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The Virome of two *Fusarium* spp. Collections and its Potential for Controlling Fusarium Disease

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Abstract

We know little about viruses compared to other lifeforms. Recently, metagenomics has helped us to gain a better understanding of the virosphere. This research aims to discover the virome diversity and composition in *Fusarium poae* and *Fusarium proliferatum* collections, characterize the mycovirus that may have an effect on host pathogenicity to provide potential materials for the biological control of *Fusarium* spp. pathogens.

Next-Generation Sequencing (NGS) analysis of 30 *F. poae* isolates revealed an extreme diversity of mycoviruses. Bioinformatic analysis shows that contigs associated with viral genome belong to the families: *Hypoviridae*, *Mitoviridae*, *Partitiviridae*, *Polymycoviridae*, proposed *Alternaviridae*, proposed *Fusagraviridae*, proposed *Fusariviridae*, proposed *Yadokariviridae*, and *Totiviridae*. The complete genomes of 12 viruses were obtained by assembling contigs and overlapping cloning sequences.

Moreover, all the *F. poae* isolates analyzed are multi-infected. Fusarium poae partitivirus 1 appears in all the 30 strains, followed by Fusarium poae fusagravirus 1 (22), Fusarium poae mitovirus 2 (18), Fusarium poae partitivirus 3 (16), and Fusarium poae mitovirus 2 and 3 (11).

Using the same approach, the virome of *F. proliferatum* collections resulted in lower diversity and abundance. The identified mycoviruses belong to the family *Mitoviridae* and *Mymonaviridae*. Interestingly, most *F. proliferatum isolates* are not multi-infected. The complete genomes of four viruses were obtained by assembling contigs and overlapping cloning sequences.

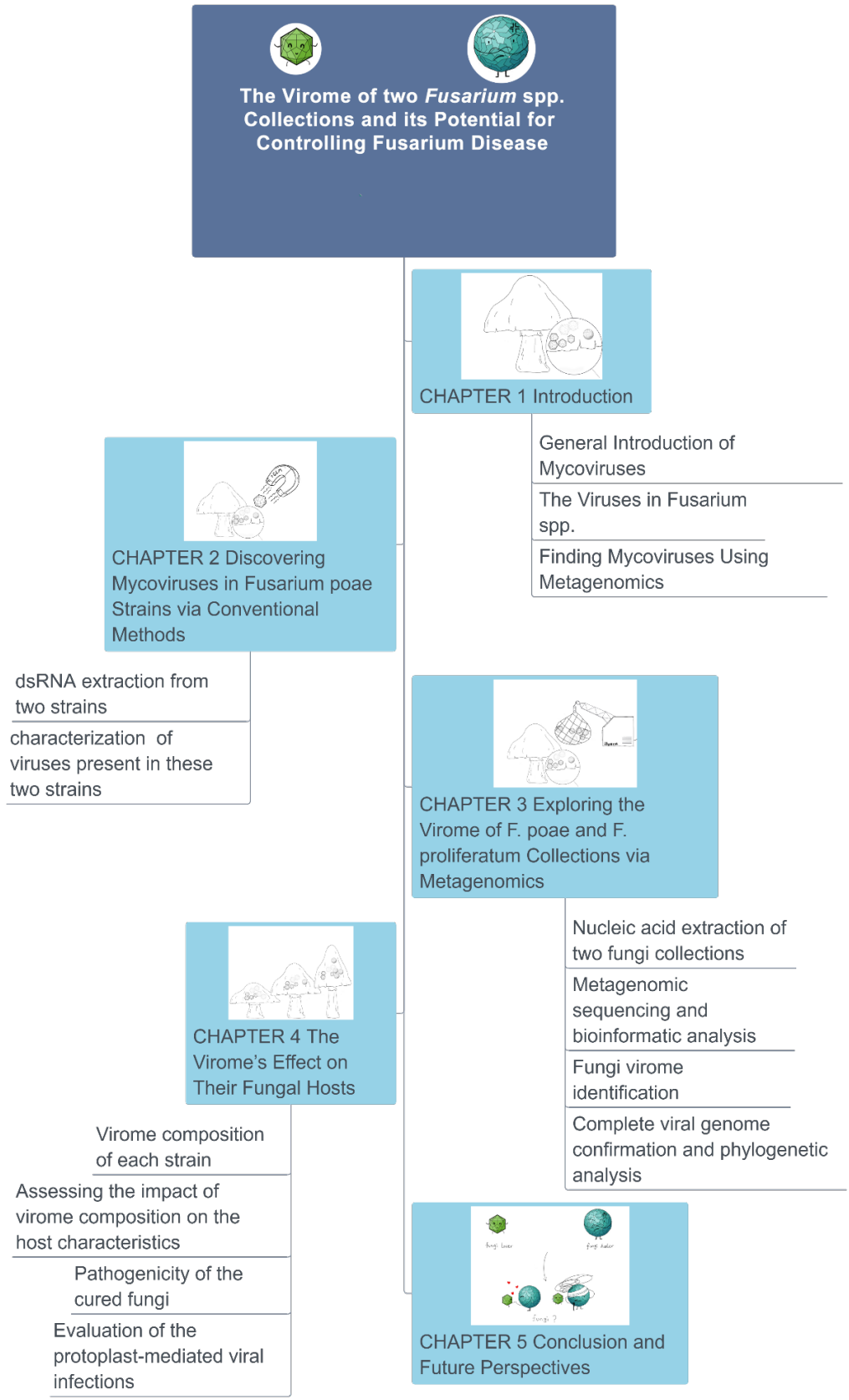
By multiple liner regression of the virome composition and growth rate of 30 *F. poae*, Fusarium poae mitovirus 3 is significantly correlated with the growth rate among *F. poae* collection. Furthermore, the principal component analysis of the virome composition from 30 *F. poae* showed that the presence of Fusarium poae mitovirus 3 and other two viruses could increase the *F. poae* growth rate.

The curing experiment and pathogenicity test in Petri indicated that Fusarium poae hypovirus 1 might be associated with the host hypovirulence phenotype, while Fusarium poae fusagravirus 1 and Fusarium poae partitivirus 3 may have some beneficial effect on host pathogenicity.

From the results obtained so far, it is interesting to further explore the differences between the two fungal collections and the virus-virus interaction in a single fungus.

Key words Mycovirus, virome, *Fusarium poae*, *Fusarium proliferatum*, NGS, biological control

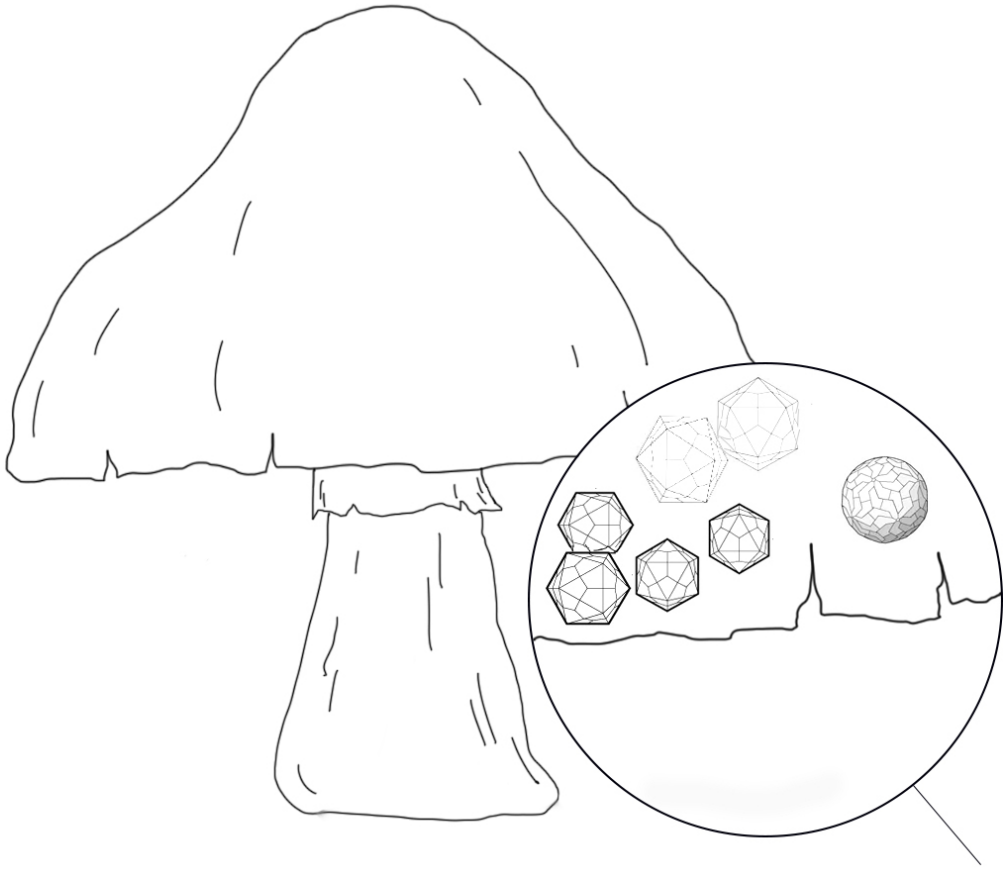
Thesis Conceptual Flow



Index

CHAPTER 1

INTRODUCTION



General Introduction of Mycoviruses

1. Mycovirus Origin

It is always fascinating to think about the origin of life, and ubiquitous viruses are hard to be ignored. Recently accumulated virus data via new technologies have deeply reformed our understanding of the virosphere and other life forms. For example, The last universal cellular ancestor (LUCA) was suggested to have a remarkably complex virome, and the virome, in turn, proved the genomic complexity of LUCA itself (Krupovic, Dolja, and Koonin 2020). Although new technologies have accelerated the process of our exploration, the origin remains hypotheses.

Mycoviruses are widespread in all major taxa of fungi, and as the other viruses, they are believed to have originated in ancient times (Ghabrial 1998; Holmes 2011). Its origin hypotheses are mainly divided into two: "ancient coevolution hypothesis" (Wang *et al.,* 2018) and "plant virus hypothesis" (Abbas 2016). Because there are diverse types of mycoviruses (Fig. 1.1), they may have different origins, or even plant viruses could partially derive from a plant-associated fungus, like ourmiaviruses (Rastgou *et al.,* 2009; Nerva *et al.,* 2017).

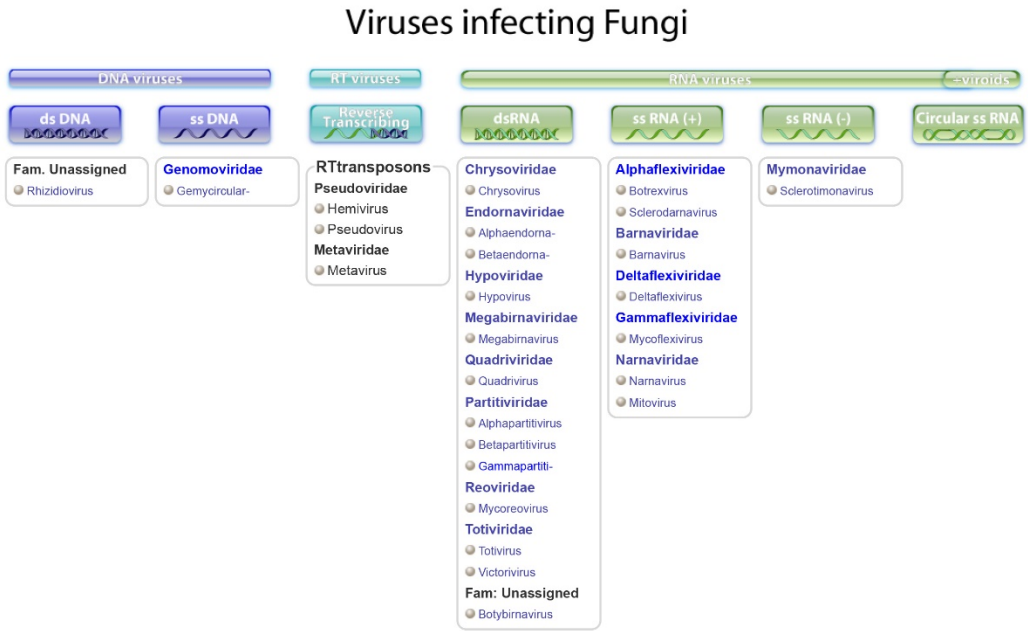


Figure 1.1 Relevant families/genera that host fungi under the Baltimore index. (https://viralzone.expasy.org/8337)

2. Taxonomy and Evolution of Mycoviruses

The Baltimore classes (BCs), based on virus genome, have classified viruses into seven

classes (Baltimore 1971; Koonin *et al.*, 2020):

Ⅰ Double-stranded DNA (dsDNA) viruses.

Ⅱ Single-stranded DNA (ssDNA) viruses.

Ⅲ Double-stranded RNA (dsRNA) viruses.

Ⅳ Positive-sense single-stranded RNA ((+)ssRNA) viruses.

Ⅴ Negative-sense single-stranded RNA ((-)ssRNA) viruses.

Ⅵ RNA reverse-transcribing (RT-RNA) viruses.

Ⅶ DNA reverse-transcribing (RT-DNA) viruses.

Fungal viruses are distributed in almost all Baltimore classes (Fig. 1.1), the replicate and expression strategy among each class is diverse, lacking universal conserved genes make it hard to analyze the evolution of all mycoviruses together. However, it is possible to use RNA-dependent RNA polymerase (RdRp) or reverse transcriptase (RT) sequences to study the phylogeny (Fig. 1.2) and evolution among RNA viruses, the dominant genome type of mycoviruses (Wolf *et al.*, 2018; Koonin *et al.*, 2020).

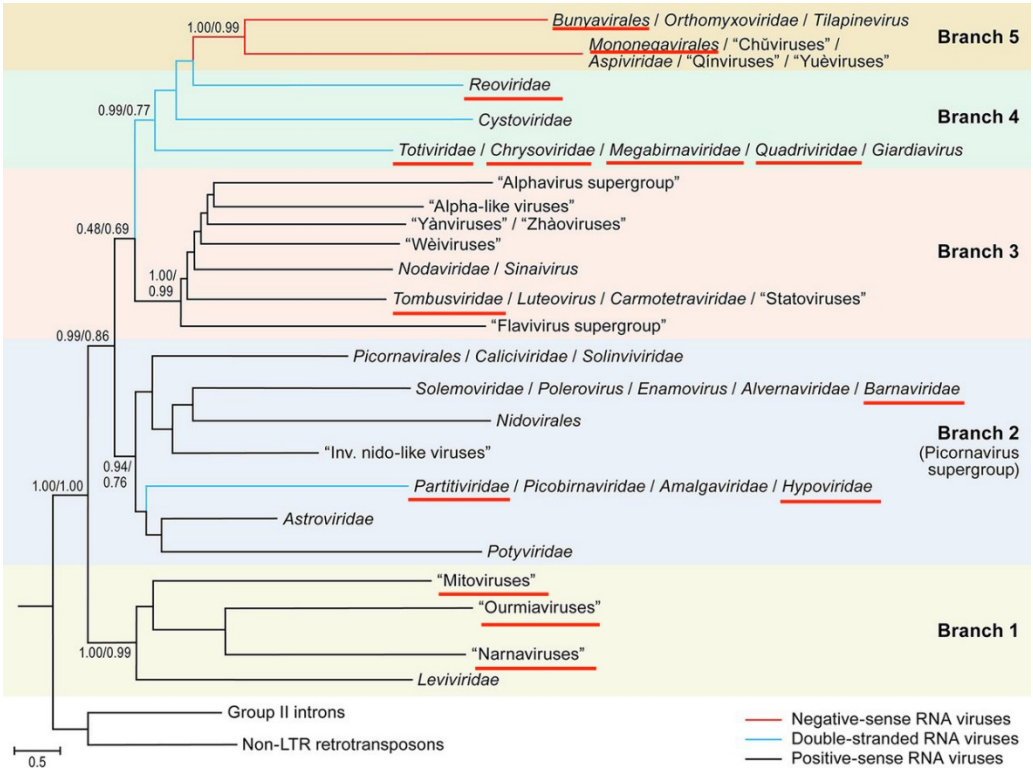


Figure 1.2 The phylogenetic analysis of RNA viruses using RdRp sequences (Wolf *et al.*, 2018). Taxonomy groups that previously reported contained mycovirus species are marked with a red underline.

The newly organized megataxonomy showed RNA viruses containing RdRp, or RT are

grouped in the *Riboviria* realm, RNA viruses in *Orthornavirae* kingdom can be grouped into five branches, and the *Pararnavirae* kingdom has one branch as far as we discovered. Families that previously reported contained mycovirus species are marked with red underline in Figure 1.3 (Marzano *et al.,* 2016; Koonin *et al.,* 2020; Abbas 2016).

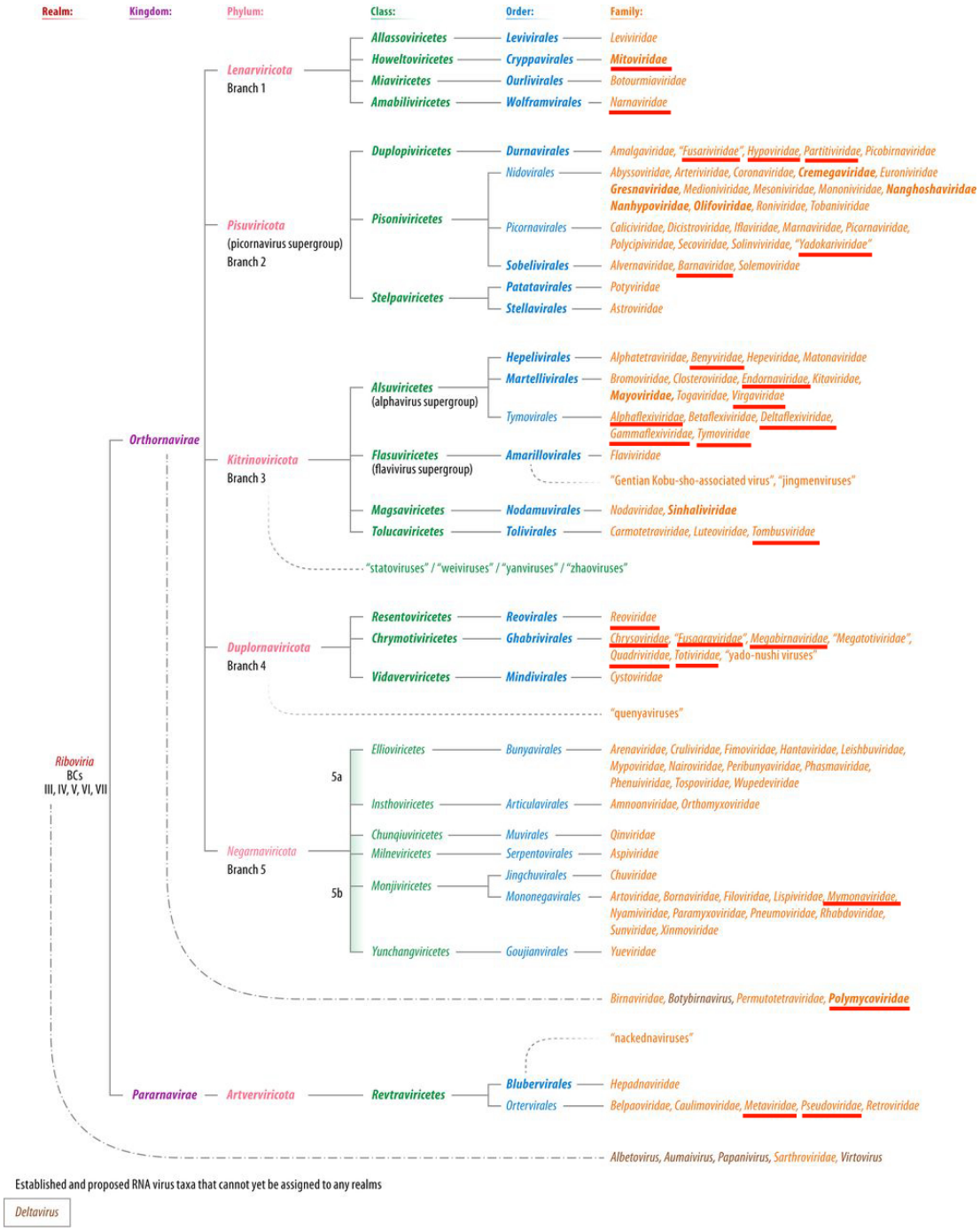


Figure 1.3 the megataxonomy of RNA viruses from realm to family rank (Koonin *et al.,* 2020). Families that previously reported contained mycovirus species are marked with a red underline

As we can see from figure 1.2 and 1.3, the reported mycoviruses are distributed in all the branches, indicating a diverse origin and evolutionary mode. Moreover, their dominant mode

of virus diversification is codivergence, which is evident in the two large mycovirus families, *Partitiviridae* and *Totiviridae* (Göker *et al.,* 2011).

