification of Virology include eight families: Alphaflexiviridae, Gammaflexiviridae, Deltaflexiviridae, Barnaviridae, Botourmiaviridae, Narnaviridae, Hypoviridae and Endornaviridae (King et al., 2012). Fusariviridae is a recently proposed family of + ssRNA viroviridae. The genome size of Fusariviruses is 6–10 kbp, and the larger ORF encodes replicase-related proteins.

In recent decades, a number of phytopathogenic and plant-associated fungi have been screened for viruses from a few perspectives, such as virological control practices (biological use of viruses) and basic virus research. The tested fungi were shown to be infected by viruses at different incidence rates, ranging from a few to over 90% (Hillman et al., 2018). These studies have revealed the great diversity of fungal viruses and provided insights into their evolutionary histories (Xie and Jiang, 2014; Marzano and Domier, 2016; Marzano et al., 2016). For example, dsRNA analysis has revealed that of the 79 R. necatrix isolates tested (62 Spanish, 1 Italian and 16 Israeli), 11 tested positive for the presence of dsRNA (Arjona-Lopez et al., 2018). Luo isolated 13 B. dothidea strains in Wuhan city of Hubei Province. After dsRNA detection, 12 were found to carry mycoviruses, and the carrying rate was as high as 92.31% (Luo et al., 2019). All 31 Entoleuca sp. isolates showed mycovirus infection. New virus-carrying Entoleuca sp. isolates were obtained, which are capable of controlling avocado white root rot (Arjona-Lopez and Lopez-Herrera, 2021). All P. vexans isolated from eggplant in this study also carried mycoviruses.

Eggplant is an economically important crop with a long cultivation history. It is widely planted in China, India, Pakistan, Menggara, the Philippines and other countries (Kumar et al., 2011). Eggplant brown streak (Phomopsis blight of eggplant) caused by P. vexans is an important disease that limits eggplant production (Rangaswami and Madevan, 1998; Khan, 1999; Akhtar and Chaube, 2006; Jayaramiah et al., 2013), causing leaf and stem death or fruit rot. The disease was shown to decrease the production of eggplant by 20–50% in one season (Akhtar and Chaube, 2006; Pandey, 2010). The pathogen of eggplant brown streak can pass through the infected seed coat, cotyledon and hypocotyl (Thippeswamy et al., 2012), resulting in a low seed
germination rate and poor seed vigor. The seed yield is often reduced or even lost in breeding fields due to the spread of the disease (Das, 1998; Beura et al., 2008; Udayashankar et al., 2019). Eggplant brown streak was first reported in Nanjing, China in 1932 (Teng, 1932). At present, the disease has spread to most parts of the world (Smith et al., 1988) (Supplementary Figure SEQ Figure \textsuperscript{1}). In addition to infection of eggplant, Phomopsis comprises more than 100 different species and can live in more than 70 different families of herbaceous and woody plants and cause serious disease in soybean and sunflower oil crops, such as vegetables and fruit trees. These disease include soybean satisfactory stem rot (Jackson et al., 2009), Phomopsis stem cStem anker of sunflower Sunflower (Hulke et al., 2019), asparagus stem blight (Zhang et al., 2012), and die-back disease of neem (Nagendra Prasad et al., 2010), a very important plant pathogenic fungus. Mycoviruses reported in P. vexans include only P. vexans RNA virus 1 (PvRV1) (Zhang et al., 2015) and P. vexans partitivirus 1 (PvPV1) (Zhang et al., 2021). In this study, a number of P. vexans strains were isolated 58 eggplant planting areas in eight provinces of China. All P. vexans isolated in this study carried mycoviruses, which contained not only rich mycovirus resources but also the possibility of mycoviruses that could be used as plant vaccines. The genome size of the mycovirus varied from 1 to 15 kbp, and most of the strains could be used as plant vaccines. The genome size of the mycoviruses varied from 1 to 15 kbp, and most of the strains could be used as plant vaccines.
3 Results

3.1 Isolation and purification of *P. vexans* strains

From 2017 to 2019, 162 leaf and fruit samples with typical symptoms of eggplant brown streak were collected from 58 eggplant planting areas in Hunan, Hubei, Jiangxi, Sichuan, Zhejiang, Fujian, Guangdong and Anhui Provinces. The location and geographical distribution of the samples in the field are shown in Figure 1, and the symptoms of eggplant brown streak are shown in Figure 2.