3. Fungi-viruses Interactions

The detailed studies let us know more about the diverse interactions among virus and fungal hosts. Most infections are asymptomatic and complex, depending on the host genotype and environmental conditions (Hillman, Annisa, and Suzuki 2018).

There are few examples in which the virus infections caused a beneficial or harmful effect on the fungal hosts.

In the case of *Curvularia protuberia* and its Curvularia thermal tolerance virus, the virus can confer heat tolerance to the fungal host and even the plant (Lathem *et al.,* 2007); the virulence, sporulation, laccase activity, and pigmentation levels of fungal *Nectria radicicola* can be up-regulated by its dsRNA mycovirus (Ahn and Lee 2001).

What attracts our attention most is the mycoviruses that could confer hypovirulence to the fungal hosts. The most famous case is *Cryphonectria parasitica* and Cryphonectria parasitica hypovirus 1 (CHV1) (Dawe and Nuss 2013).

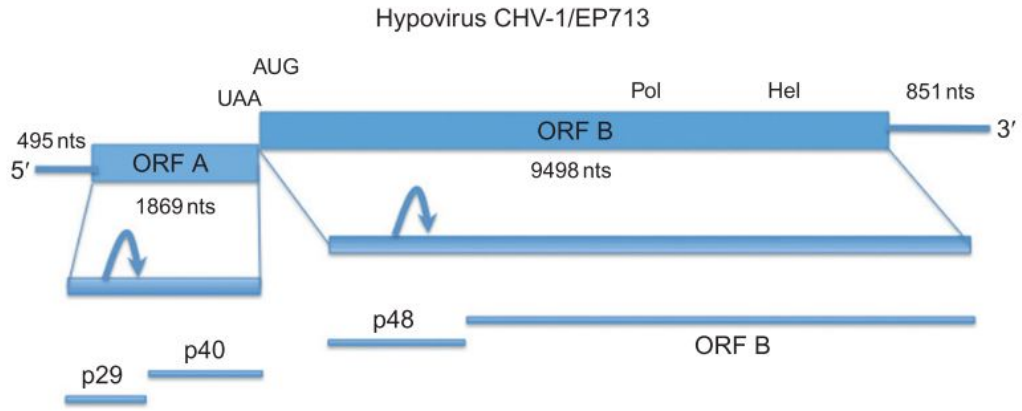


Figure 1.4 Genome organization and expression strategy of CHV-1/EP713 (Nuss 2011)

The development of the hypovirus reverse genetics system makes it possible to study the virus-host interaction in the lab. Figure 1.4 shows the CHV1 genome, its coding RNA have 12,712 nucleotides excluding a poly-A tail and contains two major coding domain, ORF A and ORF B (R Shapira *et al.,* 1991). The junction sequence between ORF A and B is "5'-UAAUG-3'" in which UAA works as the terminal codon and AUG is working as the initial codon (Guo *et al.,* 2009). ORF A encodes p29, which has a papain-like protease activity and could mediate the polyprotein p69 (ORF A) and a large polyprotein (ORF B) to release p29, p40, and p48 (Roni

Shapira, Choi, and Nuss 1991).

Most fungal hosts use programmed cell death (PCD) among vegetative incompatible strains and RNA silencing as weapons to defense against virus infection. In turn, a negative correlation between PCD and the CHV1 infection was reported (Biella *et al.,* 2002); and a variety of viral suppressors (VSR) were found to suppress the fungal RNA silencing pathways (Wu, Wang, and Ding 2010), p29 in CHV1 is a possible VSR that could enhance viral RNA accumulation and suppressed RNA silencing in *C. parasitica* (Sun, Nuss, and Suzuki 2006; Segers *et al.,* 2006).

4. Mycoviruses in Biological Control

Biological control of mycoviruses depends on their ability to confer hypovirulence to phytopathogenic fungi. The first case is *C. parasitica* and CHV1 that mentioned above, the hypovirulent strains were applied to control the outbreak of chestnut cryphonecrosis in the United States and Europe (Anagnostakis 1982).

Apart from this, a DNA virus (SsHADV-1) in *Sclerotinia sclerotiorum* (X. Yu *et al.,* 2013), Rosellinia necatrix megabirnavirus 1 (RnMBV1) in *Rosellinia necatrix* (Kondo, Kanematsu, and Suzuki 2013), and Botrytis cinerea RNA virus 1 (BcRV1) in *Botrytis cinerea* (L. Yu *et al.,* 2015) were reported that can induce hypovirulence in the host.

5. Conclusion

Mycoviruses that could induce hypovirulence are potential biological control agents. By screening the fungal virome, we will find more suitable mycoviruses.

It is also worth study the viruses that do not have a significant effect on the host. They may become a suitable candidate as a gene vector and a good material for fundamental mechanism studies.

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Finding Mycoviruses Using Metagenomics

1. Metagenomic Studies Revealed a Diverse Virus World

The development of NGS and other new technologies changed the traditional virology research model, and avoided many time-consuming processes to detect new viruses, such as complex isolation and culturing procedures (Zhang, Shi, and Holmes 2018).

The "virome" in fungi refers to the whole genome sequences of all viruses present in one fungal strain. Metagenomics is to study the genetic materials from the environment. Viral metagenomic studies involve the large-scale RNA sequencing of the virome from single tissue or mixed samples.

Viral metagenomics has revealed a large number of viruses, expanded the diversity of existing viral families, and discovered some new lineages (Zhang, Shi, and Holmes 2018; Shi *et al.,* 2016; Sutela *et al.,* 2020; Charon, Murray, and Holmes 2021). Figure 1.5 is an RNA virus's phylogenic diversity tree. It shows different ways to explore the virosphere using virus culture, consensus PCR, and metagenomics (Zhang, Shi, and Holmes 2018).

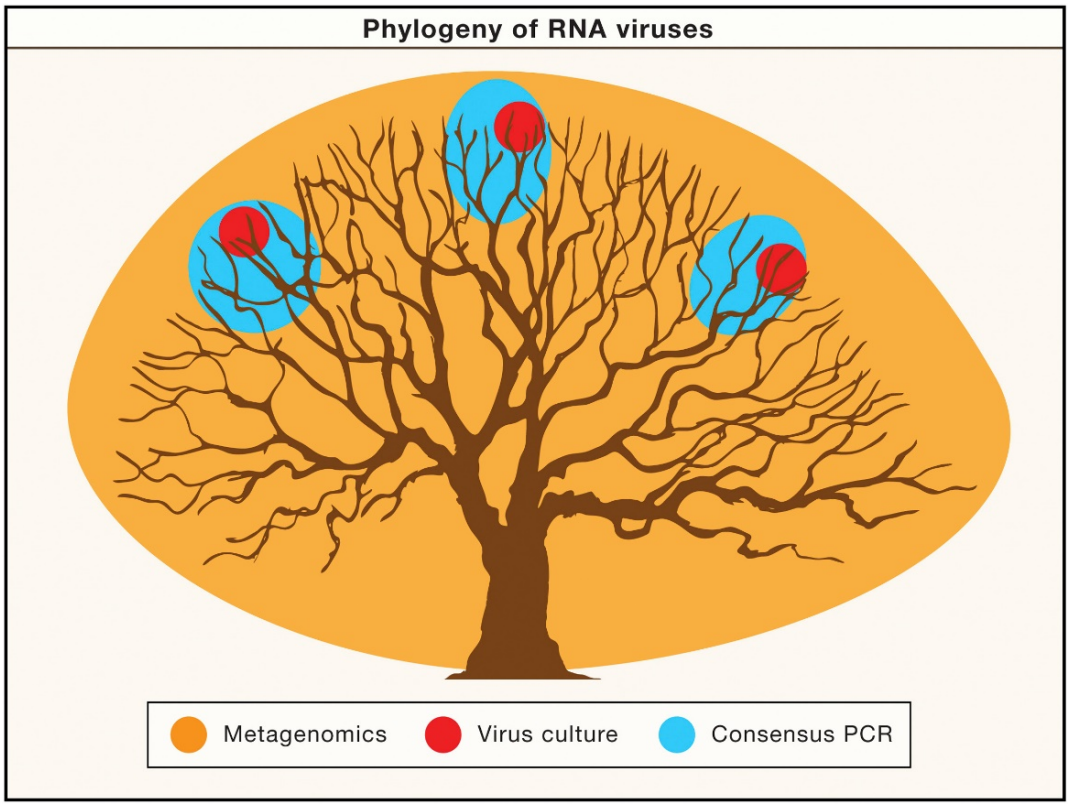

Figure 1.5 Different ways to explore the virosphere (Zhang, Shi, and Holmes 2018).

The booming discovery of new viruses enrich the phylogenetic diversity and indicate new

viral genome structures and possible evolutionary processes (Fig. 1.6). With enough virus data obtained, we can see that lateral gene transfer, gene loss, recombination, duplication, and other genome rearrangement processes frequently happen among RNA viruses, and segmentation is a flexible process that is not strong enough to be a taxa-defining trait *(Shi et al., 2016; Ladner et al.,* 2016).

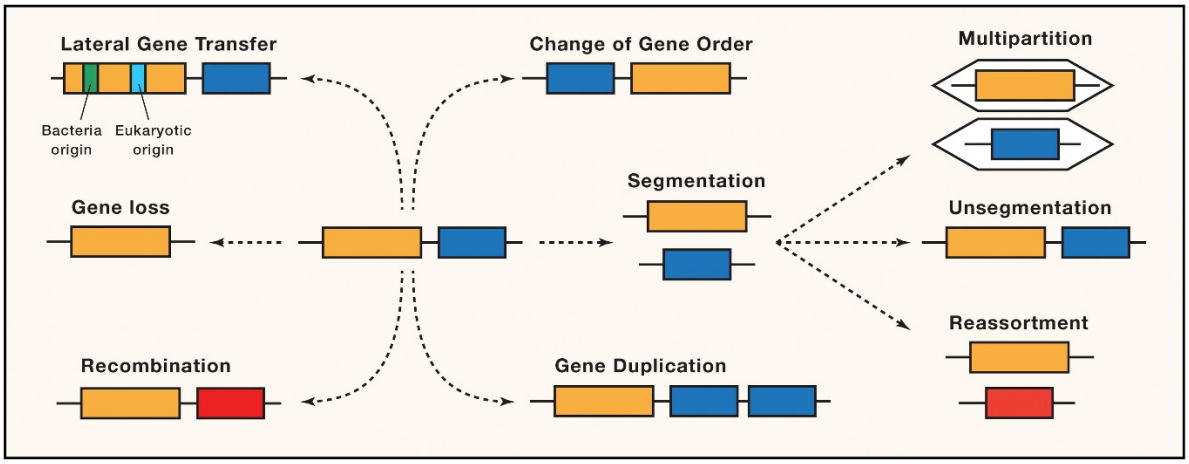

Figure 1.6 Genome evolution mechanisms of RNA viruses (Zhang, Shi, and Holmes 2018).

2. Bioinformatics Analysis and Tools

The procedure of metagenomic studies, including sample collection, library preparation, sequencing, data quality control, and data analysis, is presented in figure 1.7, the flow chart of general data analysis (Zhang *et al.,* 2021).

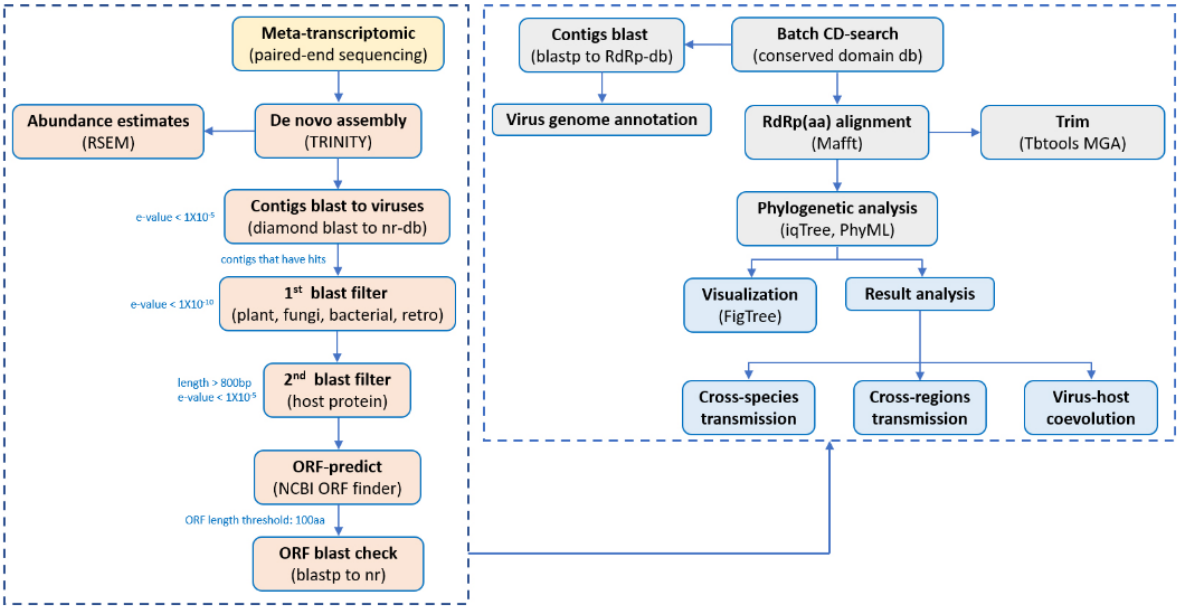

Figure 1.7 Virus analysis and identification flow chart (Zhang *et al.,* 2021).

First is sample collection and nucleic acid extraction. For viral metagenomic studies, we

can (1) extract the total DNA or RNA directly from samples and remove the sequences of the host or other organisms during the data quality control process. This method is suitable for digging the virome of the samples (Sieradzki *et al.,* 2019), but we may lose some low abundance viruses during the data processing. (2) Another way is to enrich viral particles first and then extract the nucleic acid from the viral materials. This method may help to reduce the background noise sequences from the host and environment in the raw data and amplifies the viral signal. It is suitable for viral sequences identification (Džunková *et al.,* 2019) but also generates biases associated with the used viral particle enrichment method. Extracting double-strand RNA (dsRNA) to prepare the library can amplify the viral signal, similar to the method (2), but many viruses do not accumulate dsRNA.

After sequencing, raw reads are generated, and several steps of the quality control process should be conducted to get the clean reads; then, taxonomic profiling and functional profiling are made based on the clean reads or assembled contigs. Many tools are made for this, such as kneadData, which includes Trimmomatic and Bowtie2 to trim and filter the raw data (McIver *et al.,* 2018); assembler metaSPAdes (Prjibelski *et al.,* 2020; Nurk *et al.,* 2017); and VirSorter2 (Guo *et al.,* 2021), VirFinder (Ren *et al.,* 2017) , which are suitable for identifying viral signals.

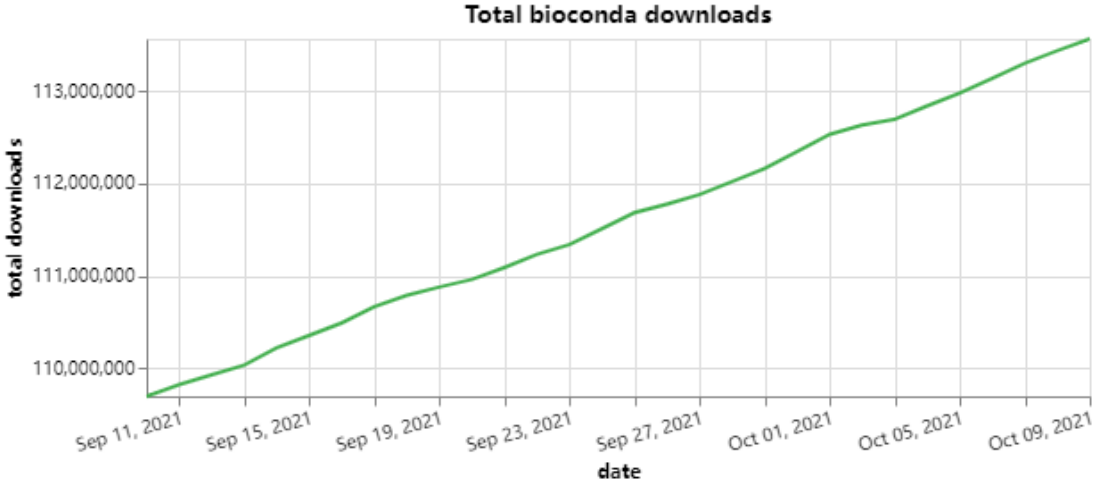

Figure 1.8 The increasing downloads of bioconda (https://bioconda.github.io/)

Bioinformatic tools are written in different programming languages and require different installation methods, making them complex to use and often report errors. After introducing all the valuable tools, a software distribution, Bioconda, must be mentioned (Dale *et al.,* 2018). Conda has overcome these challenges and becoming popular (Fig. 1.8). It can create separate environments for the packages, the tools mentioned above are all included in the Bioconda

channel.

3. Explore Fungi Virome via Metagenomics

Metagenomics shed new light on mycoviruses, including the virome of widely distributed phytopathogens such as *Colletotrichum truncatum*, *Cryphonectria parasitica*, *Fusarium graminearum*, *Macrophomina phaseolina*, *Diaporthe longicolla*, *Rhizoctonia solani*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum*, as well as the virome of endomycorrhizal fungi, were detected in previous researches (Marzano *et al.,* 2016; Ruiz-Padilla *et al.,* 2021; Chu *et al.,* 2002; Forgia *et al.,* 2021; Sutela *et al.,* 2020).

By metagenomic analysis of 275 fungal isolates from *C. truncatum*, *M. phaseolina*, *D. longicolla*, *R. solani*, and *S. sclerotiorum*, 66 new mycoviruses were identified, they showed an affinity with 15 distinct lineages: *Barnaviridae*, *Benyviridae*, *Chrysoviridae*, *Endornaviridae*, *Fusariviridae*, *Hypoviridae*, *Mononegavirales*, *Narnaviridae*, *Ophioviridae*, *Ourmiavirus*, *Partitiviridae*, *Tombusviridae*, *Totiviridae*, *Tymoviridae*, and *Virgaviridae*, and it is the first time that mycoviruses were found in these three families: *Benyviridae*, *Ophioviridae*, and *Virgaviridae* (Marzano *et al.,* 2016).

Another large-scale experiment was conducted on 248 *B. cinerea* isolates. The identified mycoviruses showed remarkable diversity, dsRNA, ssRNA+, ssRNA-, and ssDNA genome types were found in the virome (Ruiz-Padilla *et al.,* 2021).

In addition to discovering new potential biological control mycoviruses, these metagenomic results expand our view of virus diversity, fungus-virus coevolution, horizontal transfers, etc.

4. Conclusion and Future Perspective

Metagenomic studies indeed expanded our knowledge of the virosphere, and new tools based on different methods are born to analyze high-throughput data.

However, massive sequencing data also proposed new challenges in virus taxonomy and classification. The complex viral genome structures raise more questions about driving evolutionary selective pressures and how they can clarify interacting with the host.

In the future, more protocols that are used to enrich the viral materials for NGS should be explored, and the biases associated with different methods should be minimized (Callanan *et al.,* 2021).

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The Viruses in *Fusarium* spp.

Fusarium spp. is an important phytopathogen widely distributed in soil and water and associated with several mycotoxins (Mielniczuk and Skwaryło-Bednarz 2020). Thanks to the developed technologies, the detected mycoviruses in *Fusarium* spp. have increased these years rapidly.

1. Diverse Mycoviruses in Fusarium spp.

To date, 17 *Fusarium* species were reported that contain mycoviruses, including *Fusarium andiyazi*, *F. asiaticum*, *F. boothii*, *F. circinatum*, *F. coeruleum*, *F. equiseti*, *F. globosum*, *F. graminearum*, *F. incarnatum*, *F. langsethiae*, *F. oxysporum*, *F. poae*, *F. pseudograminearum*, *F. sambucinum*, *F. solani*, *F. verticillioides*, and *F. virguliforme* (P. Li *et al.,* 2019; Jacquat *et al.,* 2020; Mizutani *et al.,* 2021; Mahillon *et al.,* 2021).

Mycoviruses in *Fusarium* spp. have different genome types, dsRNA, ssRNA+, ssRNA-, and ssDNA. Table 1.1 shows the identified *Fusarium*-infecting mycoviruses to date.

Table 1.1 Identified *Fusarium*-infecting mycoviruses to date

Mycovirus	Proposed family	Genome type	Host	Accession (RNA1)	Method	Reference
FaMV1-162	*Mitoviridae*	(+)ssRNA	*F. andiyazi* strain 162	MT506025	Illumina/total RNA of single strain 162	(Jacquat *et al.,* 2020)
FaVV1	*Totiviridae*	dsRNA	*F. asiaticum* strain F16176	MH615042	Illumina/dsRNA of single strain F16176	(W. Li *et al.,* 2019)
FbMV1	*Mitoviridae*	(+)ssRNA	*F. boothii* strain Ep-BL13	LC425112	Illumina/dsRNA of single strain Ep-BL13	(Mizutani *et al.,* 2018)
		(+)ssRNA	*F. boothii* strain Ep-BL14	LC425113	Conventional cloning and sanger sequencing	
		(+)ssRNA	*F. boothii* strain Ep-N28	LC425114	Conventional cloning and sanger sequencing	
FbLFV1	Unassigned	(+)ssRNA	*F. boothii* strain Ep-BL13	LC425115	Illumina/dsRNA of single strain Ep-BL13	
FcMV1	*Mitoviridae*	(+)ssRNA	*F. circinatum* strain FcCa070	KF803546	Conventional cloning and sanger sequencing	(Martínez-Álvarez *et al.,* 2014)
FcoMV1	*Mitoviridae*	(+)ssRNA	*F. coeruleum* MAFF No. 235976	LC006129	Conventional cloning and sanger sequencing	(H Osaki *et al.,* 2015)
FePV1	*Partitiviridae.*	dsRNA	*F. equiseti* strain 020FO1-18	MT659123	Illumina/dsRNA of single strain 020FO1-18	(Mahillon *et al.,* 2021)
FgMV1	*Mitoviridae*	(+)ssRNA	*F. globosum* MAFF No. 237511	LC006128	Conventional cloning and sanger sequencing	(H Osaki *et al.,* 2015)

Table 1.1 Identified *Fusarium*-infecting mycoviruses to date (continued 1)

Mycovirus	Proposed family	Genome type	Host	Accession (RNA1)	Method	Reference
FgV-ch9	*Chrysoviridae*	dsRNA	*F. graminearum* strain China 9	HQ228213	Conventional cloning and sanger sequencing	(Darissa *et al.,* 2011)
FgV2	*Chrysoviridae*	dsRNA	*F. graminearum* strain 98-8-60	HQ343295	Conventional cloning and sanger sequencing	(Chu *et al.,* 2004)
AV1	*Alternaviridae*	dsRNA	*F. graminearum* strain AH11	MG254901	Conventional cloning and sanger sequencing	(He *et al.,* 2018)
FgV3	*Fusagraviridae*	dsRNA	*F. graminearum* strain DK3	NC_013469	Conventional cloning and sanger sequencing	(Yu *et al.,* 2009)
FgV4	Unassigned	dsRNA		NC_013470		
FgV5	Unassigned	dsRNA	*F. graminearum* strain HN1	KX380787	Conventional cloning and sanger sequencing	(Luan Wang *et al.,* 2017)
FgDFV1	*Deltaflexiviridae*	(+)ssRNA	*F. graminearum* strain BJ59	KX015962	Conventional cloning and sanger sequencing	(Chen *et al.,* 2016)
FgHV1	*Hypoviridae*	(+)ssRNA	*F. graminearum* strain HN10	KC330231	Conventional cloning and sanger sequencing	(S. Wang *et al.*,2013)
FgHV2	*Hypoviridae*	(+)ssRNA	*F. graminearum* strain JS16	KP208178	Conventional cloning and sanger sequencing	(P. Li *et al.,* 2015)
FgV1	*Fusariviridae*	(+)ssRNA	*F. graminearum* strain DK21	NC_006937	Conventional cloning and sanger sequencing	(Chu *et al.*,2002)
FgMTV1	*Tymoviridae*	(+)ssRNA	*F. graminearum* strain SX64	KT360947	Conventional cloning and sanger sequencing	(P. Li *et al.,* 2016)
FgNSRV-1	*Mymonaviridae*	(-)ssRNA	*F. graminearum* strain HN1	MF276904	Conventional cloning and sanger sequencing	(Luan Wang *et al.,* 2018)
FgGMTV1	*Genomoviridae*	ssDNA	*F. graminearum* strain HB58	MK430076	Conventional cloning and sanger sequencing	(P. Li *et al.,* 2020)
FiAV1	*Alternaviridae*	dsRNA	*F. incarnatum* strain LY003-07	MH899114	Conventional cloning and sanger sequencing	(Zhang *et al.,* 2019)
FlHV1	*Hypoviridae*	(+)ssRNA	*F. langsethiae* strain AH32	KY120321	Conventional cloning and sanger sequencing	(P. Li *et al.,* 2017)
FodV1	*Chrysoviridae*	dsRNA	*F. oxysporum* f. sp. dianthi strain 116	KP876629	Conventional cloning and sanger sequencing	(Lemus-Minor *et al.,* 2018)
FpV1	*Partitiviridae*	dsRNA	*F. poae strain* A-11	NC_003883	Conventional cloning and sanger sequencing	(Compel *et al.,* 1999)
FpV2	*Fusagraviridae*	dsRNA	*F. poae strain* SX63	KU728180	Conventional cloning and sanger sequencing	(L Wang *et al.,* 2016)
FpV3	*Fusagraviridae*	dsRNA		KU728181		
FpMV1	*Mitoviridae*	(+)ssRNA	*F. poae* MAFF 240374	LC150564	Illumina/dsRNA of strain MAFF 240374	(Hideki Osaki *et al.,* 2016)
FpMV2	*Mitoviridae*	(+)ssRNA		LC150565		
FpMV3	*Mitoviridae*	(+)ssRNA		LC150566		
FpMV4	*Mitoviridae*	(+)ssRNA		LC150567		
FpNV1	*Narnaviridae*	(+)ssRNA		LC150604		
FpNV2	*Narnaviridae*	(+)ssRNA		LC150605		

Table 1.1 Identified *Fusarium*-infecting mycoviruses to date (continued 2)

Mycovirus	Proposed family	Genome type	Host	Accession (RNA1)	Method	Reference
FpV1-240374	*Partitiviridae*	dsRNA	*F. poae* MAFF 240374	LC150606	Illumina/dsRNA of strain MAFF 240374	(Hideki Osaki *et al.,* 2016)
FpPV2	*Partitiviridae*	dsRNA		LC150608		
FpVV1	*Totiviridae*	dsRNA		LC150610		
FpFV1	*Fusariviridae*	(+)ssRNA		LC150611		
FpHV1	*Hypoviridae*	(+)ssRNA		LC150612		
FpAV1	*Alternaviridae*	dsRNA		LC150613		
FpMyV1	*Fusagraviridae*	dsRNA		LC150616		
FpMyV2	*Yadokariviridae*	(+)ssRNA		LC150617		
FpNSV1	*Ophioviridae*	(-)ssRNA		LC150618		
FpNSV2	Unassigned	(-)ssRNA		LC150619		
FpgMBV1	*Megabirnaviridae*	dsRNA	*F. pseudograminearum* strain FC136-2A	MH057692	Conventional cloning and sanger sequencing	(Zhang *et al.,* 2018)
FsamHV1	*Hypoviridae*	(+)ssRNA	*F. sambucinum* strain FA1837		MinION/dsRNA of strain FA1837	(Mizutani *et al.,* 2021)
FsamHV2	*Hypoviridae*	(+)ssRNA	*F. sambucinum* strain FA2242		MinION/dsRNA of strain FA2242	(Mizutani *et al.,* 2021)
FsamVV1	*Totiviridae*	dsRNA				
FsamMV1	*Mitoviridae*	(+)ssRNA				
FsamMV2	*Mitoviridae*	(+)ssRNA				
FsamMV3	*Mitoviridae*	(+)ssRNA				
FsamMV4	*Mitoviridae*	(+)ssRNA				
FsamMV5	*Mitoviridae*	(+)ssRNA				
FsV1	*Partitiviridae*	dsRNA	*F. solani* f. sp. robiniae strain SUF704	NC_003885	Conventional cloning and sanger sequencing	(M. Nogawa *et al.,* 1993)
FsPV2	*Partitiviridae*	dsRNA	*F. solani* f.sp. pisi RNA1	LC006130	Conventional cloning and sanger sequencing	(H Osaki *et al.,* 2015)
FvMV1	*Mitoviridae*	(+)ssRNA	*F. verticillioides* strain Sec505	MT506024	Illumina/total RNA of single strain Sec505	(Jacquat *et al.,* 2020)
FvV1	*Fusagraviridae*	dsRNA	*F. virguliforme*	JN671444	Illumina/total RNA of mix *F. Virguliforme* strains	(Marvelli *et al.,* 2014)
FvV2	*Fusagraviridae*	dsRNA	*F. virguliforme*	JN671443		

2. Viruses' Impact on Hosts

Mycovirus infection is symptomless in most cases, viruses FgV3, FgV4, FgHV1, FsV1, FpV1, FpV2, and FpV3 in table 1.1 were reported not to cause any changes in phenotypic, virulence, or toxin production (Lee *et al.,* 2014; S. Wang *et al.,* 2013; Masahiro Nogawa *et al.,* 1996; L Wang *et al.,* 2016).

Few viruses could act as a potential biocontrol agent. FgHV2 is associated with host hypovirulence phenotypes. It reduced fungal growth rate, conidia production, DON production and delayed the spread of pathogen on spikelet (P. Li *et al.,* 2015). FgV1 could reduce the host virulence and mycotoxin production (Chu *et al.,* 2002). FgV-ch9 and FodV1 viruses in the family *Chrysoviridae* could confer hypovirulence and cause abnormal fungal morphology also (Darissa, Adam, and Schäfer 2012; Lemus-Minor *et al.,* 2018).

3. Interaction Between Viruses and Hosts

Studying the genes regulated by mycovirus infection can help us understand pathogens and the interaction between virus and pathogen (P. Li *et al.,* 2019).

In the case of FgV1 infection (Kwon *et al.,* 2009), seven proteins were up-regulated, and 16 proteins were down-regulated by virus infection. Later, a genome-wide expression profiling of *F. graminearum* infected by FgV1 was carried out. Using a 3′-tiling microarray covering all known *F. graminearum* genes, genes associated with protein synthesis, transcription, and signal transduction were up-regulated. In contrast, genes involved in various metabolic pathways and genes required for transport systems localizing to transmembranes were down-regulated (Cho *et al.,* 2012). The up-regulated genes seem to be related to virus replication, while the down-regulated ones appear related to the host defense and virulence system (Cho *et al.,* 2012).

F. graminearum uses RNA silencing as a counterattack, encoding two dicer proteins: FgDicer1 and FgDicer2; 2 Argonaute proteins: FgAgo1 and FgAgo2; and 5 RdRp proteins: FgRdRp1 to 5 (P. Li *et al.,* 2019). FgDicer2 is related to the small RNA transcription and micro-like RNA generation in fungi and is the primary dicer-like component for defense against artificial infection with viroid (Wei *et al.,* 2019). FgDicer1 and FgAgo2 could mediate the sex-specific RNA silencing pathway (Son *et al.,* 2017). These studies revealed a complex RNA silencing system of *F. graminearum* against viruses.

4. Future Perspective

Fusarium spp. contain large quantities of mycoviruses of different genome types. Thus, it is suitable for studying mycovirus structure, function, evolution, etc. (P. Li *et al.,* 2019).

Furthermore, because the genome of *F. graminearum* strain PH-1 is sequenced and there are many studies about *Fusarium* spp., *F. graminearum*-mycovirus host-virus interaction system has become one of the four best study models for mycovirus-host interactions; the other three are *C. parasitica*–mycovirus, *S. sclerotiorum*–mycovirus, and *R. necatrix*–mycovirus.

Future studies should continue to screen mycoviruses and focus on identifying viral and host factors involved in the interactions and mycotoxin production pathway.

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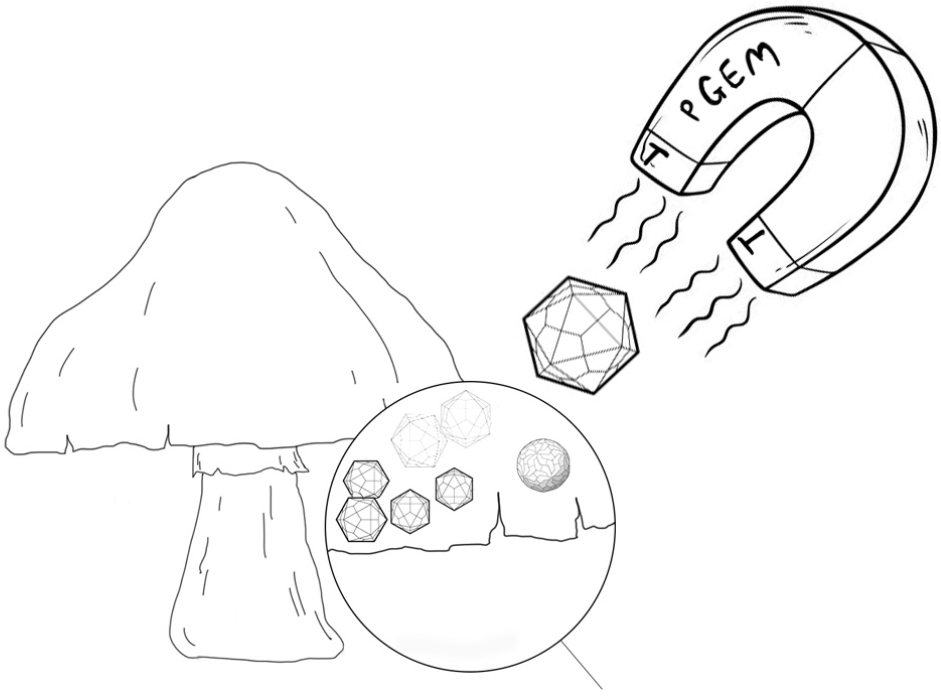
CHAPTER 2

Discovering Mycoviruses in *Fusarium poae* Isolates via Conventional Methods



1. Introduction

Fusarium head blight (FHB), a global wheat disease caused by *Fusarium* spp., mainly *F. graminearum*, could cause severe yield losses and release mycotoxins in the grains. A consortium of pathogens causes FHB, and *F. poae* is expected in the FHB complex. Its participation complicates the development of infection and disease (Tan *et al.,* 2020). Previous results indicated that co-inoculation of *F. graminearum* and *F. poae* could inhibit disease development. Pre-inoculation of *F. poae* could reduce both symptoms and mycotoxin levels compared to the single inoculation of *F. graminearum*. *F. poae* takes advantage of its co-occurrence with *F. graminearum* (Tan *et al.,* 2020).

F. poae seems a "foe" to *F. graminearum*, but does the proverb "The enemy of my enemy is my friend" also work in agriculture biological control? Recent research indicated that with the deepening of our understanding about *F. poae*, its role has changed from "bystander" to "saboteur" to "accomplice." The biocontrol effect of *Streptomyces* spp. against *F. graminearum* could be hampered by the presence of *F. poae* (Tan *et al.,* 2021).

Considering the impact of mycoviruses could help us obtain a comprehensive understanding of infection, interaction, and defense processes among the FHB complex.

Mycovirus are rich in *Fusarium* species and may infect and confer hypovirulence to the fungus to control the disease (Mahillon *et al.,* 2021). Chu et al. reported a dsRNA virus in *F. graminearum* that could reduce its host's growth rate and pathogenicity (Chu *et al.,* 2002). Recently more and more mycoviruses have been characterized, but few of them could induce host hypovirulence (Li *et al.,* 2019).

This chapter aims to characterize more *F. poae* mycoviruses and explore a suitable method for describing the *F. poae* virome.

2. Materials and Methods

2.1 Fungal mono-conidium colonies and culture conditions

F. poae F.456, F.1080 were isolated from wheat in Italy and are stocked in the Mycology lab of The University of Bologna.

In order to obtain mono-conidium colonies, conidium solutions were spread on water agar plates, discarded the redundant water, and incubated the plates at 28℃ for 2 d. Then select the mono-conidium colony from the Petri and transfer it to potato dextrose agar (PDA) medium.

The plates were maintained at 28℃ in the dark for 5 d. Mycelia were cultured in liquid V8 medium at room temperature for 7 d in the dark and collected for nucleic acid extractions. Fungi were stocked on PDA and synthetic low nutrient agar (SNA) in tubes at 4℃ in the dark.

2.2 Fungal dsRNA extraction, purification, and dsRNA bands determination and purification

2.2.1 dsRNA extraction

The protocol for the dsRNA rapid extraction was described before with some modifications more suitable for fungi materials (Okada *et al.,* 2015). Grind up to 1 g of dry material with liquid nitrogen. Immediately add 5 ml of extraction buffer (EB: 0.2 M NaCl; 0.1 M Tris-HCl; 0.004 M EDTA pH 8.0; 2% SDS) and 5 ml of phenol: chloroform: isoamyl alcohol (PCI) solution (V/V) 25:24:1 (or 5 ml chloroform: isoamyl alcohol (CI) solution (V/V) 24:1 and 100 µl β-mercapto-ethanol). Mix by vortex in 15 ml falcon.

Incubate by shaking for 15 min. Centrifuge at 3000 g for 15 min, save the supernatant, add 5 ml of PCI, mix by vortex in 15 ml falcon. Repeat the step using 5 ml of CI. Mix and centrifuge at 3000 g for 15 min, aliquot 1680 µl of supernatant in two tubes (2 ml). Add 320 µl of 100% EtOH and 25 mg of advantec·C cellulose powder 300 mesh. Incubate the samples for 1 h in a tissue rotator.

Centrifuge at max speed for 3-5 min. After 4-5 washing steps, resuspend cellulose with 500-800 µl STE (0.1 M NaCl; 0.05 M tris; 0.001 M EDTA pH 8.0, Adjust pH to 6.8 with concentrate HCl) with 16% EtOH, centrifuge at max speed for 3 min and remove supernatant. If necessary, perform additional steps until PCI is completely removed. Dry the cellulose completely under vacuum and resuspend it using 50 µl of nuclease-free water in each tube (30 µl could be collected). Incubate from 15 min to overnight at room temperature to elute dsRNA, then centrifuge at max speed for 5 min. Multiple elutions can be performed to increase the amount of dsRNA.

2.2.2 Double digestion of dsRNA

Prepare the 10× double digest buffer (10×DD buffer: 0.9 M NaOAC; 0.3 M NaCl; 0.15 M $MgCl_2$; 0.03 M $ZnSO_4$), and dsRNA double digest mixture (10 µl 10× DD buffer, 5 µl RQ1 RNase-Free DNase (Promega M6101, 1 u/µl), 0.5 µl S1 Nuclease (Promega M5761, 85 u/µl), 27 µl sample dsRNA (180 ng/µl), then add H2O to the total volume 100 µl)

The mixture was incubated at 37℃ for 2 h, add 600 µl H2O and 700 µl PCI, vortex, and

centrifuged at 12,000 rpm for 10 min, add 650 µl CI to the supernatant, vortex, and centrifuge at 12,000 rpm for 10 min, take the supernatant and add 600 µl iso-propanol and 60 µl 3 M NaOAC to precipitate at -80℃ for 20 min, then centrifuge at 12,000 g for 30 min. Wash with 500 µl 70% ethanol twice and dry up. Resuspend the double digested dsRNA in 50 µl nuclease-free water.

2.2.3 dsRNA bands determination and purification

Gel electrophoresis was conducted using 1% agarose gel and loaded dsRNA with Orange/blue loading dye 6× (Promega G1881). Dye the gel in ethidium bromide solution (Promega H5041) and cut the dsRNA bands under UV light.

Transfer the gel in a 1.5 ml tube, add 400 µl H_2O, grinding with plastic pestle very well. Transfer the mixture into a column with cotton. Centrifuge at 12,000 rpm for 1 min and save the filtrate. Add 400 µl PCI, vortex, centrifuge at 12,000 rpm for 10 min at 4℃. Take the upper part and add 400 µl iso-propanol, 40 µl 3 M NaOAC to precipitate at -80℃ for 20 min, then centrifuge at 12,000 g for 30 min at 4℃. Wash with 70% ethanol twice, vacuum dry, and resuspend in 10 µl H_2O. The purified dsRNAs are ready for the following reverse transcription (RT) and PCR steps.

2.3 cDNA synthesis and RT-PCR, molecular cloning, and sequencing

2.3.1 RT and PCR

The ImProm-II™ Reverse Transcriptase kit (Promega A3801) and dN6 universal primer were used to conduct the RT reaction.

The pre-mix was prepared (2 µl dsRNA (180 ng/µl), 1 µl dNTPs (10 mM), 2 µl dN6 primer (10 µM)) and incubated in a thermocycler at 90℃ for 5 min, then set it immediately on ice and add the rest RT-mix (4 µl 5×Impron Ⅱ buffer, 1 µl RT enzyme, 1.2 µl MgCl2 (25 mM), 8.8 µl H2O), put the tube of RT-mix in a thermocycler and run the program (25℃ 10 min, 37℃ 45 min, 42℃ 15 min, 80℃ 5 min).