A total of 87 *P. vexans* strains were isolated, which included some Colletotrichum and Alternaria, but none of them were our target strains. *P. vexans* colonies were first white and then became yellowish, ultimately resulting in the formation of black pycnidium. By observing the colony morphology, colonies typical of *P. vexans* were selected for purification. The morphology of the isolated *P. vexans* strains is shown in Figure 3.

3.2 Observation of the spore carrier and spore morphology of *P. vexans* strains

The purified strains were identified with ITS4/ITS5 primers. Sequence homology comparison identified 87 strains as *P. vexans*. The pycnidium of *P. vexans* was induced and cultured on three kinds of media. The results showed that the number of pycnidium was greater on OMA medium than on PDA and eggplant decoction medium in the dark at 25 °C. However, the eggplant tissue culture medium was most suitable for spore production; many pycnidia emerged after 5-7 days of cultivation, and the sporulation load of a single pycnidium was the largest (Figures 4A–C). The α-type and β-type conidia of *P. vexans* were observed in the pycnidium (Figures 4D, E), which was consistent with the morphological characteristics of *P. vexans*.

3.3 *P. vexans* contain abundant dsRNAs

A representative *P. vexans* strain was selected from each eggplant planting area, making up all 58 strains, to detect dsRNA viruses (Supplementary Table 1). Nucleic acid extraction resulted in a number of nucleic acid bands (Figure 5) that were resistant to DNase I and S1 nuclease digestion, indicating that the presence of dsRNA-like nucleotides was 100%, and the results showed abundant mycoviral resources in the *P. vexans* strain. The 58 isolated strains all had detectable mycoviruses. The genome size of the mycoviruses varied from 1 to 15 kbp, and most of the strains showed mixed infection. Only 6 strains were infected with a single dsRNA virus (PV_05, PV_06, PV_14, PV_15, PV_25, PV_37), accounting for 10.3% of the tested strains. According to the statistical analysis of dsRNA bands of different sizes, 77.6% of the tested strains were infected with nucleic acid bands of approximately 5.0 kb in size (45/58), demonstrating the highest carrying rate. Nucleic acid bands greater than 10 kb in size (43/58) accounted for 74.1% of the tested strains. There were differences in the detection rate of dsRNA among strains isolated from different provinces. The top three average carrying rates of dsRNA were from Anhui, Sichuan and Hunan, while the lowest was isolated from Guangdong (the number of strains from Jiangxi and Hubei was less than 3, which was not biologically significant and thus was not taken into account (Table 1). Nucleic acid bands of approximately 3.0 kb in size were usually accompanied by other mycoviruses.

3.4 Identification of novel mycoviruses

High-throughput sequencing was carried out using a mixture of 10 strains in 6 batches. In this study, we identified diverse viral segments from *P. vexans* strains using high-throughput transcriptome sequencing. Some viruses discovered in our analysis were nearly full length and included 5 unreported
viruses. 5 whole viral genomes were obtained by random primer construction library and ligase-mediated end amplification. Their complete nucleotide sequences were assembled by DNAMAN. The predicted amino acid sequences of the putative viral genomes showed significant sequence identity with described viruses from several distinct lineages, including one novel virus in the family Botourmiaviridae, named *P. vexans* ourmia-like virus 1 (PvOLV1), one novel virus in the family Fusariviridae, named *P. vexans* fusarivirus 1 (PvFV1), one novel virus in the family Endornaviridae, named *P. vexans* endornavirus 2 (PvEV2), one novel virus in the family Partitiviridae, named *P. vexans* partitivirus 1 (PvPV1) and one novel virus in the family Totiviridae in which an unconventional victorivirus consisted of two segments of nucleic acid, named *P. vexans* lexivirus L1 (PvVVL1).

### 3.4.1 Analysis of the PvOLV1 genome structure

Sequence analysis of the full-length cDNA indicated that the PvOLV1 genome (Figure 6A) was 2452 nt in length and had a C +G content of 54.73%. ORF prediction revealed that PvOLV1 consisted of a unique ORF of 1904 nt (nt positions 68–1972), which encoded a 634-amino-acid (aa)-residue polypeptide with a calculated molecular mass of 72.04 kDa. The 5’-untranslated region (UTR) of PvOLV1 was determined to be 67 nt long. The 3’-UTR was relatively long, at 480 nt. BLASTp alignment of its RdRp indicated that PvOLV1 showed the highest aa identity (E-value: 3e-110; query cover: 95%; identity: 38.7%) with *Plasmopara viticola*-associated ourmia-like virus 52 (PvLaOLV52). A search of the conserved domain database (CDD) and multiple protein alignment confirmed that the predicted RdRp domains contained eight conserved motifs (I...
to VIII), including the GDD motif, a typical characteristic of mycoviral RdRps (Figure 6B). We have named this novel mycovirus, the first reported in this fungal pathogen, *P. vexans* ourmiavirus-like virus 1 (PvOLV1). The virus sequence has been deposited in GenBank under accession number MZ044287.