When the program is finished, take out the tube and add 30 µl H_2O to the RT product. Then it is ready for the following PCR.

The following PCR was conducted using GoTaq® reaction buffers (Promega M7911) and dN6-Tag primer, the 12.5µl PCR mixture (2.5 µl 5× GoTag buffer, 0.5 µl MgCl2 (25 mM), 0.25 µl dNTPs (10 mM), 0.5 µl dN6-Tag primer (10 µM), 0.125 µl DNA Polymerase (5 u/µl), 6.125

µl H2O, cDNA 2.5 µl) was put in thermocycler under 50℃ to 55℃ annealing temperature.

2.3.2 Molecular cloning

(1) Ligation

Run the RT-PCR products in 1% agarose gel, cut and purified the bands or smears follow the protocol in 2.2.3 (the ethidium bromide sometimes affect the efficiency of ligation, so it should be avoided to use or reduce the dying time), vacuum dry the sample and resuspend in 3µl H_2O for ligation using pGEM®-T Easy Vector Systems (Promega A1360).

Culture the ligation mix at 4℃ overnight and clean ligation and precipitate the DNA with phenol and NaCl, similar as described in 2.2.3. In the end, resuspend in 3 µl H_2O and take 1.5 µl of the ligation for electroporation.

(2) Electroporation

For electroporation, wash and dry the cuvette before use, take 25 µl of the competent *E. coli* 1022 cells from -80℃ and leave it to melt on ice. Set 2500 volts (Eppendorf® Electroporator 2510) to transfect 25 µl *E. coli* 1022 with 1.5 µl ligation. Do not mix them with pipetting but take all the mixture at once and transfer it into the cuvette. After electroporation, add 500 µl LB medium into the cuvette, mix, and transfer it to a 1.5 ml Eppendorf. Water bath at 37℃ for 20 min without shaking. Spread all on the LB plate containing iPTG, AMP, and xgal (100 ml LB contains 500 µl 0.1 M iPTG; 100 µl 100 mg/ml AMP; and 100 µl 80 mg/ml xgal, N,N-Dimethylformamide solution).

(3) Screening

Incubate the plate at 37℃ overnight and pick the milky white colonies with a sterilized toothpick, draw streak on LB plates containing iPTG, AMP, and xgal. Then dip the toothpick in PCR mixture using GoTag kit and M13 forward and reverse primers (2.5 µl 5×GoTag Buffer, 0.25 µl dNTPs (10 mM), 0.5 µl MgCl2 (25 mM), 0.5/0.5 µl Primers M13F/R (10 µM), 0.125 µl enzyme, 8.125 µl H2O), set annealing temperature at 55℃.

(4) Extract the plasmid and sequence analysis

Check the screening PCR by agarose gel and select the colonies that harbor the target insert. Grow the selected colonies in 5 ml LB containing 0.1 mg/ml AMP in a 50 ml falcon at 37℃ overnight and extract the plasmid using Wizard® Plus SV Minipreps DNA Purification Systems (Promega A1460).

Measure the plasmid concentration and cut the extracted plasmid with the EcoR1 enzyme. Run a gel with the cut plasmid to confirm that the clone is successful. Send at least four independent clones per one purified dsRNA band to Sanger sequencing (Eurofins Genomics) with the M13 forward primer.

Proceed searching the sequence in the NCBI database using BLAST (Altschul et al. 1990). Once the viral sequences are confirmed, design specific primers to complete the viral genome.

2.4 Complete the viral genome with RT-PCR and Rapid Amplification of cDNA Ends (RACE)

To get the complete viral genome, specific primers were used in RT-PCR for the coding region, and RACE was used for the sequence terminal.

2.4.1 RT-PCR with specific primers

Specific primers were designed on the known sequences to fill the gap of the genome. Diluted primers (10mM), ImProm-II™ Reverse Transcriptase kit and GoTag kit were used for the RT-PCR. Then, the amplified nucleotide sequences were sent to sanger sequencing directly after being purified from PCR product using the Wizard® SV Gel and PCR Clean-Up System (Promega A9282).

2.4.2 Confirming the terminal sequence

(1) dsRNA denaturation

Incubate the 90 µl DMSO and 10 µl dsRNA mixture at 65℃ for 20 min, immediately add 10 µl NaOAC and 100 µl iso-propanol to precipitate as described in 2.2.3. vacuum dry the denatured dsRNA.

(2) Poly(A) Tailing

Add a poly(A) tail to the 3’ termini of RNA using Poly(A) Polymerase Tailing Kit (Lucigen Cat. No. PAP5104H), follow the “Alternate protocol” in the manual. Stop the reaction by phenol/chloroform, precipitate and vacuum dry the RNA with poly(A) tail.

(3) RT and PCR

Dissolve the dry polyadenylated RNA directly in the RT mix. After the reaction, add 30 µl of H_2O to the 20 µl RT product (4 µl 5×ImproⅡ buffer, 1 µl dNTPs, 1 µl RT enzyme, 1.2 µl MgCl2, 2 µl Oligo(dT) 21 primer, 10.8 µl H2O). And it is ready for the RT-PCR (2.5 µl 5×GoTag buffer, 0.5 µl MgCl2, 0.25 µl dNTPs, 0.25 µl Oligo(dT) 21 primer, 0.25 µl Specific F or R, 0.125 µl Enzyme, 6.125 µl H2O, 2.5 µl cDNA).

(4) Molecular cloning

The following step is similar as described in 2.3.2. Send selected plasmids to Sanger sequencing, and the terminal of dsRNA viral genome is obtained. For each sequence, send at least four plasmids from different colonies.

(5) Confirming the terminal of poly(A)-tailed viruses

For the virus that have poly(A) at the 3' end, the RLM-RACE was used, after dsRNA denaturation, a 3' RACE adopter was ligated (3 µl 3' RACE adopter, 5.75 µl 10×T4 RNA ligase buffer, 6 µl ATP, 3 µl 0.1% BSA, 31.25 µl 40% PEG-6000, 1 µl T4 RNA ligase enzyme) at 16℃ overnight, then add 350 µl DMSO and the mixture was incubated at 65℃ for 20 min and was precipitated and dried up. The dried product was used for the RT with 3'RACE 1st strand primer, and RT-PCR with 3'RACE 2nd strand primer. The following cloning steps was the same as in 2.4.2 (4).

(6) The 5' end amplification of ssRNA viruses

Specific reverse primers were used for RT, two RT products and 10 µl H2O were mixed to have the total volume of 50 µl mixture. Then 1 µl RNase T1 and 1µl Ribonuclease H were added to the mixture, and incubated at 35-37℃ for 30-45 min. The cDNA was precipitated and resuspend in 16.5 µl H2O for cDNA CAP. The mixture containing 16.5 µl cDNA, 5 µl Terminal transferase 5× buffer, 2.5µl dCTP (2mM) was incubated at 94℃ for 3 min and set immediately on ice. Then add 1 µl terminal deoxynucleotidyl transferase and incubated at 37℃ for 30 min, then 65℃ for 10 min to inactivated enzyme. Oligo(dG) primer and specific reverse primer were used for RT-PCR. The following cloning steps was the same as in 2.4.2 (4).

2.5 Phylogenetic analysis

After obtaining the full-length sequences, the conserved region coding RNA dependent RNA polymerase (RdRp) was extracted and translated to the amino acid sequence. Multiple alignment with other sequences acquired from BLASTp searches was conducted using Clustal Omega (Sievers et al. 2011). Phylogenetic trees were generated with MrBayes (version 2.2.4) (Huelsenbeck and Ronquist 2001), using the metropolis-coupled Markov chain monte Carlo (MCMC) sampling approach to calculate posterior probabilities; all other parameters were set as defaults.

2.6 viral particle extraction and observation

2.6.1 Viral particle extraction

Virus particles were purified as described before (Crawford *et al.,* 2006; Jamal, Bignell, and Coutts 2010) with a little modification.

Start with 40 g mycelium. Grind mycelium with liquid nitrogen and resuspend in 400ml (10× of the weight volume) extraction buffer (0.25 M potassium phosphate buffer, pH 7 and 0.5% thioglycolic acid, 10mM EDTA), use the blender to mix for 2 min and filter the mixture with cheesecloth.

Centrifuge the filtrate at 12,000 rpm at 4℃ for 30 min to remove the cellular debris, the supernatant was then transferred into a bottle, and add 1% Triton X100, 10% (w/v) PEG6000, 1% (w/v) sodium chloride, stirred at 4℃ for 2 h to precipitate the virus.

Collect virus precipitation by 12,000 rpm centrifuge for 30 min. The pellet was resuspended in 0.25 M potassium phosphate buffer. The crude virus suspension was centrifuged at 12,000 rpm for 30 min to remove unsuspended material. The supernatant was collected for ultracentrifuge (Beckman L7-65).

Prepare the sucrose cushion (20% sucrose in 0.25 M potassium phosphate buffer). For each 60 ml tube, put 50 ml supernatant and 10 ml sucrose cushion (slowly add with the glass tube, load at the bottom). Ultracentrifuge at 35,000 rpm for 2 h 30 min, discard the supernatant, resuspend pellet in 250 μl of 0.25 M potassium phosphate buffer and shake overnight at 4℃.

Prepare the sucrose gradient (10% to 50%), put it in a 4℃ fridge overnight.

The next day, crush the resuspended pellet with the glass tissue homogenizer and wash the tubes with another 250 μl buffer. Transfer 500 μl liquid to sucrose gradient, start the rate zonal centrifugation.

Centrifuge in sucrose gradient, 41,000 rpm, 1 h 5 min. The blue-grey, opalescent virus band was removed with a syringe and centrifuged with 5 ml sucrose cushion at 60,000 rpm for 2 h 5 min, load 500 μl H2O in the rotator. Collect the pellet.

Resuspend the pellet in 20 μl of 0.01 M potassium phosphate buffer first and wash with another 30 μl 0.01 M buffer; the total volume is 50 μl in a 1.5 ml tube. Wash with another 100 μl to save more particles on the tube wall. 12,000 rpm for 10 min to spin down the dirt, and the samples are stored at -80℃.

2.6.2 Viral particle observation using Transmission Electron Microscope (TEM)

(1) Preparation of support films for the grids

Use the finest copper grids for mycovirus, dip a glass slide in the solution of 0.3% formvar in chloroform three times. Let it dry with one side touching the filter paper for 7 min. Shave the side of the slide with a knife. Dip the slide with water and wait for the film to float. Put the grids on the film and take the film out with parafilm. Dry very well.

(2) Preparation of samples for observation

Load 30 μl samples on the plastic board, put the grids upside down on the drop for 5 min, take the grids with tweezers, wash with ultrapure water drops three times, and dry with filter paper.

Put on the uranyl acetate for 30 s to 2 min, dry by paper, and leave the grids to dry under the hood.

3. Results and Discussion

3.1 Detection and complete genome of viruses in *F. poae isolates*

F. poae F.456 and F.1080 were isolated from wheat and were found to be dsRNA positive. Agarose gel electrophoresis (Fig. 2.1-A) showed a 2,300 bp dsRNA band in F.456, and multiple bands (two bands higher than 10,000; 9,000; 2,300; and 600 bp) in F.1080.

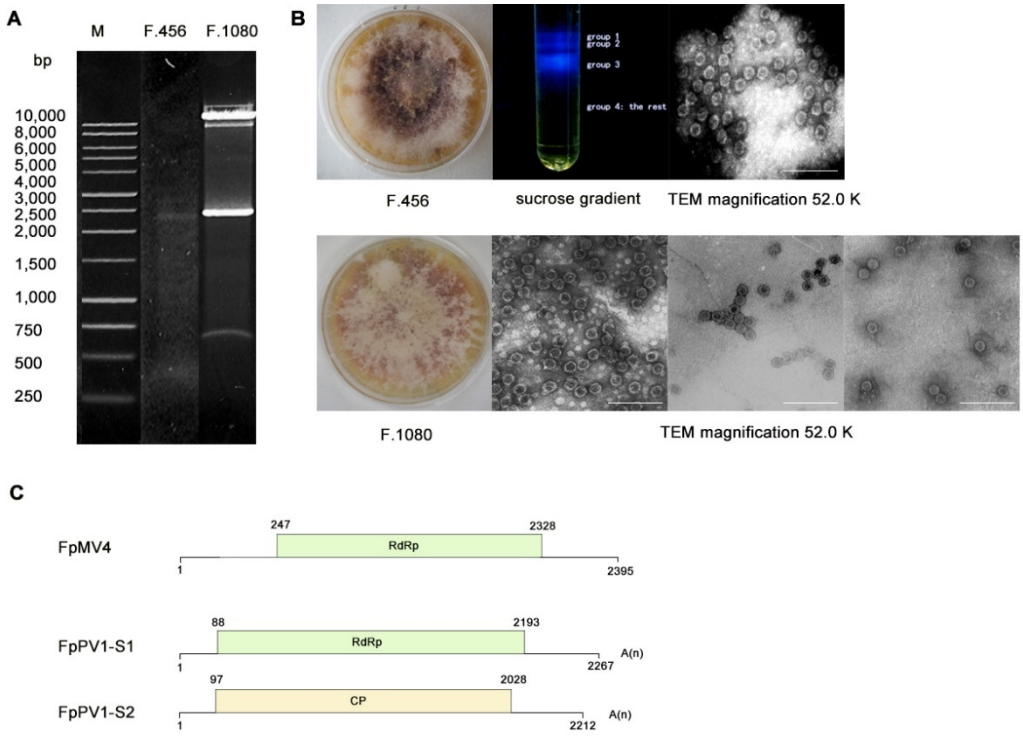


Figure 2.1 The viruses in *F. poae* F.456 and F.1080 strains. **(A)** dsRNA profile of F.456 and F.1080, a 2000 bp band was observed in F.456; 3 bands higher than 8000 bp, one 2000 bp band, and a 600 bp band were observed in F.1080. **(B)** The colonies of F.456 and F.1080, and the viral particles observed by TEM, the bar refers to 200nm. Particles were purified by ultracentrifuge and the sucrose gradient, bands were observed in sucrose gradient tube under LED light. **(C)** Genome size and organization of the characterized viruses: FpMV4 and FpPV1. Boxes on the genome indicate the position and size of ORFs.

By overlapping sequencing, the virus genomes were obtained. From the F.456's 2,300 bp dsRNA band, a 2395 nt complete genomic sequence was found (Fig. 2.1-C), the GC% is 42%, A BLASTX search of the whole nucleotide sequence showed that the highest amino acid identity (91.45%) was to the RNA-dependent RNA polymerase (RdRp) of Fusarium poae mitovirus 4 (YP_009272901). Based on the result, this virus was tentatively named Fusarium poae mitovirus 4-F.456 (FpMV4-F.456).

From the F.1080's dsRNA bands, only one virus was detected (Fig. 2.1-C). The BLASTX search showed that the highest amino acid identity (99.71%) was to the RdRp of Fusarium poae virus 1-240374 (YP_009272951.1), a partitivirus. So, this virus was named Fusarium poae partitivirus 1-F.1080 (FpPV1-F.1080). It has two segments, RNA1 is 2106 bp long, GC% is 43.8%, and encodes the RdRp; RNA2 is 2212 bp long with 45.7% GC content and encodes the capsid protein (CP). Both segments have a poly (A) tail at the 3' terminal. The sequence results of F.1080 500bp band showed that this was an FpPV1 fragment collection (the sequence data are not shown). Multiple individual clones showed that the fragments are disorderly distributed in the FpPV1 genome in segments 1 and 2, that might be due to the host defense process.

The viral particles in F.456 and F.1080 were purified (Fig. 2.1-B). Different bands appeared in their sucrose gradient, and several particles were observed under TEM. The virions are about 40nm in diameter.

3.2 phylogenetic analysis based on the RdRp gene

Based on the RdRp sequence, Bayesian trees were constructed (Fig. 2.2 and 2.3).

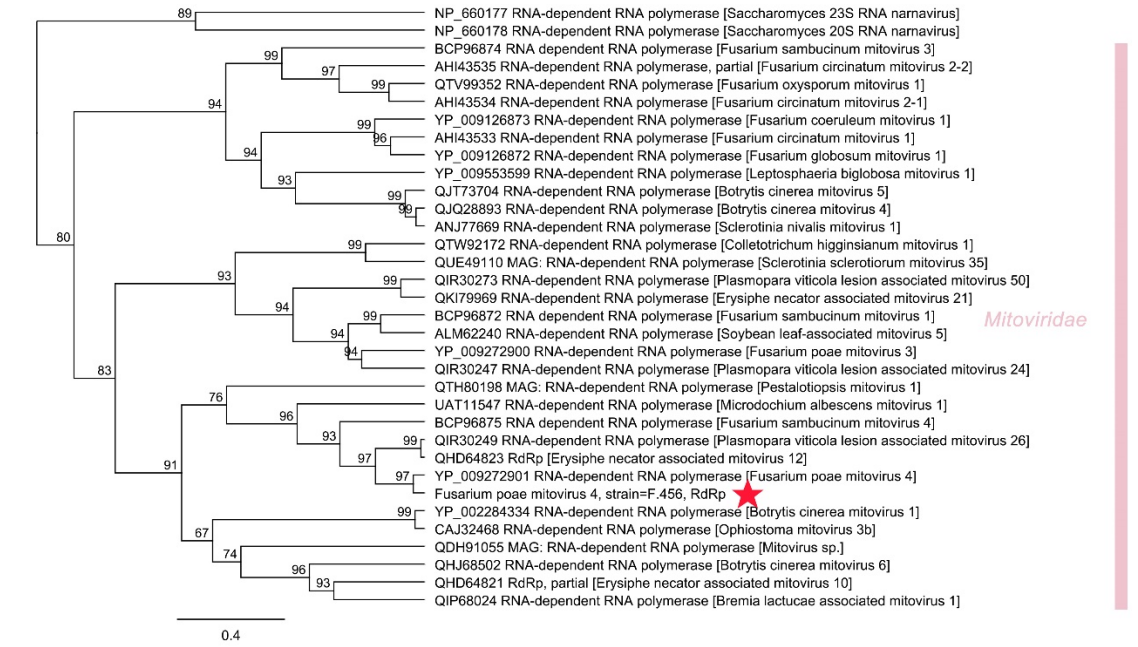


Figure 2.2 Phylogenetic tree of FpMV4-F.456. The Bayesian tree was constructed based on multiple amino acid alignment of RdRp. FpMV4 and FpMV4-F.456 form clades in *Mitoviridae* with posterior probabilities of 97%. The tree is rooted with the narnavirus clade as an outgroup. Accession numbers of protein sequences are shown in the figure.

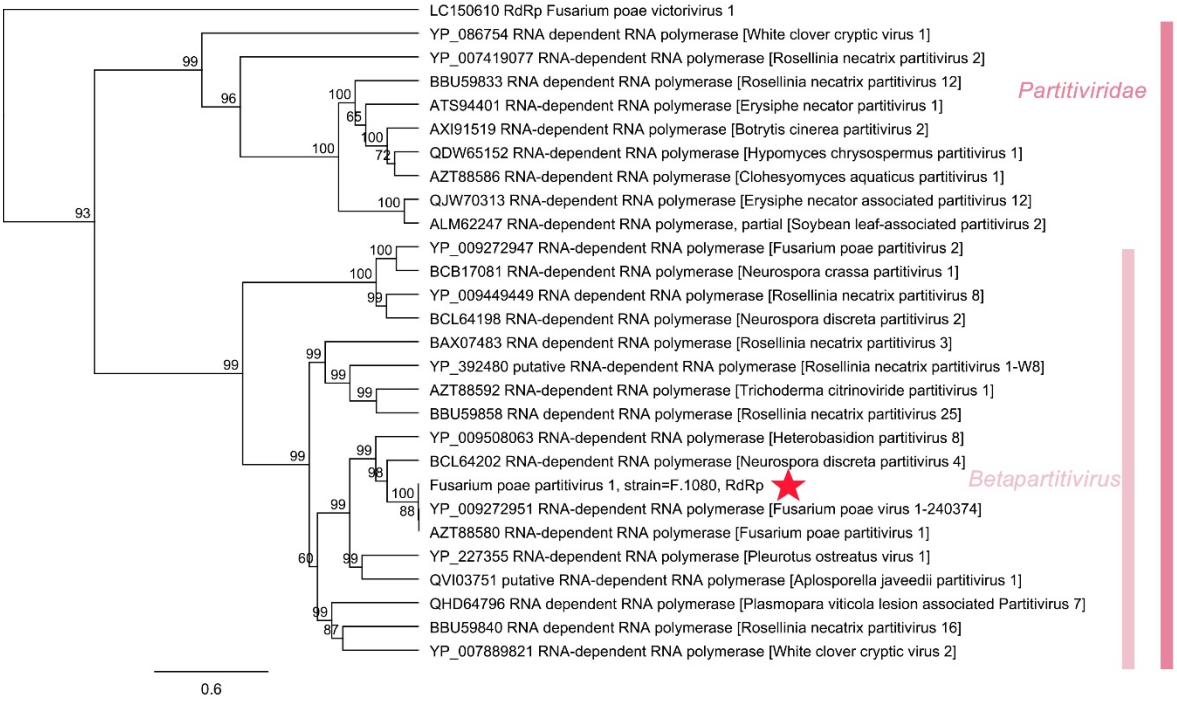


Figure 2.3 Phylogenetic tree of FpPV1-F.1080. The Bayesian tree was constructed based on multiple amino acid alignment of RdRp. FpV1-240374 and FpPV1-F.1080 form clades in *Partitiviridae* *Betapartitivirus* with posterior probabilities of 100%. The tree is rooted with the *Totiviridae* clade as an outgroup. Accession numbers of protein sequences are shown in the figure.

The Phylogenetic analysis confirms again that the viruses found in F.456 belong to the family *Mitoviridae* (Fig. 2.2), and FpPV1-F.1080 belongs to the genus *Betapartitivirus* in

family *Partitiviridae* (Fig. 2.3).

4. Conclusion and Future Perspective

FpMV4 and FpPV1 were found in two *F. poae isolates* using the conventional method. According to the BLAST results and phylogenetic analysis, they belong to the family *Mitoviridae* and *Partitiviridae*.

The viral particle photos taken by TEM revealed an interesting sight. From the dsRNA profile of F. 456, only one band at 2300 bp was observed. Furthermore, by cloning and sequencing, a mitovirus FpMV4 was characterized. As we know, mitovirus does not have true virion and structural proteins, and its genomes are associated with their RdRp in the cytoplasm. So, the viral particles we saw in TEM were another virus that might be some non-dominant viruses or does not accumulate dsRNA and were ignored during the amplifying and cloning.

Only the complete genome of FpPV1 was obtained in F.1080 strain, but multiple dsRNA bands appeared in the gel, multiple bands showed in the sucrose gradient, and the F.1080 virions' appearances, such as size and outline, under TEM are slightly different, which indicates the diversity of its virome.

Overall, we can conclude that the conventional method is not enough to detect mycoviruses when co-infection occurs. Thus, we introduced new technologies to better screen and detect our fungi virome, displayed in Chapter 3.

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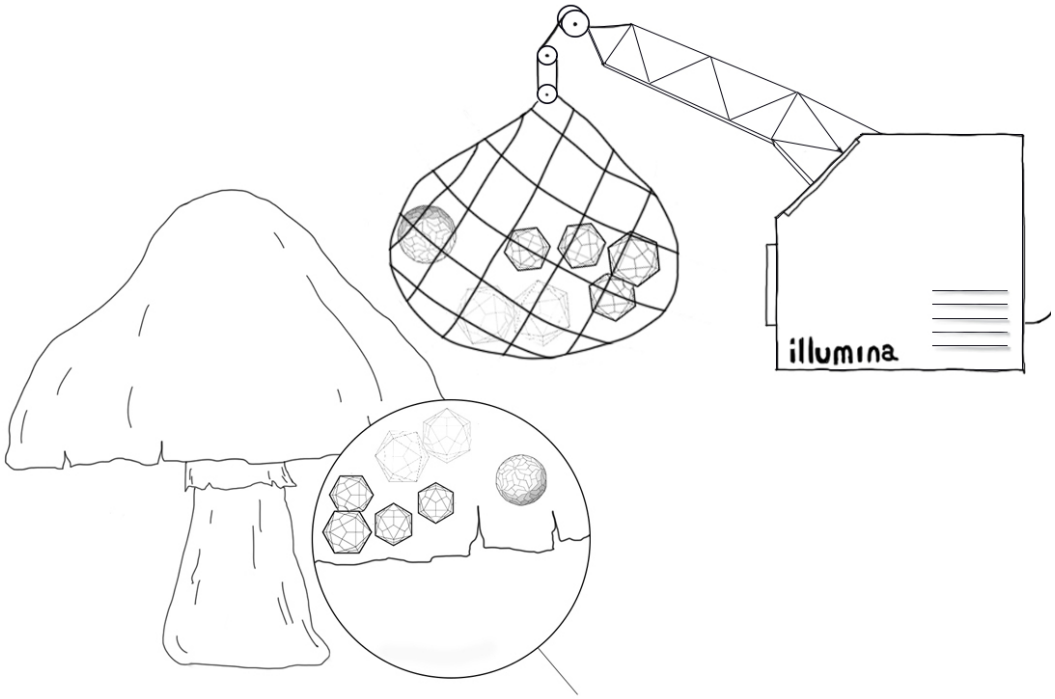
CHAPTER 3

Exploring the Virome of *F. poae* and *F. proliferatum* Collections via Metagenomics



1. Introduction

Viruses are ubiquitous in all environments on the earth and can infect or host other organisms, such as animals, plants, microorganisms, and even other viruses (Y. Z. Zhang, Shi, and Holmes 2018; R. Zhang *et al.,* 2016). Since the virus was first described in 1898 (Beijerinck 1898), research focused mainly on the pathogenic viruses that caused animal and plants disease. The specified research helped us better understand about the pathogenic viruses but ignored the other viruses, the abundant and diverse genetic material, that existed since life developed on our planet.

Our limited understanding of the virosphere is not only due to our interest in pathogenic viruses but also the virus' unculturable and parasitic characteristics. Fortunately, metagenomic has overcome these limitations, and new studies are expanding the virosphere characterization (Mizuno *et al.,* 2013; Shi *et al.,* 2016; Marzano *et al.,* 2016; Geoghegan *et al.,* 2021; Huang *et al.,* 2021).

Recent studies had revealed a remarkable number of mycoviruses, and new viral taxa were proposed, such as *Fusagraviridae* (L Wang *et al.,* 2016), *Alternaviridae* (He *et al.,* 2018), *Yadokariviridae* (Hisano *et al.,* 2018), *Fusariviridae* (R. Zhang *et al.,* 2014), etc. Most mycoviruses contain double-stranded RNA (dsRNA) (Nibert *et al.,* 2014) or positive-sense single-stranded RNA ((+)ssRNA) (Osaki *et al.,* 2016; Jia *et al.,* 2021). Others also contain negative-sense single-stranded RNA ((-)ssRNA) (Luan Wang *et al.,* 2018), reverse-transcribing (RT virus) (Pearson *et al.,* 2009), double-stranded DNA (dsDNA) (Dawe and Kuhn 1983), or single-stranded DNA (ssDNA) (Ruiz-Padilla *et al.,* 2021; Krupovic *et al.,* 2016).

Some research of single mycovirus effects on its host fungus revealed that few mycoviruses could confer hypovirulence on their fungal hosts (Xiao *et al.,* 2014; Chu *et al.,* 2002; Nuss 2005); more studies showed that many fungal strains are multi-infected and most mycoviruses are not hypovirulence-associated viruses (Flores-Pacheco *et al.,* 2017; Osaki *et al.,* 2016). Since it is normal to have multi-infected fungi, the virome approach becomes necessary. It is the basis of an accurate and comprehensive understanding of virus-host and virus-virus interaction.

Fusarium spp. as a world-spread pathogen has obtained great attention, unlike *F. graminearum* and *F. oxysporum*, *F.poae* and *F. proliferatum* are regarded as moderately or

even weakly aggressive pathogen (Tan *et al.,* 2020; Stępień, Koczyk, and Waśkiewicz 2011). However, their importance cannot be ignored.

F. poae was reported to contain diverse mycoviruses; a single *F. poae* strain was infected by 16 different mycoviruses (Osaki *et al.,* 2016). However, its role in the Fusarium head blight (FHB) complex is ambiguous (Tan *et al.,* 2021). *F. proliferatum* is important for its wide range of hosts and distribution. *F. proliferatum* is unlike *F. poae*, does not harbor multiple viruses. The dsRNA profile of 100 *F. proliferatum isolates* showed that only four strains contain dsRNA (Heaton and Leslie 2004).

In this chapter, the virome of 30 *F. poae* and 21 *F. proliferatum isolates* will be explored to provide some basic information for future studies of mycovirus biocontrol effect on FHB and interaction between virus-host and virus-virus.

2. Materials and methods

2.1 Fungal isolates cultivation and nucleic acid extraction

Thirty *F. poae isolates* and 21 *F. proliferatum isolates* were isolated from different hosts and are stocked in the Mycology lab of the University of Bologna. More information is shown in table 3.1.

Table 3.1 Basic information about *F. poae* and *F. proliferatum isolates*

Order	Name	Host	species	Location	year
1	242	`	*F. poae*	/	2002
2	419	wheatear	*F. poae*	Ancona	2006
3	456	wheat	*F. poae*	Baricella	2006
4	481	wheat	*F. poae*	Baricella	2006
5	504	wheat	*F. poae*	Baricella	2006
6	541	wheat	*F. poae*	Idice	2006
7	570	wheat	*F. poae*	Urbino	2006
8	630	wheat kernel	*F. poae*	Taglio di Po-Rovigo	2007
9	703	wheat kernel	*F. poae*	Bari	2006
10	1066	wheat kernel	*F. poae*	Umbria	2009
11	1067	wheat kernel	*F. poae*	Umbria	2009
12	1080	wheat kernel	*F. poae*	Umbria	2009
13	1085	wheat kernel	*F. poae*	Umbria	2009
14	1099	wheat kernel	*F. poae*	PSB×Cadriano	2011
15	1103	wheat kernel	*F. poae*	PSB×Cadriano	2011
16	1104	wheat kernel	*F. poae*	PSB×Cadriano	2011
17	1111	wheat kernel	*F. poae*	Verona	2011
18	1112	wheat kernel	*F. poae*	Verona	2011

Table 3.1 Basic information about *F. poae* and *F. proliferatum isolates* (Continued)

Order	Name	Host	species	Location	year
19	1136	durum wheat	*F. poae*	Palermo	`
20	1188	Dressed barley	*F. poae*	Ferrara	2012
21	1189	Dressed barley	*F. poae*	Ferrara	2012
22	1191	Dressed barley	*F. poae*	Ferrara	2012
23	1193	Dressed barley	*F. poae*	Ferrara	2012
24	1194	Dressed barley	*F. poae*	Ferrara	2012
25	1196	Bare Barley	*F. poae*	Bologna	2012
26	1197	Bare Barley	*F. poae*	Bologna	2012
27	1214	Barley kernel	*F. poae*	/	2012
28	1226	Barley kernel	*F. poae*	/	2012
29	1278	Barley kernel	*F. poae*	Perugia	2014
30	1284	durum wheat	*F. poae*	/	2013
1	CREA 13	Welsh onion	*F. proliferatum*	Umbria, Italy	2016
2	CREA 17	Welsh onion	*F. proliferatum*	Marche, Italy	2016
3	CREA 19	Welsh onion	*F. proliferatum*	Marche, Italy	2016
4	F.1002	Wheat kernels	*F. proliferatum*	Nasreyyeh, Syria	2010
5	F.1129	Garlic grown	*F. proliferatum*	China	2012
6	F.1300	Maize kernels	*F. proliferatum*	/	2014
7	F.1304	Onion	*F. proliferatum*	Cesena, Italy	2014
8	F.1316	Garlic soil	*F. proliferatum*	Voghiera, Italy	2014
9	F.1320	Garlic	*F. proliferatum*	Campania, Italy	2014
10	F.1353	Durum wheat kernels	*F. proliferatum*	Cadriano, Italy	2015
11	F.1383	Wheat kernels	*F. proliferatum*	Cervia, Italy	2015
12	F.1384	Maize	*F. proliferatum*	/	2015
13	F.1385	Barley	*F. proliferatum*	/	/
14	F.1398	Maize LaRAS	*F. proliferatum*	Cadriano, Italy	2016
15	F.1452	Welsh onion	*F. proliferatum*	/	2016
16	F.1489	Durum wheat kernels	*F. proliferatum*	Perugia, Italy	2017
17	F.1504	Onion	*F. proliferatum*	Calabria, Italy	2017
18	S.444	Maize kernels	*F. proliferatum*	Serbia	2017
19	S.446	Maize kernels	*F. proliferatum*	Serbia	2017
20	R.96	Kashkan river sediments	*F. proliferatum*	Lorestan, Iran	2018
21	R.422	Kashkan river water	*F. proliferatum*	Lorestan, Iran	2018

Note: All the 30 *F. poae isolates* were sent for the first NGS; the 9 *F. proliferatum isolates* in the blue shade were selected for the second NGS, and the rest 12 *F. proliferatum isolates* were sent for the third NGS, and its data analysis is still ongoing.

Mono-conidium colonies were cultured on PDA plates at 28℃, mycelia were cultured in liquid V8 medium at room temperature for 7 d in the dark and collected for nucleic acid extractions. Fungi were stocked on PDA and synthetic low nutrient agar (SNA) in tubes at 4℃ in the dark.

The dsRNA of *F. poae* were extracted as described before (Okada *et al.,* 2015); more details are written in Chapter 2 (2.2.1 dsRNA extraction).

Moreover, the total RNAs of 21 *F. proliferatum* were extracted using Spectrum plant total

RNA kit (Sigma-Aldrich, St. Louis, MO, USA), following the manual. Measure the RNA concentration and OD values with Q-bit and Nano-drop. Run an agarose gel to check the RNA integrity.

2.2 Metagenomic sample sequencing and Bioinformatics analysis

The *F. poae* dsRNAs and *F. proliferatum* total RNAs were extracted and pooled in two samples maintaining an equal proportion of RNA from each isolate. Samples were then sequenced by Ion Torrent and Illumina platform, respectively.

F. poae dsRNA mix was denatured for 20min at 65℃, and cDNA was synthesized using ImProm Ⅱ RT kit and dN6 universal primers. Random amplicons were obtained from RT-PCR with dN6-Tag primer and PCR with hexamer primer and Large (Klenow) Fragment DNA polymerase Ⅰ. The library was prepared using Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific Inc.) and sequenced with Ion 314 v2 chip (Thermo Fisher Scientific Inc.) More details are described in the thesis of Matteo Calassanzio (Calassanzio 2020).

For the final RNA mix sample of *F. proliferatum*, the RNA quantity ≥ 3 µg, concentration ≥ 250 ng/µl, RNA amount OD 260/280: 1.8 - 2.0; OD 260/230: 2.0 - 2.2; RIN or RQI value ≥ 8. Then the sample was sent to Eurofins genomics Illumina platform.

The bioinformatic pipeline adopted is well described in previous study (L Nerva, Varese, and Turina 2018). Raw reads were trimmed, and host sequences were removed using kneadData (McIver *et al.,*2018). Clean reads were assembled using the metaSPAdes assembler (Prjibelski *et al.,* 2020; Nurk *et al.,* 2017). The obtained transcriptome was blasted against the viral database using the DIAMOND blastn function (Buchfink, Xie, and Huson 2015) and VirFinder (Ren *et al.,* 2017). Mapping contigs on the reference viral genomes and ORF predictions were performed using Geneious Prime software (version 2021.2). For *F. proliferatum* sample, the pipeline is similar but with an additional step to filter the host genome after filtered reads and assembly. See the workflow chart in figure 3.1.

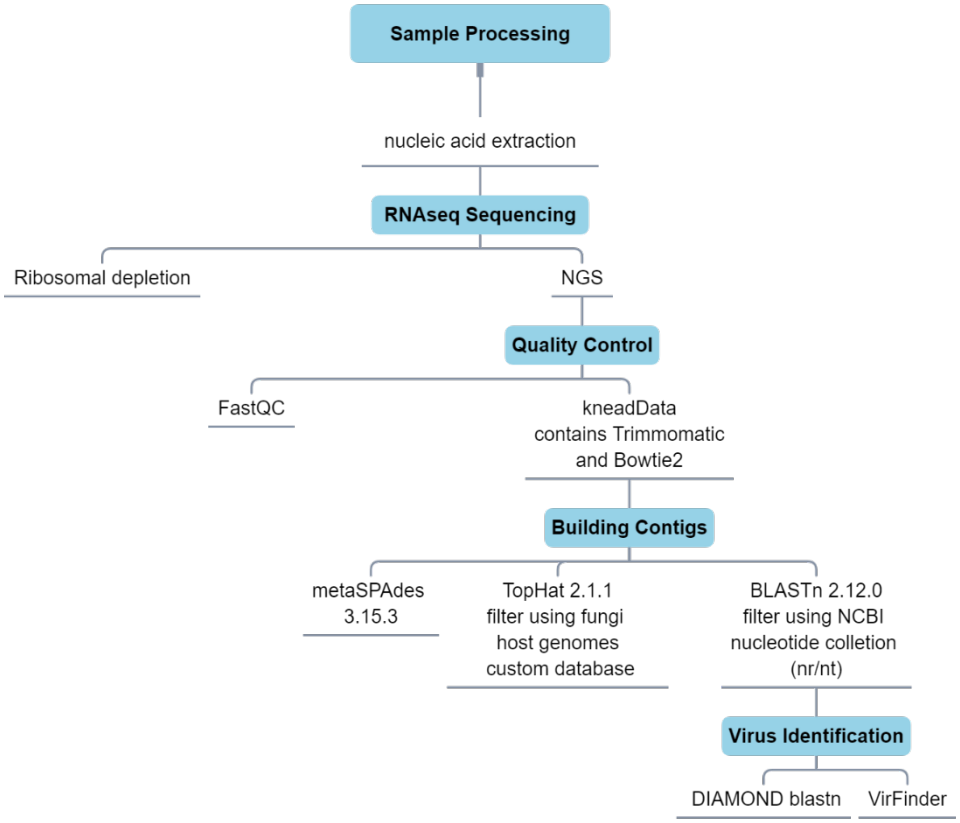


Figure 3.1 Workflow chart for the characterization of the virome of *F. prolifetarum*. More filter steps are introduced in the workflow because the cleaned reads still contain many host genomes.

2.3 Fungi virome identification

According to the consensus of viral contigs and references viral genome obtained by bioinformatic analysis, specific primers were designed using Geneious Prime® v2021.2.2 to associate the detected viral contigs to their fungal hosts.

Random primed cDNA synthesis was conducted with dsRNA and total RNA samples, and cDNAs were subjected to the RT-PCR using specific primers (2.5µl GoTag 5× buffer, 0.5µl MgCl2, 0.25µl dNTPs, 0.25/0.25µl Specific primers F/R, 0.125µl Enzyme, 6.125µl H2O, 2.5µl cDNA). The annealing temperatures were adjusted according to the T_m of specific primers.

Purify the amplicons with the Wizard® SV Gel and PCR Clean-Up System (Promega A9282). And send the purified PCR product or agarose gel to Sanger sequencing (Eurofins genomic) to confirm the viral sequence presence.

2.4 Virus whole genomes confirmation and phylogenetic analysis

The whole genomes of the viruses are confirmed by overlapping sequences with RT-PCR

and RACE as described in Chapter 2 (2.4.1 and 2.4.2).

Briefly, RT-PCR was conducted to obtain the missing viral genomes from infected fungi using ImProm-II™ Reverse Transcription System (Cat. No. A3800) and GoTaq® DNA Polymerase kit (Cat. No. M3005) follow the manual, and its products were purified with the Wizard® SV Gel and PCR Clean-Up System (Cat. No. A9282) and sequenced.

To obtain the terminal sequences, RACE was performed, more details are described in Chapter two 2.4.2; and cloning follow the instruction of pGEM-T easy vector system (Promega, Cat. No. A1360); then screened by M13 forward and reverse primers and purified for sequencing.

After obtained the viral genome, the conserved region coding RNA dependent RNA polymerase (RdRp) was extracted and translated to an amino acid sequence. Multiple alignment with other sequences acquired from BLASTp searches was conducted using Clustal Omega (Sievers *et al.,* 2011). Phylogenetic trees were generated with MrBayes (version 2.2.4) (Huelsenbeck and Ronquist 2001), using the metropolis-coupled Markov chain monte Carlo (MCMC) sampling approach to calculate posterior probabilities; all other parameters were set as defaults.

3. Results and Discussion

3.1 Fungal isolates collection and nucleic acid extraction

Strains were collected from different locations; 30 *F. poae* and 21 *F. proliferatum* are shown in table 3.1 and figure 3.2. The first yellow square contains all the 30 *F. poae* whose dsRNAs were sent to Ion Torrent sequencing, the second and third squares contain 9 and 12 *F. proliferatum* who's total RNAs were sent to Illumina sequencing separately.