A phylogenetic tree was constructed based on the deduced aa sequence of the putative RdRp region encoded by PvOLV1 using the NJ method in MEGA (version 7.0). The results showed that PvOLV1 was clustered with ourmiavirus in the family Botourmiaviridae (Figure 7).

### 3.4.2 Analysis of the genomic structure of PvFV1

Sequence analysis of the full-length cDNA indicated that the PvFV1 genome (Figure 8A) was 6007 nt in length, excluding the poly(A) structure at the end of the 3′ noncoding region, and had a C+G content of 42.86%. The 5′ and 3′ noncoding regions were 93 nt and 27 nt long, respectively. Fusariviridae is a new, recently proposed + ssRNA family comprising members that typically have 6 to 8 kbp genomes, with one larger ORF encoding putative polyproteins of replicases and one to three smaller ORFs.
encoding hypothetical proteins (Zhang et al., 2014). The genome of PvFV1 also contained two ORFs that did not overlap. ORF1 encoded RdRp, which was predicted to encode a protein of 1540 aa with a molecular weight of 174.75 kDa. ORF2 was predicted to encode a 402-aa protein with a molecular weight of 45.78 kDa. CD-Search in NCBI indicated that ORF1 contained a conserved RdRp and an RNA unwinding domain, and ORF2 contained a DUF3084 (e-value, 4.31e-04) domain with unknown function. BLASTp alignment of its ORF1 revealed the highest aa identity (E-value: 0; query cover: 94%; identity: 51.7%) with the RdRp of the Fusariviridae family, Fusarium graminearum dsRNA mycovirus-1 (FgV-ch). CD search showed that the ORFs contained eight conserved domains of the Fusariviridae family virus (Figure 8B). The virus sequence was deposited in GenBank under accession number MZ044286.

The phylogenetic tree of PvFV1 encoding RdRp aa was constructed using MEGA7 software, the NJ method and the Poisson model. The results showed that PvFV1 and Fusariviridae were clustered together (Figure 9).

3.4.3 Genomic structure analysis of PvEV2

The sequences showed similarity to members of the family Endornaviridae. Viruses in the Endornaviridae family have linear ssRNA genomes ranging in length from approximately 10 kb to more than 17 kb and containing an ORF encoding a single long polyprotein. The polyprotein encoded by

### TABLE 1 Table of dsRNA bands in P. vexans.

| Provinces of strain isolation | Number of strains | Total number of dsRNA | The average number of dsRNA |
|------------------------------|-------------------|-----------------------|----------------------------|
| Anhui                        | 3                 | 21                    | 7                          |
| Sichuan                      | 7                 | 33                    | 4.7                        |
| Hunan                        | 27                | 118                   | 4.37                       |
| Zhejiang                     | 6                 | 23                    | 3.83                       |
| Fujian                       | 7                 | 25                    | 3.57                       |
| Guangdong                    | 5                 | 6                     | 1.2                        |
| Jiangxi                      | 2                 | 7                     | –                          |
| Hubei                        | 1                 | 4                     | –                          |

The phylogenetic tree of PvFV1 encoding RdRp aa was constructed using MEGA7 software, the NJ method and the Poisson model. The results showed that PvFV1 and Fusariviridae were clustered together (Figure 9).
FIGURE 7
Phylogenetic analysis of PvOLV1. Phylogenetic analysis of PvOLV1 based on aa alignments of RdRp and other viruses related to these proteins. A phylogenetic tree was constructed by the neighbor-joining algorithm using MEGA7, with 1000 bootstrap replications. The percentage of bootstrap values supporting the branches in phylogenetic trees are indicated on the nodes. The genetic distance is represented by the scale bar of 0.2 aa substitutions per site. The novel virus PvOLV1 is indicated by a green square. The names and database accession numbers of other related viruses analyzed are indicated in the tree.

FIGURE 8
Diagrammatic representation of the genome organization and multiple alignment of PvFV1. (A) Schematic representation of the PvFV1 genome organization, which contained a single open reading frame (ORF) encoding a putative RNA-dependent RNA polymerase (RdRp) and 5' and 3' untranslated regions (UTRs). (B) Multiple alignment of the aa sequences of the RdRp domains of PvFV1 and other similar Fusariviruses using the ClustalX program and highlighted using the GeneDoc program. The conserved motifs in these RdRps are indicated by Roman numerals I to VIII. Virus names and accession numbers are as follows: FgV-ch, Fusarium graminearum dsRNA mycovirus-1 (AAT07067.2); PrSRV1, Penicillium roqueforti ssRNA mycovirus 1 (AII99895.1); PvLaFV1, Plasmopara viticola lesion associated fusarivirus 1 (QHD64725.1); EnFV1, Erysiphe necator-associated fusarivirus 1 (QHD64833.1); HfFV1, Helminthosporium maydis fusarivirus 1 (QRI93681.1); BCL64192.1; MpSRV1, Macrophomina phaseolina single-stranded RNA virus 1 (ALD89094.1).
endornaviruses often includes conserved domains, such as viral RNA helicases and RdRps (Ghabrial et al., 2015). We designated the virus *P. vexans* endornavirus 2 (PvEV2). The full-length PvEV2 genome was 11015 nt, excluding the poly(C) structure at the end of the 3' noncoding region, which contained a single ORF encoding a 3597-aa polyprotein with a molecular weight of 397.10 kDa (Figure 10). The BLASTP alignment information of the four conserved domains of PvEV2 with other Endornaviruses are shown in Table 2. Domain searches revealed that this polyprotein contained the conserved domains of viral methyltransferase (Met), putative DEXDc, viral helicase (Hel) and RdRp. Consistent with the homology search, phylogenetic analysis based on the conserved RdRp domain suggested that PvEV2 was a new putative species in the family Endornaviridae. The virus sequence was deposited in GenBank under accession number MZ044288.

A phylogenetic tree was constructed using the neighbor-joining algorithm in MEGA7, with 1000 bootstrap replications (Figure 11). PvEV2 and Betaendornavirus in the family Endornaviridae were clustered together. There was a poly(C) structure at the end of the 3' noncoding region of PvEV2 and a methyltransferase domain rather than a glycosyltransferase domain. It was inferred that PvEV2 was a betaendornavirus.

### 3.4.4 Structural analysis of the PvPV1 genome

Sequence analysis of *P. vexans* partitivirus 1 (PvPV1) revealed two independent segments (dsRNA1 and dsRNA2), each with a single open reading frame (ORF) (Figure 12A). The
FIGURE 11
Phylogenetic analysis of PvEV2. Phylogenetic analysis illustrating the evolutionary classification of PvEV2 was conducted using the method described in Figure 7. The genetic distance is represented by the scale bar of 0.2 aa substitutions per site. The novel virus PvEV2 is indicated by black dots. The names and database accession numbers of other related viruses analyzed are indicated in the tree.

TABLE 2 Percent aa sequence identity of the 2 conserved domains of *P. vexans* endornavirus compared with those of other endornaviruses.