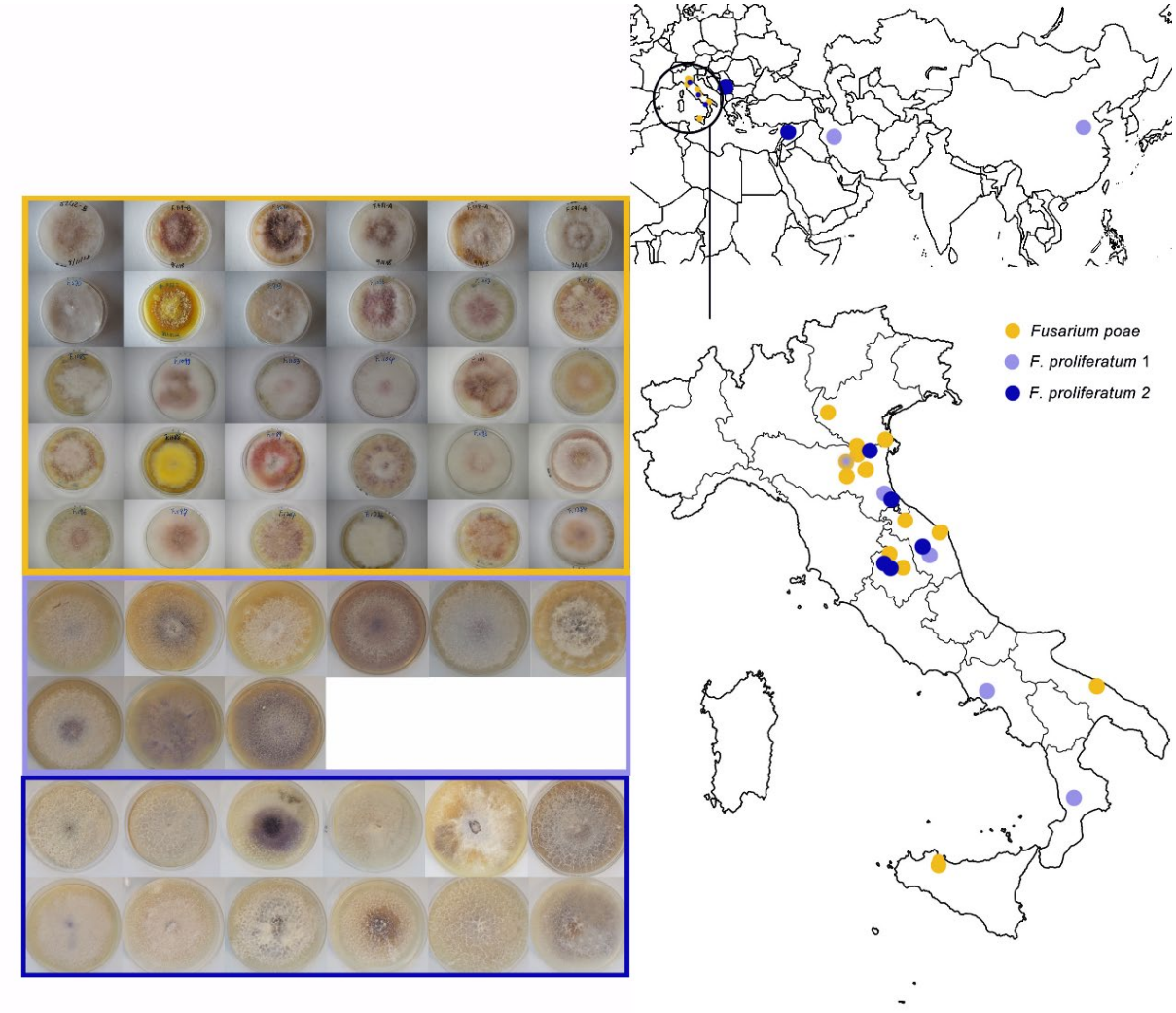


Figure 3.2 Collected location and colony morphology of Fusarium spp. Different squares refer to different batches of NGS sequencing.

3.2 The virome diversity in *F. poae* and *F. proliferatum*

Ion Torrent produced 460,566 reads from *F. poae* collection and were assembled into 5,953 contigs up to 12,775 nt in length. After analyzing, putative viral contigs have been identified belonging to families *Hypoviridae*, *Mitoviridae*, *Partitiviridae*, *Polymycoviridae*, proposed *Alternaviridae*, proposed *Fusagraviridae*, proposed *Fusariviridae*, proposed *Yadokariviridae*, and *Totiviridae* (Fig. 3.3-A&C).

By filter and assemble the 7,421,810 reads from 9 *F. proliferatum isolates*' Illumina sequencing data, 3,009 contigs up to 9,471 nt in length were obtained. After analyzing, viral contigs belonged to families *Mitoviridae* and *Mymonaviridae* (Fig. 3.3-B&C).

The reads abundance among different virus families in the two *Fusarium* NGS data is shown in Figure 3.3-C. In *F. poae* collection, the *Partitiviridae* is the dominant virus family; next are *Totiviridae* and proposed *Fusagraviridae*.

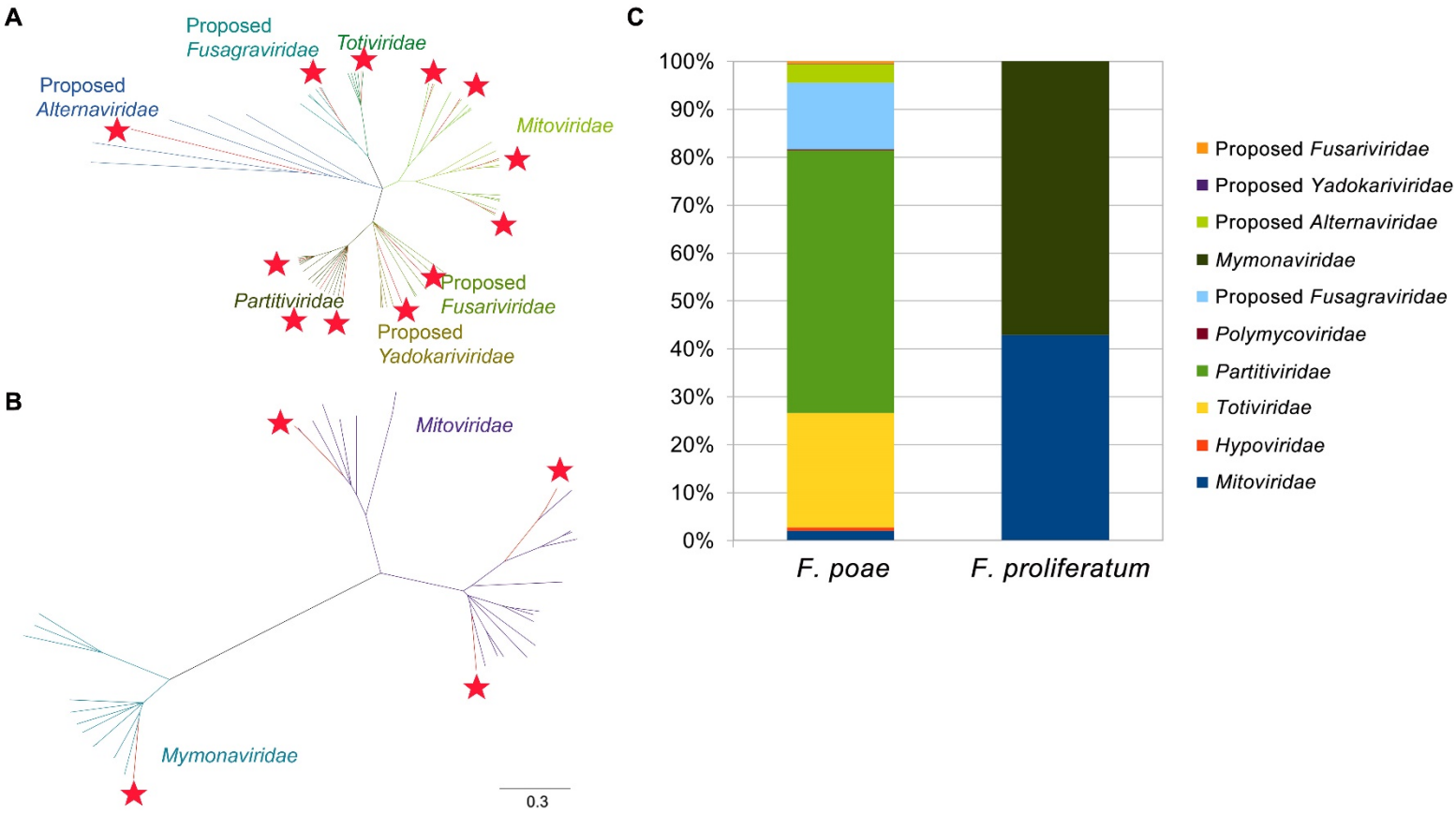


Figure 3.3 The virome diversity in *F. poae* and *F. proliferatum*. (A) The viruses in *F. poae*, which have completed the genome and are grouped at the family level. Red stars refer to the virus identified in this study. (B) The viruses in *F. proliferatum*, which have completed the genome and are grouped at the family level. Red stars refer to the virus identified in this study. (C) The reads abundance among different virus families in the two *Fusarium* NGS data.

3.3 Identification of mycoviral sequences in 30 *F. poae isolates* and 9 *F. proliferatum isolates*

By assembling the contigs and sequences obtained by cloning, 19 viruses were found in *F. poae* and *F. proliferatum* collections. The detailed information is shown in table 3.2. Among the 19 viruses, 3 are partial genomes, and the others' genomes were complete and confirmed.

Table 3.2 Obtained virus sequences with similarity to the viruses by Blastp

Name of putative viruses	Accession No.	Genome status	Blastp best hits	% aa identity	e-value	Genome type	Family	reference
Fusarium poae victorivirus 1 strain F.1194 (FpVV1-F.1194)	OK524179	full length	YP_009272905 RdRp Fusarium poae victorivirus 1	96%	0.0	dsRNA	*Totiviridae*	(Osaki *et al.*, 2016)
Fusarium poae partitivirus 1 strain F.1080 (FpPV1-F.1080)	OK524180-1	full length	YP_009272951.1 RdRp Fusarium poae virus 1-240374	99%	0.0	segmented dsRNA	*Partitiviridae*	(Osaki *et al.*, 2016)
Fusarium poae partitivirus 2 strain F.419 (FpPV2-F.419)	OK524182-3	full length	YP_009272947.1 RdRp Fusarium poae partitivirus 2	99.60%	0.0	segmented dsRNA	*Partitiviridae*	(Osaki *et al.*, 2016)
Fusarium poae fusagravirus 1 (FpFgV1)	OK524184	full length	YP_009253997.1 RdRp Fusarium poae dsRNA virus 3	91.66%	0.0	dsRNA	Proposed *Fusagraviridae*	(L Wang *et al.*, 2016)
Fusarium poae alternavirus 2 (FpAV2)	OK524185-7	full length	YP_009449439.1 Polyprotein Fusarium graminearum alternavirus 1	94.75%	0.0	segmented dsRNA	Proposed *Alternaviridae*	(He *et al.*, 2018)
Fusarium poae mitovirus 5 (FpMV5)	OK524188	full length	YP_009272898.1 RdRp Fusarium poae mitovirus 1	63.97%	0.0	(+)ssRNA	*Mitoviridae*	(Osaki *et al.*, 2016)
Fusarium poae mitovirus 3 strain F.1278 (FpMV3-F.1278)	OK524189	full length	YP_009272900.1 RdRp Fusarium poae mitovirus 3	95%	0.0	(+)ssRNA	*Mitoviridae*	(Osaki *et al.*, 2016)
Fusarium poae partitivirus 3 (FpPV3)	OK524190-1	full length	AXI91519.1 RdRp Botrytis cinerea partitivirus 2	94.13%	0.0	segmented dsRNA	*Partitiviridae*	(Kamaruzzaman *et al.*, 2019)
Fusarium poae mitovirus 4 strain F.1196 (FpMV4-F.1196)	OK524192	full length	YP_009272901.1 RdRp Fusarium poae mitovirus 4	91.45%	0.0	(+)ssRNA	*Mitoviridae*	(Osaki *et al.*, 2016)
Fusarium poae mitovirus 2 strain F.570 (FpMV2-F.570)	OK524193	full length	YP_009272899.1 RdRp Fusarium poae mitovirus 2	96.60%	0.0	(+)ssRNA	*Mitoviridae*	(Osaki *et al.*, 2016)

Table 3.2 Obtained virus sequences with similarity to the viruses by Blastp (continued)

Name of putative viruses	Accession No.	Genome status	Blastp best hits	% aa identity	e-value	Genome type	Family	reference
Fusarium poae yadokarivirus 1 (FpYV1)	OK524194	full length	YP_009272910.1 RdRp Fusarium poae mycovirus 2	88.49%	0.0	(+)ssRNA	Proposed *Yadokariviridae*	(Osaki *et al.*, 2016)
Fusarium poae fusarivirus 2 (FpFV2)	OK524195	full length	YP_009272906.1 RdRp Fusarium poae fusarivirus 1	83.35%	0.0	(+)ssRNA	Proposed *Fusariviridae*	(Osaki *et al.*, 2016)
Fusarium poae Polymycovirus 1 (FpPmV1)		partial	QDB74985.1 RdRp Phaeoacremonium minimum tetramycovirus 1	57.92%	8.00E-122	segmented dsRNA	proposed *Polymycoviridae*	(Luca Nerva *et al.*, 2019)
Fusarium poae hypovirus 2 (FpHV2)	OK524196	partial	YP_009011065.1 Hypothetical protein Fusarium graminearum hypovirus 1	71.67%	0.0	(+)ssRNA	*Hypoviridae*	(S. Wang *et al.*, 2013)
Fusarium poae hypovirus 1 strain F.1080 (FpHV1-F.1080)		partial	BAV56305.1 polyprotein Fusarium poae hypovirus 1	90.58%	0.0	(+)ssRNA	*Hypoviridae*	(Osaki *et al.*, 2016)
Fusarium proliferatum mitovirus 1 (FprMV1)	OK524197	full length	QIP68024.1 RdRp Bremia lactucae associated mitovirus 1	93.57%	0.0	(+)ssRNA	*Mitoviridae*	unpublished
Fusarium proliferatum mitovirus 2 (FprMV2)	OK524198	full length	YP_009272898.1 RdRp Fusarium poae mitovirus 1	74.93%	0.0	(+)ssRNA	*Mitoviridae*	(Osaki *et al.* 2016)
Fusarium proliferatum mitovirus 3 (FprMV3)	OK524199	full length	QDH86886.1 RdRp Mitovirus sp.	61.90%	0.0	(+)ssRNA	*Mitoviridae*	(Starr *et al.* 2019)
Fusarium proliferatum mymonavirus 1 (FprMnV1)	OK524200	full length	ALM62220.1 RdRp Soybean leaf-associated negative-stranded RNA virus 1	60.71%	0.0	(-)ssRNA	*Mymonaviridae*	(S. Y. L. Marzano and Domier 2016)

Here below, identified viruses are described and grouped based on the genome type. The phylogenetic trees were constructed by MrBayes (version 2.2.4) (Huelsenbeck and Ronquist 2001), using the metropolis-coupled Markov chain monte Carlo (MCMC) sampling approach to calculate posterior probabilities; all other parameters were set as defaults.

3.3.1 dsRNA virus

A total of 7 mycoviruses with dsRNA genome were identified in the *F. poae* collection. They belong to *Partitiviridae* (n=3), *Polymycoviridae* (n=1), Proposed *Alternaviridae* (n=1), Proposed *Fusagraviridae* (n=1) and *Totiviridae* (n=1).

3.3.1.1 *Partitiviridae*

Partitiviridae virus has a linear segmented genome composed of two RNAs (Nibert et al. 2014), three partitivirus in this study named FpPV1-F.1080, FpPV2-F.419, and FpPV3 belong to *Betapartitivirus* and *Alphapartitivirus*, respectively (Fig. 3.4).

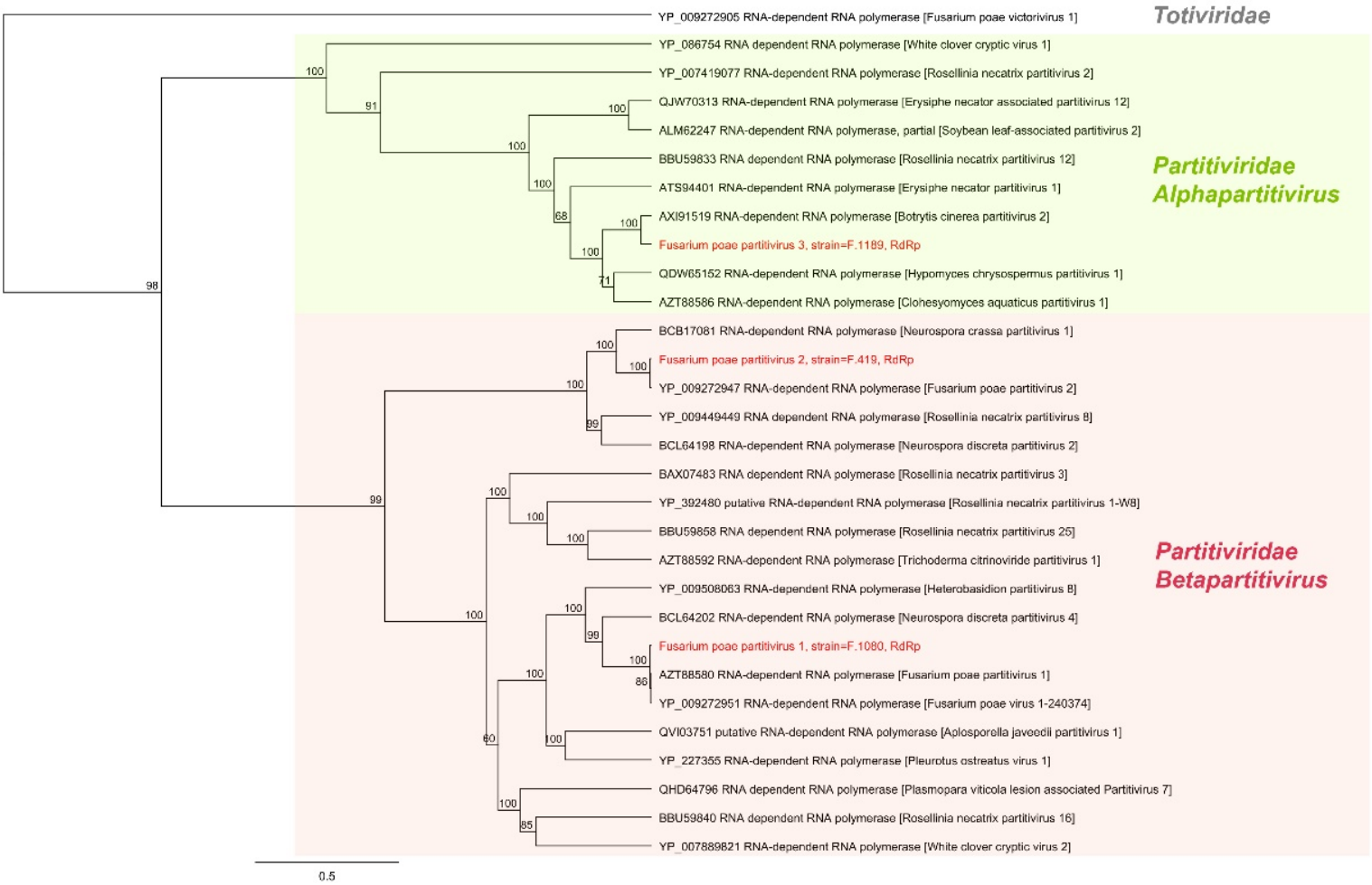


Figure 3.4 *Partitiviridae* phylogenetic tree. The Bayesian tree was constructed based on multiple amino acid alignment of RdRp. FpV1-240374 and FpPV1-F.1080, FpPV2, and FpPV2-F.419 form clades in *Partitiviridae Betapartitivirus* with posterior probabilities of 100%. BcPV2 and FpPV3 form

clades in *Partitiviridae Alphapartitivirus* with posterior probabilities of 100%. The tree is rooted with the *Totiviridae* clade as an outgroup. Accession numbers of protein sequences are shown in the figure.

FpPV1 segment 1 (2267 bp) has a single ORF coding for RdRp of 702 aa; segment 2 (2212 bp) contains an ORF that encodes capsid protein of 644 aa. Similar genome organization appeared in FpPV2 and FpPV3, in FpPV2, segment 1 (2393 bp) encodes RdRp (756 aa) and segment 2 (2403 bp) encodes CP (651 aa). FpPV3 has a smaller genome than the other two *partitivirus*, its segment 1 (1909 bp) coding RdRp of 580 aa and segment 2 (1885 bp) coding CP of 530 aa.

FpPV1 and FpPV2 share 99% aa identity with the known partitivirus found in *F. poae*. While FpPV3 might be a new partitivirus that shares 94% aa identity with Botrytis cinerea partitivirus 2 (Kamaruzzaman *et al.*, 2019).