| Protein | Organism name | Accession number | Identify | e-value |
|---------|---------------|------------------|----------|---------|
| RdRp    | *Botrytis cinerea* betaendornavirus 1 | YP_009315910.1 | 73.86%   | 4E-86   |
|         | *Sclerotinia sclerotiorum* endornavirus 2-A | AWY10955.1 | 74.43%   | 2E-85   |
|         | *Gremmeniella abietina* type B RNA virus XL1 | YP_529670.1 | 73.86%   | 2E-84   |
|         | *Gremmeniella abietina* type B RNA virus XL2 | ARB73306.1 | 75%      | 7E-85   |
|         | *Sclerotinia sclerotiorum* endornavirus 2 | AND83000.1 | 73.86%   | 2E-84   |
| Hel     | *Gremmeniella abietina* type B RNA virus XL1 | YP_529670.1 | 36.36%   | 1E-37   |
|         | *Gremmeniella abietina* type B RNA virus XL2 | ARB73306.1 | 35.93%   | 2E-37   |
|         | *Sclerotinia sclerotiorum* endornavirus 2 | AND83000.1 | 33.91%   | 4E-35   |
|         | *Sclerotinia sclerotiorum* betaendornavirus 1 | AIF43939.1 | 33.48%   | 1E-34   |
|         | *Sclerotinia sclerotiorum* betaendornavirus 1 | AIF43939.1 | 33.48%   | 1E-34   |
|         | *Sclerotinia sclerotiorum* betaendornavirus 1 | AIF43939.1 | 33.48%   | 1E-34   |
|         | *Sclerotinia sclerotiorum* betaendornavirus 1 | AIF43939.1 | 33.48%   | 1E-34   |
|         | *Sclerotinia sclerotiorum* betaendornavirus 1 | AIF43939.1 | 33.48%   | 1E-34   |
| Met     | *Sclerotinia sclerotiorum* endornavirus 2 | AND83000.1 | 54.31%   | 1E-78   |
|         | *Sclerotinia sclerotiorum* betaendornavirus 1 | AIF43939.1 | 53.45%   | 2E-77   |
|         | *Botrytis cinerea* betaendornavirus 1 | YP_009315910.1 | 53.88%   | 2E-76   |
|         | *Sclerotinia sclerotiorum* endornavirus 1 | AIF43939.1 | 53.02%   | 3E-76   |
|         | *Gremmeniella abietina* type B RNA virus XL2 | ARB73306.1 | 49.79%   | 7E-76   |
| DexDc   | *Gremmeniella abietina* type B RNA virus XL2 | ARB73306.1 | 44.79%   | 4E-32   |
|         | *Gremmeniella abietina* type B RNA virus XL1 | YP_529670.1 | 44.79%   | 4E-32   |
|         | *Sclerotinia sclerotiorum* endornavirus 1 | AIF43939.1 | 40.65%   | 4E-32   |
|         | *Sclerotinia sclerotiorum* endornavirus 1 | YP_009315910.1 | 41.29%   | 5E-32   |
|         | *Sclerotinia sclerotiorum* endornavirus 1 | YP_009315910.1 | 41.29%   | 5E-32   |
|         | *Sclerotinia sclerotiorum* endornavirus 1 | YP_009315910.1 | 38.71%   | 1E-29   |
|         | *Sclerotinia sclerotiorum* endornavirus 2 | AND83000.1 | 38.06%   | 1E-28   |

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full-length dsRNA1 was 1662 nt, containing an ORF encoding a 518-aa protein of RDRP with a molecular weight of 59.8 kDa. The 5’ and 3’ untranslated regions (UTRs) of dsRNA1 were 45 nt and 60 nt, respectively. BLASTp alignment of the ORF encoded by dsRNA1 indicated that the ORF encoded by dsRNA1 was most similar to RdRp in the family Partitiviridae, including diatom colony-associated dsRNA virus 14 (DcaRV14; identity, 48.23%; query cover, 92%; E-value, 4e-148), pepper cryptic virus 1 (PcV1; identity, 36.92%; query cover, 86%; E-value, 6e-93), Raphanus sativus cryptic virus 2 (RsCV2; identity, 37.50%; query cover, 86%; E-value, 1e-89), and Cucumis melo cryptic virus (CmCV; identity, 36.12%; query cover, 84%; E-value, 37 1e-88). The results of a conservative structure database (CDD) search and multiple alignment (Figure 12B) revealed 6 (III to VIII) conserved domains. The sequence of dsRNA1 was deposited in GenBank under accession number MT711194.

The full-length dsRNA2 was 1628 nts and had a C+G content of 42.86%, and the base numbers of the 5’ and 3’-UTRs were 168 nt and 263 nt, respectively. In addition, it contained an ORF (169 nt-1366 nt) encoding a hypothetical protein with a molecular weight of 44.5 kDa. BLASTp alignment of the ORF encoded by dsRNA2 indicated that the ORF of PvPV1 was most similar to RdRp in the family Partitiviridae, including diatom colony-associated dsRNA virus 14 (DcaRV14; identity, 48.23%; query cover, 92%; E-value, 4e-148), pepper cryptic virus 1 (PcV1; identity, 36.92%; query cover, 86%; E-value, 6e-93), Raphanus sativus cryptic virus 2 (RsCV2; identity, 37.50%; query cover, 86%; E-value, 1e-89), and Cucumis melo cryptic virus (CmCV; identity, 36.12%; query cover, 84%; E-value, 37 1e-88). The results of a conservative structure database (CDD) search and multiple alignment (Figure 12B) revealed 6 (III to VIII) conserved domains. The sequence of dsRNA2 was deposited in GenBank under accession number MT711195.

A phylogenetic tree based on PvPV1 encoding RdRp was constructed by the neighbor-joining algorithm using MEGA7, with 1000 bootstrap replications (Figure 13). The blue square represents the identified virus in the fungus, and the green triangle represents the identified virus in the plant. Phylogenetic analysis showed that PvPV1 and deltapartitivirus in the family Partitiviridae were clustered together. In addition, it has been reported that the hosts of deltapartitivirus and crypsovirus viruses are plants and protozoa (Nibert et al., 2009; Vainio et al., 2018). This is the first report to describe deltapartitivirus virus in fungi.

### 3.4.5 Structural analysis of the PvVV L1 genome

Sequence analysis of P. vexans partitivirus 1 (PvVV L1) revealed two independent segments (dsRNA1 and dsRNA2), each with a single open reading frame (ORF) (Figure 14). The full-length dsRNA1 was 2996 nts, and the GC content was 56.4%, containing an ORF encoding an 855-aa protein of RDRP with a molecular weight of 95.2 kDa. The 5’ and 3’ untranslated regions (UTRs) of dsRNA1 were 209 nt and 219 nt, respectively. BLASTp alignment indicated that the ORF encoded by dsRNA1 was most similar to RdRp of victorivirus in the family Totiviridae, including lebolus microspores totivirus 1 (TmTV1; identity, 45.42%; query cover, 24.10%; E-value, 0.0), Umbelopsis ramanniana virus 2 (UmRV2; identity, 64.24%; query cover, 58.11%; E-value, 0.0).
query cover, 76%; E-value, 0.0. *Tolypocladium ophioglossoides* totivirus 1 (ToTV1; identity, 41.68%; query cover, 76%; E-value, 0.0; *Ustilaginoidea virens* RNA virus 5 (UvRV5; identity, 41.03%; query cover, 76%; E-value, 7e-175, *Sclerotinia nivalis* victorivirus 1 (SnVV1; identity, 37.95%; query cover, 76%; E-value, 6e-158, *Penicillium aurantiogriseum* totivirus 1 (PaTV1; identity, 38.28%; query cover, 76%; e-value, 5e-157). The conservative structure database (CDD) was searched, and multiple aa comparisons were carried out. The results showed that there were 8 (I to VIII) conserved domains of the monotypic Viridae family (Figure 15).

The full-length dsRNA1 was 2901 nts, and the GC content was 59.6%, containing an ORF encoding a 689 aa protein of coat protein (CP) with a molecular weight of 73.5 kDa. The 5’ and 3’ untranslated regions (UTRs) of dsRNA1 were 540 nt and 291 nt, respectively. BLASTp alignment indicated that the ORF encoded by dsRNA2 was most similar to CP in the family Totiviridae, including *Thelebolus microsporus* totivirus 1 (TmTV1, *Tolypocladium ophioglossoides* totivirus 1 (ToTV1, *Fusarium asiaticum* victorivirus 1 (FaVV1, *Sclerotinia nivalis* victorivirus 1 (SnVV1, *Sphaeropsis sapinea* RNA virus 1 (SsRV1.

A phylogenetic tree based on PvVV1L1 encoding RdRp and CP aa was constructed by the neighbor-joining algorithm using MEGA7, with 1000 bootstrap replications (Figures 16, 17). The results showed that PvVV1L1 and victorivirus of the family Totiviridae were clustered into the same group. Combined
with the results of multiple aa alignment of RdRp, PvVVL1 was found to belong to the victoriviruses based on sequence similarity and phylogenetic analysis.