3.3.1.2 *Totiviridae* and Proposed *Fusagraviridae*

The identified *Totiviridae* virus FpVV1-F.1194 in *F. poae* belongs to genus *Victorivirus* (Fig. 3.5). It has a genome of 5133 bp and encodes two large overlapping ORFs, the first one encodes CP of 805 aa, and the second one encodes RdRp of 787 aa. The ORFs junction sequence is TAATG; the “TAA” is the stop codon of ORF1, and the “ATG” is the start codon of ORF2. It shares 96% aa identity with a victorivirus found in *F. poae* (Osaki *et al.*, 2016).

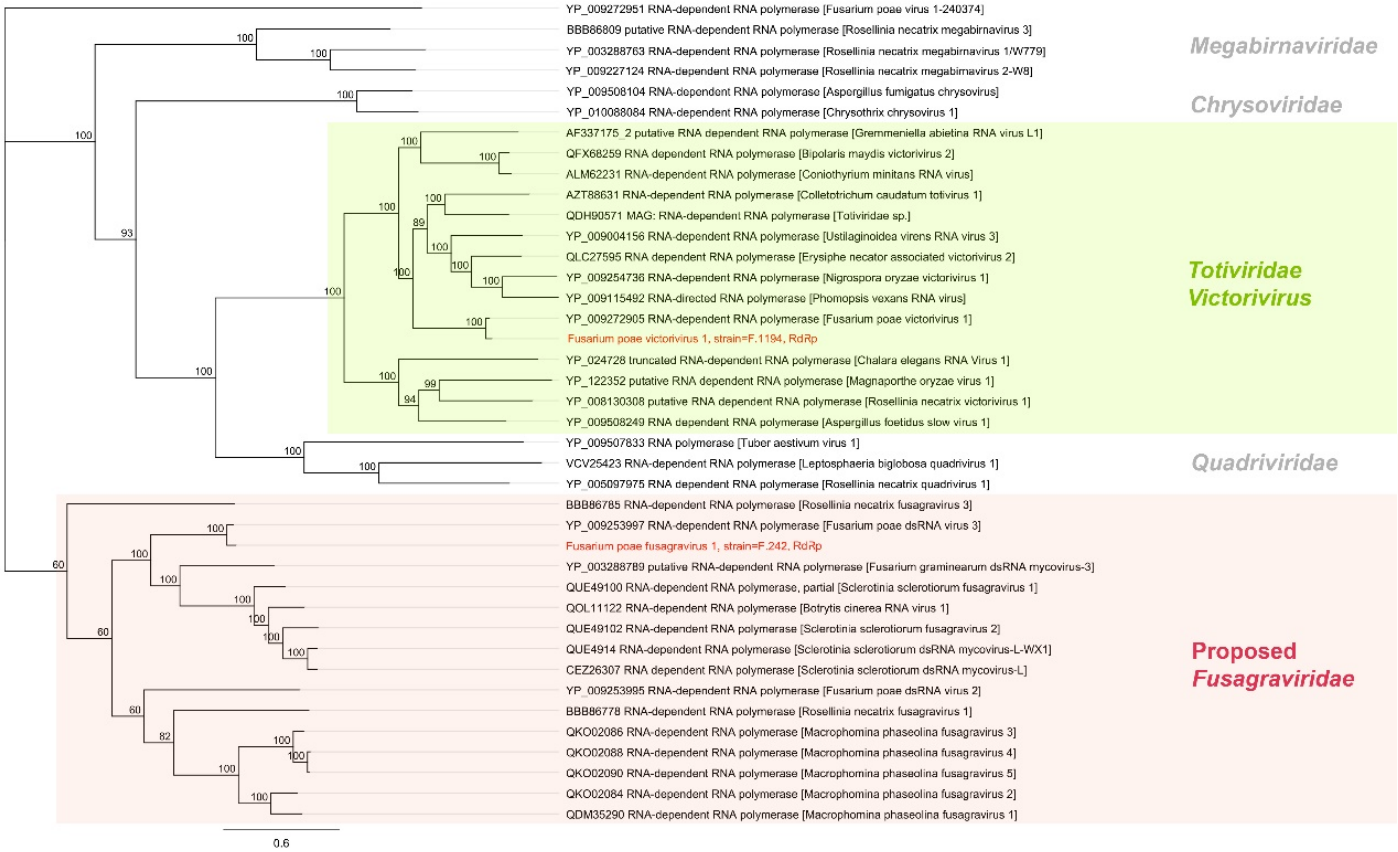


Figure 3.5 *Totiviridae* and proposed *Fusagraviridae* phylogenetic tree. The Bayesian tree was constructed based on multiple amino acid alignment of RdRp. FpVV1 and FpVV1-F.1194 form clades in *Totiviridae Victorivirus* with posterior probabilities of 100%. FpdsV3 and FpFgV1 form clades in the proposed *Fusagraviridae* with posterior probabilities of 100%. The tree is constructed with the other genus in *Totiviridae* as outgroups. Accession numbers of protein sequences are shown in the figure.

FpFgV1 might be a new virus that belongs to the proposed family *Fusagraviridae* (Fig. 3.5); it has a 9438 bp length genome that encodes two large ORFs and is not overlapped: the ORF1 coding 1332 aa CP and ORF2 coding 1356 aa RdRp. The blastp analysis showed homology with Fusarium poae dsRNA virus 3 isolate SX63 (YP_009253997.1), and the % aa identity is 91.66% (Fig. 3.5).

3.3.1.3 Proposed *Alternaviridae*

The *Alternaviridae* virus FpAV2 found in *F. poae* might be a new virus that has a linear segmented genome composed of three RNAs, dsRNA1 (3559 bp), dsRNA2 (2499 bp), and dsRNA3 (2484 bp). Each contained a single ORF, ORF1 (position 83→3454) of dsRNA1 was found to encode a putative protein of 1124 aa, with a conserved domain corresponding to RdRp; ORF2 (position 147→2354) in dsRNA2 and ORF3 (position 79→2310) in dsRNA3 coding putative proteins of 833 aa and 744 aa, respectively.

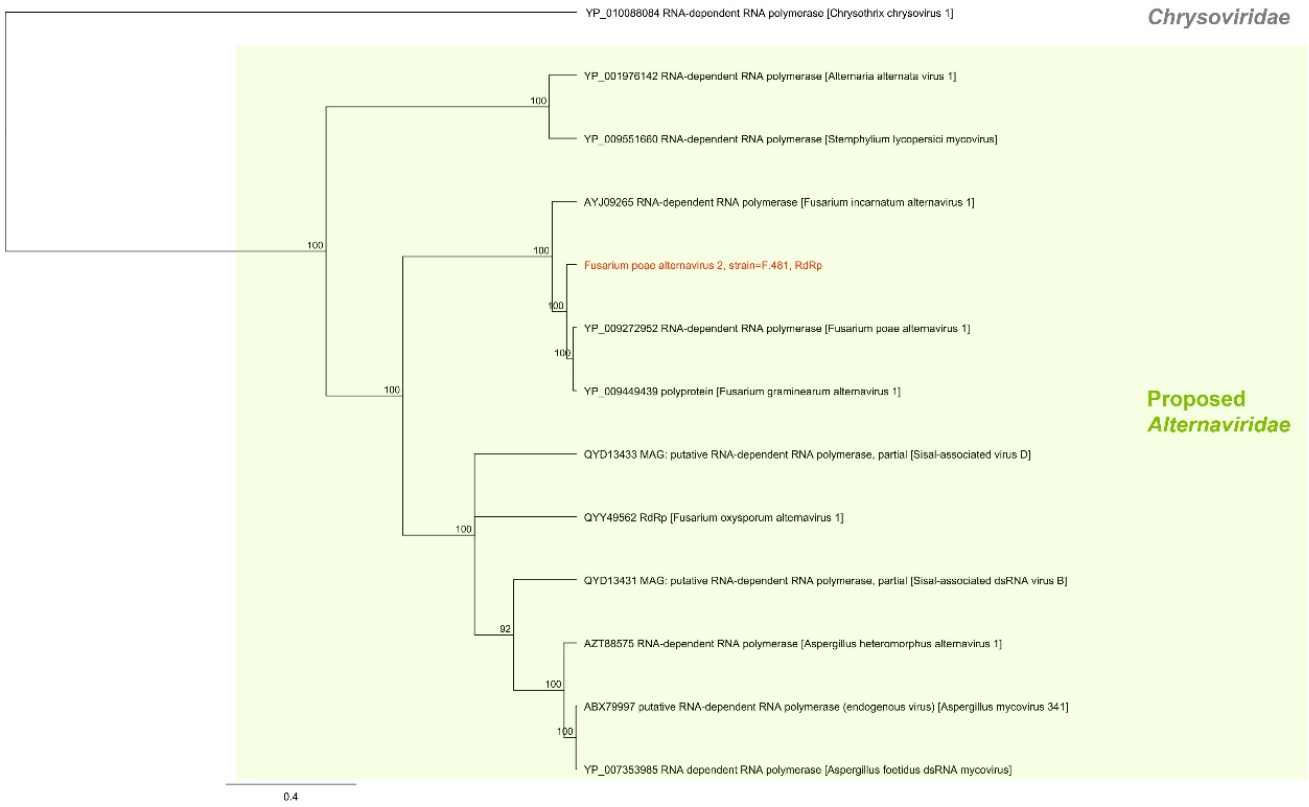


Figure 3.6 Proposed *Alternaviridae* phylogenetic tree. The Bayesian tree was constructed based on multiple amino acid alignment of RdRp. FgAV1, FpAV1, and FpAV2 form clades in proposed

Alternaviridae with posterior probabilities of 100%. The tree is rooted with the *Chrysoviridae* clade as an outgroup. Accession numbers of protein sequences are shown in the figure.

The blastp analysis showed homology with Fusarium graminearum alternavirus 1 (YP_009449439.1) with a 94.75% aa identity (Fig. 3.6).

3.3.1.4 proposed *Polymycoviridae*

Some viral contigs in *F. poae* showed that they belong to a novel renamed family: *Polymycoviridae* (Kotta-Loizou and Coutts 2017; Kanhayuwa et al. 2015). The blastp analysis showed homology with Phaeoacremonium minimum tetramycovirus 1 (QDB74985.1), the aa identity is 57.92%, and E-value is 8.00E-122. It has four dsRNA segments, but unfortunately, its genome was not complete.

Polymycoviridae was reported to have an unprecedented dynamic nature in terms of genomic element number and sequence (Kotta-Loizou and Coutts 2017).

3.3.2 Positive-sense RNA viruses (ssRNA+)

3.3.2.1 *Mitoviridae*

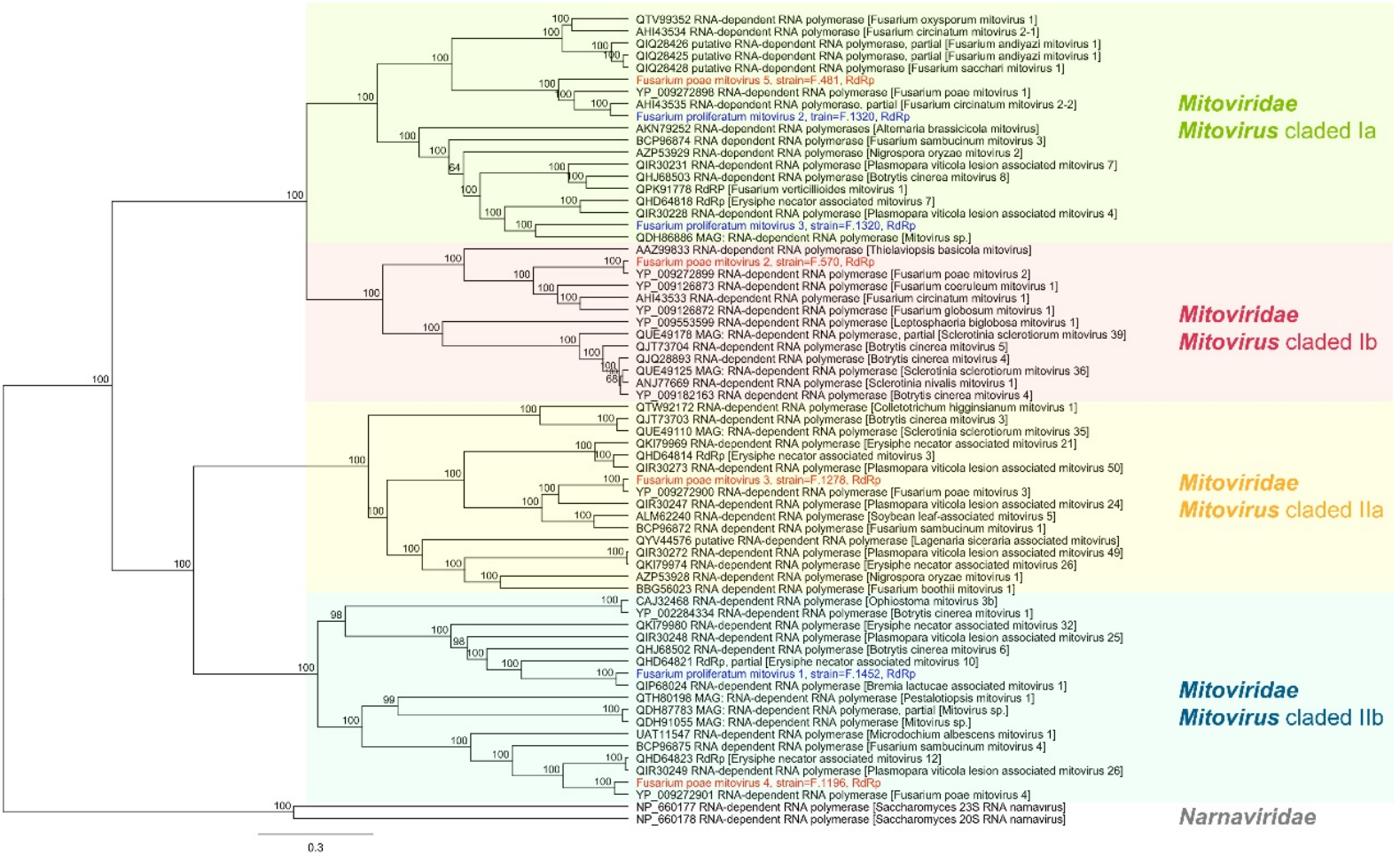


Figure 3.7 *Mitoviridae* phylogenetic tree. The Bayesian tree was constructed based on multiple

amino acid alignment of RdRp. Viruses from *F. poae* are marked in red, and *F. proliferatum* viruses are marked in blue. The tree is rooted with the *Narnaviridae* clade as an outgroup. Accession numbers of protein sequences are shown in the figure.

The majority of the ssRNA(+) mycoviruses found in this study belong to *Mitoviridae*; seven mitoviruses were identified, four in *F.poae*: FpMV2-F.570 (2414 nt, 66→2360 encodes RdRp), FpMV3-F.1278 (2719 nt, 240→2612 encodes RdRp), FpMV4-F.1196 (2395 nt, 247→2328 encodes RdRp), FpMV5 (2391 nt, 175→2352 encodes RdRp); and three in *F. proliferatum*: FprMV1 (2429 nt, 177→2420 encodes RdRp), FprMV2 (2400 nt, 180→2363 encodes RdRp), FprMV3 (2510 nt, 295→2412 encodes RdRp).

Among the seven identified mitovirus, FpMV5 and FprMV1 to 3 are new mitovirus, others were considered variants of the previously described mitovirus. FpMV5 RdRp aa sequence shares only 63.97% identity with Fusarium poae mitovirus 1 (YP_009272898.1); FprMV1 showed homology with Bremia lactucae associated mitovirus 1 (QIP68024.1), the aa identity is 93%; FprMV2 shares 75% aa identity with Fusarium poae mitovirus 1 (YP_009272898.1) and FprMV3 shares 62% aa identity with a mitovirus are found in soil sample (Starr *et al.* 2019). It is the first time that mitovirus has been found in *F. proliferatum* species (Fig. 3.7).

3.3.2.2 Proposed *Yadokariviridae*

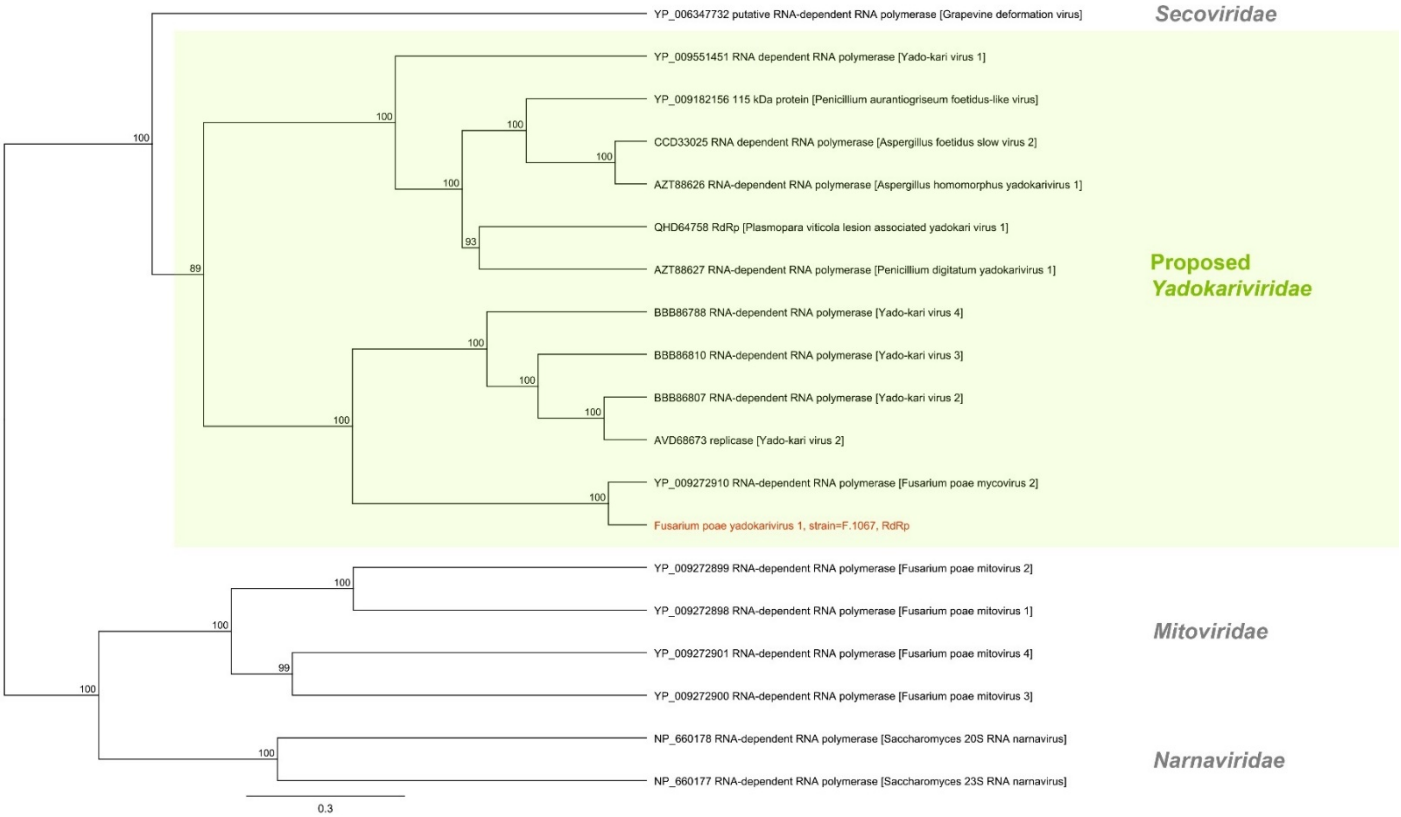


Figure 3.8 Proposed *Yadokariviridae* phylogenetic tree. The Bayesian tree was constructed based on

multiple amino acid alignment of RdRp. FpMyV2 and FpYV1 form clades in proposed *Yadokariviridae* with posterior probabilities of 100%. The tree is constructed with the other *Secoviridae* in *Pisuviricota*, and *Mitoviridae*, *Narnaviridae* in *Lenarviricota* as outgroups. Accession numbers of protein sequences are shown in the figure.

FpYV1 has a 4211 nt genome that contains a big ORF (683→3997) encoding RdRp. The blastp analysis showed homology with Fusarium poae mycovirus 2 (YP_009272910.1); the aa identity is 88.49% (Fig. 3.8).

3.3.2.3 *Hypoviridae* and Proposed *Fusariviridae*

FpFV2 has a 6386 nt genome that contains two ORFs, ORF1 (71→4585) encodes RdRp of 2128 aa, while ORF2 (4641→6089) encodes a putative protein of 483 aa. The blastp analysis showed homology with Fusarium poae fusarivirus 1 (YP_009272906.1), and the RdRp aa sequences shared 83.35% identity.