4 Discussion

The presence of mycoviruses was detected in all 58 P. vexans isolates studied, indicating abundant mycovirus resources. This study provides a potential basis for the biological control of P. vexans and increases understanding of the diversity of mycoviruses in P. vexans. In this study, we characterized five novel mycoviruses (PvFV1, PvOLV1, PvEV2, PvPV1 and PvVVL1) from P. vexans strains and identified diverse mycoviruses using high-throughput transcriptome sequencing. The mycoviruses discovered in our analysis were nearly full length. These detected mycoviruses might belong to the families Botourmiaviridae, Fusariviridae, Endornaviridae, Partitiviridae.
and Totiviridae. We conducted RT-PCR, genomic PCR and Sanger sequencing analyses using specific primers based on the obtained fragments to confirm the origins of our viral sequences. Positive amplicons were produced from only RNAs extracted from the _P. vexans_ strain by RT-PCR but not by genomic PCR, confirming that the viral sequences represented nonintegrated RNA virus sequences. To the best of our knowledge, this is the first report of a comprehensive analysis of viral diversity in _P. vexans_ strains. Thus, our results represent a step forward in exploring _P. vexans_ mycoviruses and provide insight into screening the potential of _P. vexans_ controls and understanding the mechanism of _P. vexans_ and mycoviruses coexisting with each other. To our knowledge, mycoviruses reported in _P. vexans_ include only Phomopsis vexans RNA virus 1 (PvRV1) (Zhang et al., 2015) and Phomopsis vexans partitivirus 1 (PvPV1) (Zhang et al.). There have been no reports of hypovirulence caused by mycoviruses in the pathogenic fungus _P. vexans_. As long as different fungal taxa and strains are screened for the purpose of discovering novel mycoviruses, our insight into the diversity of mycoviruses will by all means largely increase. In this context, this study provides a rich resource of mycoviruses, which may contain a large number of mycoviruses with biocontrol potential. In follow-up research, we will try to obtain virus-free strains using other methods, such as protoplast regeneration technology, to determine the impact of the four Botourmiaviruses investigated herein on host pathogenicity and the interactions among them.

In addition, as an increasing number of atypical mycoviruses were discovered, they provided a reference for the evolution of mycoviruses. There are two hypotheses regarding the origin of mycoviruses (Pearson et al., 2010): the first is that mycoviruses evolve with host fungi; the second is that mycoviruses may be isolated from the natural host plants of pathogenic fungi. Regarding PvPV1, the available information is limited, and it shows the highest homology to diatom colony-associated dsRNA virus14 deriving from marine organisms. PvPV1 is the first deltapartitivirus reported in fungi. Although PvPV1-specific primers were used to detect the virus in eggplant leaves collected in other fields, it is unfortunate that PvPV1 infection of eggplant plants was not detected. It is worth noting that the Partitiviridae genome consists of two separate dsRNA segments, which encode CP and RdRp, and the virion protein has a molecular weight of 37-77 kDa. The genome of Totiviridae is usually a single linear molecule with a size of 4.6-7.0 kbp encompassing two open reading frames. The 5' terminal ORF encodes the virus capsid protein, the 3' terminal ORF encodes a dependent RNA polymerase, and the virion protein has a molecular weight of 70-100 kDa. Expression of the downstream open reading frame is the basis of the Totiviridae classification, and the Totiviridae family has a specific translation mechanism. Victorivirus has a ribosomal insertion site (IRES) in the 5' noncoding region for CP translation, while the downstream RdRp translates two independent viral proteins through a cis-trans translation mechanism that enables a stop or restart
mediated by “AUGA” or “UAUGA”. Totivirusidae forms a fusion protein, and viral translation is mediated by the frameshift structure of “+ 1 frameshift”. Although atypical victorivirus genomic structures have been reported previously, such as a 98-nt interval between two ORFs in PrRV1 (Zhang et al., 2015) and a 2-nt interval between two ORFs of Magnaporthe oryzae virus 1 in Magnaporthe grisea (Yokoi et al., 2007). It is obvious that there are great differences in the viral genome structure and translation mechanisms between Totivirusidae and Partitiviridae. At present, viruses are mainly classified according to their RdRp aa sequences. Through the phylogenetic analysis of PrVV1, it was found that it was clustered with victorviruses in the family Totivirusidae. Multiple aa alignment analysis showed that the closely related viruses (TmTV1, UmRV2, ToTV1, UvRV5, SnVV1, PaTV1) had eight conserved motifs from the family Totivirusidae. The structure of the PrVV1 genome is composed of two separate dsRNA segments, which is similar to Partitivirusidae, indicating a certain evolutionary relationship between the two families of viruses. Ghabrial believes that partitiviruses and totiviruses may share a common ancestor (Ghabrial, 1998). The RdRp of NrV1 found in N. radicola has high homology with Partitiviridae (Ahn and Lee, 2001), but the genomic structure of the virus is a nondouble segment, and the genome size (6 kbp) is larger than that of Partitivirusidae, which seems to confirm the view of Ghabrial. At first, the virus may have only RdRp to complete replication in the host, transmission of the virus depends on the transfer of mRNA, and the transmission efficiency of the virus is not high. The virus gradually sequesters the host protein, which is used to encompass and spread RdRp in the form of a virion, forming a multisegmental virus. In recent years, viruses with atypical genomic structures have been reported in fungi. For example, Chiba reported that Narnavirus virus Aspergillus lentulus narnavirus 1 (AleNV1) with a two-segment genome was identified for the first time from Aspergillus (A. fumigatus) using segmented and primer-ligated double stranded RNA sequencing (FLDS) techniques (Chiba et al., 2021). The RdRp of RNA viruses usually encodes an open reading frame, and some viruses of Narnaviridae are often found to lack catalytic domains C and D of RdRp. Chiba found multisegmental Narnavirus viruses, Aspergillus fumigatus narnavirus 2 (AfuNV2) and Magnaporthe oryzae narnavirus 1 (MoNV1), in two different host fungi, Aspergillus and Pyricularia. The missing domains C and D were located at the N-terminus of another RNA segment of the virus. Using SWISS-MODEL software to model the homology of two ORFs, it was found that a functional enzyme could be formed between the two segments, which indicated that the two RNA segments might come from the common segment. In addition, Lin (Lin et al., 2020) and Suvi (Suvi et al., 2020) also described multisegment mycoviruses with mitotic RdRp sequences, indicating that viruses with atypical genomic structures are widely distributed in different host fungi and different countries. The study of multisegment viruses will provide materials for the evolutionary origin of the viruses. Zhang showed that a dsRNA fragment with a size of approximately 3 kbp and PrRV1 always existed simultaneously and maintained high genetic stability in virus elimination and culture, suggesting that the two dsRNA chains might be related or interact with each other (Zhang et al., 2015). This phenomenon was also observed in 58 tested strains, which confirmed that this phenomenon was not accidental, and it was found in most P. vexans strains. Although the electrophoresis results showed that dsRNA of 3 kbp in size and dsRNA of 5 kbp in size did not appear simultaneously, no single dsRNA of 3 kbp in size was observed. According to the electrophoresis results, two dsRNA segments were observed in some strains at the same position, while only one electrophoresis band was observed in some strains, which might be related to the virus type or electrophoresis environment. Therefore, it is speculated that the dsRNA segment at the 3 kbp position might be the defective RNA or satellite RNA segment of a virus. In the long-term experimental process, it was found that the mycoviruses in P. vexans could be stably transmitted to progeny through hyphae and spores with high genetic stability. No virus-free wild-type strains were found in the available studies on mycoviruses that infect P. vexans, although only 58 strains were isolated and screened, and the probability of carrying the mycoviruses was 100%. These results indicated that the mycoviruses were closely related to the species evolution of M. aubergine, and virus transmission occurred frequently among different strains. It was found that eggplant Browning streak was highly genetic conserved (Rhardwaj et al., 2014) and underwent long-distance transmission via seeds. Mycoviruses can be transmitted horizontally in homologous strains by mycelial fusion. Some specific mycoviruses can reduce heterologous incompatibility responses and facilitate virus transmission (Wu et al., 2017). There are microbes in nature that can facilitate viral transfer, and CMV has confirmed the existence of transboundary transmission (Andika et al., 2017). Therefore, it is speculated that the mycoviruses carried by the laboratory isolates of P. vexans may have a common ancestor. By infecting local P. vexans, the virus can be transmitted over a long distance along with the seed circulation of P. vexans. After the onset of disease, the virus is transferred to the local population through intermycelial fusion. However, not all viruses can be transmitted by horizontal transfer. Different hosts may have different antiviral mechanisms, and there is also antagonism between viruses. Viruses carried by the host itself will reject the invasion of alien viruses, so viruses need to evolve to complete invasion. Concurrently, there are also types of mutualism between different viruses, Zhang (Zhang et al., 2016) found that through infectious cloning and viral particle transfection, a virus-free ssRNA virus, Yado-Karivirus1 (YkV1), changes its replication pattern by hijacking the viral capsid protein Yado-Nushivirus1 (YnV1). In addition, YkV1 enhances the accumulation of YnV1 in the host. This virus changes its replication mode to suit its own survival to achieve long-term development. The transmissibility of different viruses and the possible different interactions between
them also partly explain the diversity of viral RNA bands in different strains of P. vexans.

Data availability statement

The datasets presented in this article 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

HZ, FL, QZ and JZ conceived and designed the experiments. CZ and XZ performed the experiments. FL, HZ and RZ performed the literature search and analyzed the data. FX drafted and revised the manuscript. CZ, FX and XZ contributed equally to writing the manuscript. 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

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