FpHV2 partial sequence indicated that it might be a new hypovirus in *F. poae* that contains a big ORF, which encodes a protein of 3688 aa. This aa sequence shares homology with Fusarium graminearum hypovirus 1 (YP_009011065.1), and the identity is 71.67% (Fig. 3.9).

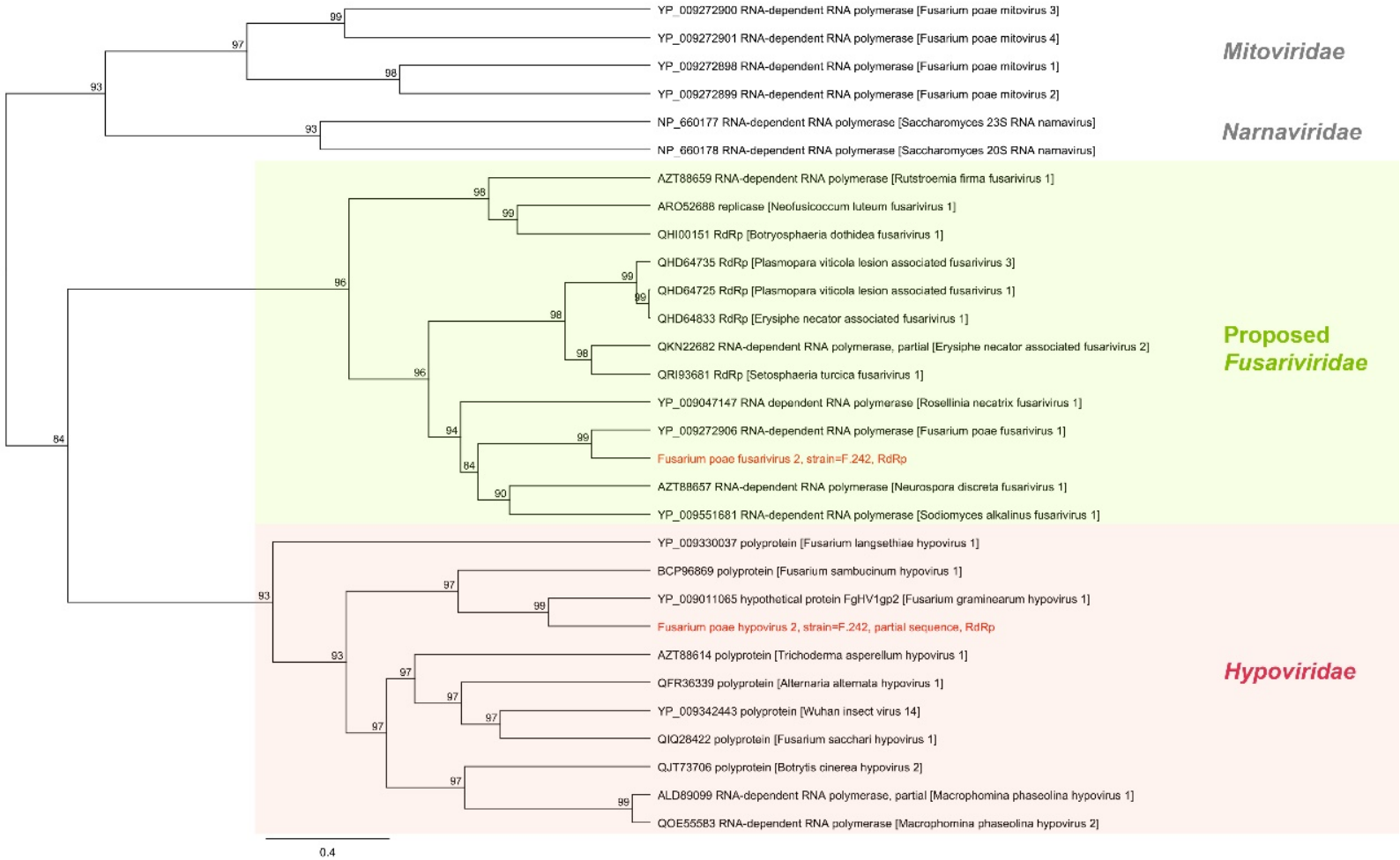


Figure 3.9 *Hypoviridae* and Proposed *Fusariviridae* phylogenetic tree. The Bayesian tree was

constructed based on multiple amino acid alignment of RdRp. FpFV1 and FpFV2 form clades in the proposed *Fusariviridae* with posterior probabilities of 99%. FgHV1 and FpHV2 form clades in *Hypoviridae* with posterior probabilities of 99%. The tree is constructed with *Mitovirudae* and *Narnaviridae* as outgroups. Accession numbers of protein sequences are shown in the figure.

3.3.3 Negative-sense RNA viruse (ssRNA-)

3.3.3.1 *Mymonaviridae*

In *F. proliferatum* collection, Fusarium proliferatum mymonavirus 1 (FprMnV1) was detected, it is a new virus and its complete genome was obtained by assembling contigs, RT-PCR, and RACE sequences. FprMnV1 is 9059 nt long and encodes four ORFs. More detailed information is shown in Fig. 3.10. The blastp analysis showed homology with Soybean leaf-associated negative-stranded RNA virus 1 (ALM62220.1), and the RdRp aa sequences shared 60.71% identity (Fig. 3.11).

Figure 3.10 Genome organization of Fusarium proliferatum mymonavirus 1 (FprMnV1)

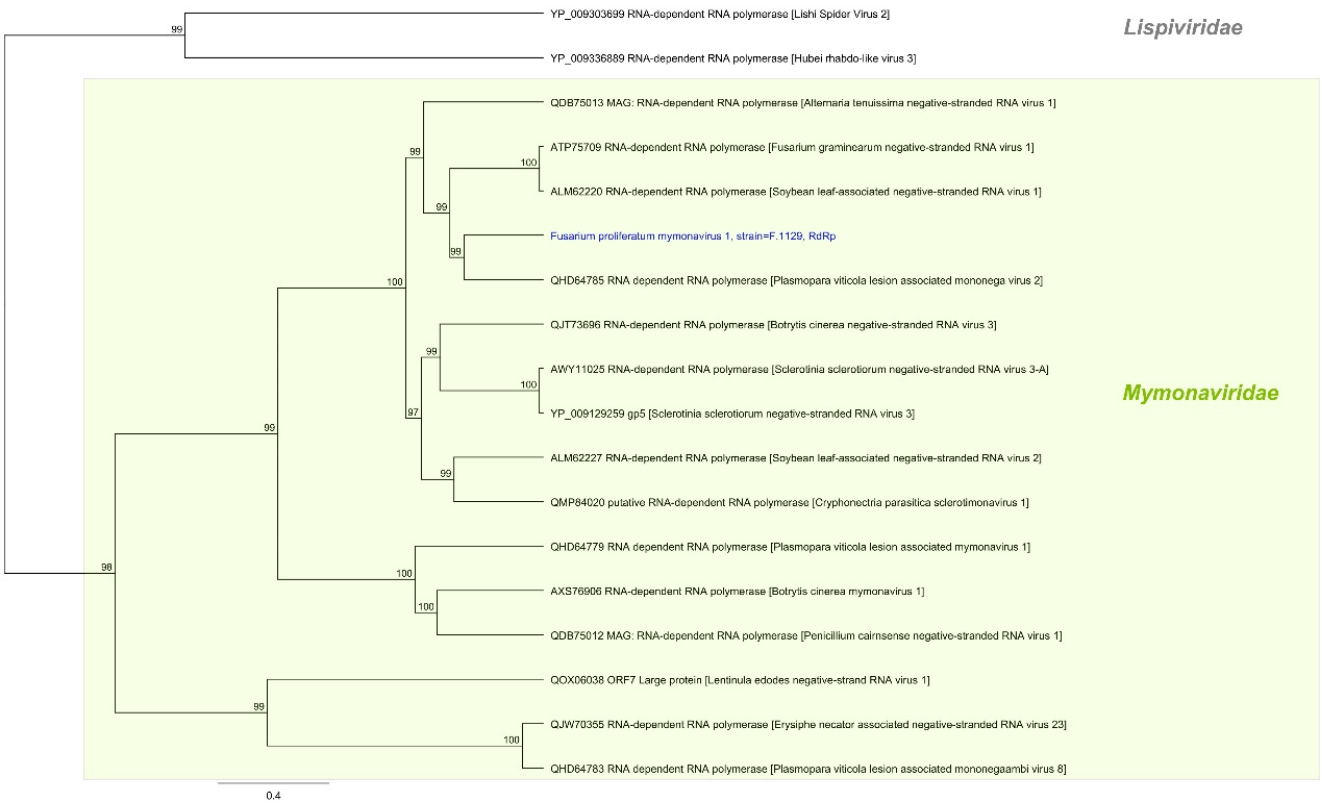


Figure 3.11 *Mymonaviridae* phylogenetic tree. The Bayesian tree was constructed based on multiple

amino acid alignment of RdRp. PvMnV1 and FprMnV1 form clades in *Mymonaviridae* with posterior probabilities of 99%. The tree is constructed with *Lispiviridae* as outgroups. Accession numbers of protein sequences are shown in the figure.

4. Conclusion and Future Perspective

The virome of *F. poae* and *F. proliferatum* collections were identified using metagenomics. Their virome diversity has a big difference; the virome of *F. proliferatum* is much simpler than *F. poae*, which might be related to the diversity of the host populations, fungi defense mechanisms or the different timescale when the two species appeared; those are assumptions and need future exploration.

In *F. poae*, dsRNA viruses are widespread, especially partitivirus; they are dominant in reads number and variety (Fig. 3.3); next are *Totiviridae* and proposed *Fusagraviridae* (Fig. 3.3-C). It is worth noting that three viruses (FpPV1 to 3) were identified in *Partitiviridae*, and FpPV1 is present in all the 30 *F. poae isolates*.

In contrast, 9 *F. proliferatum isolates* do not harbor dsRNA viruses consistent with its dsRNA gel profile result (Fig. 4.5). Instead, *F. proliferatum* has (+)ssRNA mitovirus and (-)ssRNA mymonavirus. Moreover, it is the first time that the *F. proliferatum* virome has been explored, and mitovirus and mymonavirus are found in this species.

Since we did not expect to find many viruses in the *F. proliferatum* collection, it is interesting to see that mitovirus are so widespread, and FprMV1 to 3 shared similarities with other mitovirus in *Fusarium* spp. and other fungi. Recent studies revealed mitovirus sequences' presence in fungi and plants (Bruenn, Warner, and Yerramsetty 2015). Furthermore, the origin of mitovirus and their transmission among plants and fungi have attracted attention; maybe the origin in mitochondria could give a more plausible explanation (Roossinck 2019).

When it comes to origin and evolution, the classification of viruses should also be mentioned. The *Polymycoviridae* was reported to have an unprecedented dynamic nature in terms of genomic element number and sequence (Kotta-Loizou and Coutts 2017). And in Chapter 1, figure 1.6 shows the genome evolution mechanisms of RNA viruses and indicates that segmentation is a flexible process that is not strong enough to be a taxa-defining trait (Shi *et al.,* 2016; Ladner *et al.,* 2016). According to the above, maybe we should consider better taxa-defining traits for virus taxonomy. In *F. poae* collection, a polymycovirus, FpPmV1 was detected, but unfortunately, the complete genome was not obtained; this may be the difficulty

caused by its unprecedented dynamic genome and co-infection with multiple viruses in *F. poae*.

After exploring the virome in two *Fusarium* spp. collections, we are curious to see if these "native" viruses affect their host. Please move to Chapter 4.

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CHAPTER 4

The Virome's Effect on Their Fungal Hosts (Ongoing)

1. Introduction

Previous studies revealed that hypovirulence-associated mycovirus transmission includes cytoplasmic exchange during anastomosis or by spores (Fig. 4.1) (Nuss 2005). Most mycovirus infections are asymptomatic and complex (Hillman, Annisa, and Suzuki 2018; Nuss 2005). What is more complicated is that fungi are often multi-infected by viruses (Osaki *et al.,* 2016), and it seems common in nature (Tran *et al.,* 2019; Khalifa and Pearson 2013; Sahin, Keskin, and Akata 2021; Chun and Kim 2021).

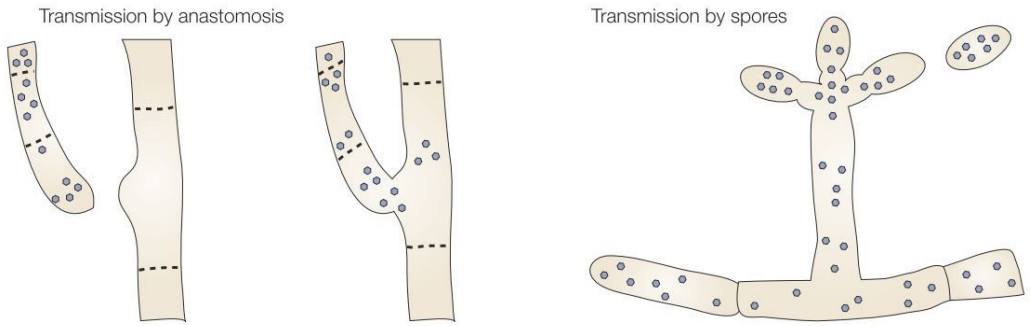


Figure 4.1 Primary modes of hypovirulence-associated mycovirus transmission (Nuss 2005).

Through recent research, a lot of paradigmatic rules of life for viruses of microorganisms are expanded. For example, viral infections can result in various outcomes for the host, and not all are bad; the virus can communicate by hijacking the host's communication system; viruses can infect other viruses (Correa *et al.,* 2021; R. Zhang *et al.,* 2016).

From the results mentioned above, we can infer that the viral community of fungi is a complicated system and will be attractive to explore. In this chapter, the virome of each fungal isolate was studied as single entity.

2. Materials and Methods

2.1 Fungal isolate cultivation

Mono-conidium colonies and cultivate conditions were described in Chapter 2 (2.1).

2.2 The virome composition of each fungal strain

The virus presence was detected using random primer cDNA synthesis and specific primer RT-PCR in all the fungal isolates. The protocol was described in Chapter 3 (2.3)

2.3 Fungal growth rate

To assess fungal growth rates, 5 mm diameter agar disks from the margins of actively growing colony of *30 F. poae* and *21 F. proliferatum* were transferred onto 9 cm diameter Petri

dishes containing 20 ml PDA and then incubated at 20°C. The diameter of *F. poae* colonies was measured at 24 h and 48 h post-inoculation (hpi). The hyphal growth rate (mm/d) of the two strains was calculated as equation 1 (L. Zhang *et al.*, 2009); this experiment was repeated three times:

$$Growth\ rate = \frac{48\ hpi\ diam. - 24 hpi\ diam.}{2} \quad (1)$$

2.4 Colony anastomosis within the selected *Fusarium* strains

Agar disks (5 mm diameter) from the margins of the actively growing colony were transferred and placed on a PDA plate, as shown in Figure 4.2 below. After 14-21 d, anastomosis appeared. It is pretty apparent to observe from the front and back sides through the light. The number of strains that could anastomose with it was counted, including itself. This experiment was conducted three times.

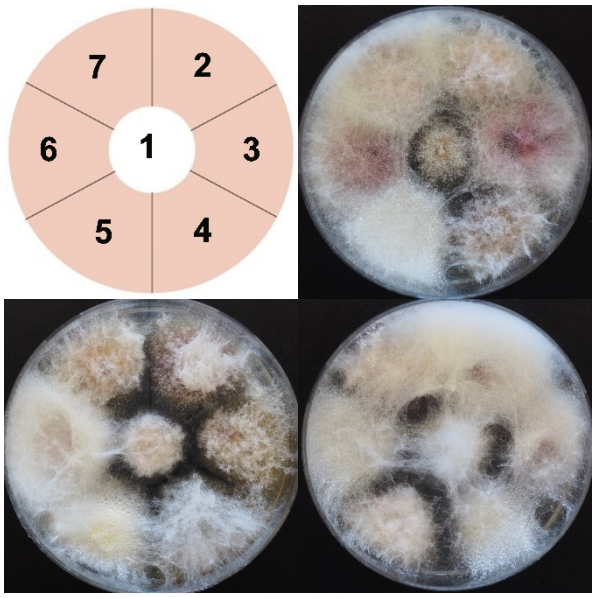


Figure 4.2 The experiment template of colony anastomosis and the plate appearance after 14-21 days

2.5 Curing fungi from viruses

Cycloheximide (200 μM) was used to eliminate mycovirus from the fungal host. The experiment workflow is shown in Figure 4.3. Based on results 3.1, there are dominant viruses in *F. poae*, and F. 1080 and F.1226 happened to harbor all these viruses. Thus F.1080 and F.1226 were chosen for the curing experiment.

Figure 4.3 The workflow of the curing experiment. Orange color refers to the medium that contains

cycloheximide (200 μM), the white refers to the pure medium. The triangle▲ refers to a liquid medium in flasks, the circle◯ refers to solid medium in plates.

The protocol used is similar to the previous study (Bhatti *et al.,* 2011), with some modifications. First, the highest temperature that strains can bear was tested, and this temperature (34℃) was used in the following cultural steps (data not shown).

Shake strain F.1080 and F.1226 for 7 days at 34℃, 125 rpm, in 20ml Bean medium containing cycloheximide 200 μM to get the conidia solution.

Load the conidia liquid on water agar (cycloheximide 200 μM), spread over the water agar medium, and discard the excess conidia liquid. Culture at 34℃ for two days.

Cut the mono-conidium colony's hypha tip by blade and place it on PDA (cycloheximide 200 μM) and culture for 1d, take the hypha tip, put it on PDA plates without cycloheximide. The mycelia were collected for the following dsRNA extraction and RT-PCR to check for virus elimination.

2.6 Pathogenicity of the selected *Fusarium* strains

First of all, a preliminary experiment was conducted with durum wheat and common wheat, and fungal conidium suspensions with different concentrations to understand the best concentration and suitable wheat material. In the end, the durum wheat and 1×10^7 suspension were chosen for the following experiment (data not shown).

Culture fungi in V8 medium for one week and count the concentration of fungi conidia. The conidia concentration was diluted to 10^7.

Surface sterilized the durum wheat seeds with 2% V/V NaOCl solution for 5min, wash with sterilized H_2O 5-6 times, and dry the seeds.

Seeds were placed in the conidial suspension added with Tween 20 (1 μl of Tween 20 every 1 ml of conidial suspension) for 2 h. Seeds immersed in sterile water and Tween 20 were used as the negative control.

A further germination test was performed in two different ways. One was using a modified version of the in vitro Petri-dish test described by (Purahong *et al.,* 2012).

Two moistened filter papers (Whatman filter paper N.1) were placed on the bottom and the lid of each plate. Seeds were placed between wet filter papers. The plates were then put into sterile plastic bags with a water-soaked cotton ball to obtain the appropriate moisture

conditions. For this trial, three plates containing ten seeds each were used as replicates for each isolate.

They were then incubated in an incubator at 22°C in the dark. The germinated seeds were counted 2 days after inoculation (DAI), and this value was set at 100% germination, and the healthy coleoptiles were counted every day from 3 to 6 DAI. The coleoptile length was measured at 6 DAI.

The second pathogenicity test was conducted as described before (Yli-Mattila *et al.,* 2018) with modification. The seeds were sterilized with 2% V/V NaOCl solution for 5min, washed with sterilized H_2O 5-6 times, and soaked for 24 h in sterile water. The swollen grains were then placed over the fungal culture grown for a week on PDA, 10 grains per Petri dish in triplicate. Placed grains on PDA without fungi as control. The measurements are the same as the test with filter papers. Experiments were repeated in duplicate.

The germination rate reduction, Coleoptile length reduction and Petri-dish aggressiveness index (APindex) were calculated as follows (Purahong *et al.,* 2012):

$$AUHPC = \frac{B1+2B2+2B3+2B4+B5}{2} \quad (2)$$

$$AUHPC_{standard} = \frac{400-\overline{AUHPC}}{350} \quad (3)$$

$$Gr = \frac{NGc-NGt}{NGc} \quad (4)$$

$$Clr = \frac{\overline{Clc}-\overline{Clt}}{\overline{Clc}} \quad (5)$$

$$A_p index = \frac{\overline{AUDPC}_{standard}+\overline{Gr}+\overline{Clr}}{3} \quad (6)$$

Equation notes:
(2) AUHPC = area under healthy tissue progress curve, B1–B5 = percent of healthy coleoptile from 2 to 6 DAI respectively (B1 always = 100%);
(3) AUDPCstandard = standardized area under disease progress curve,
(4) Gr = germination rate reduction, NGc and NGt = number of germination seed in control and F. poae treatments,
(5) Clr = coleoptile length reduction, Clc and Clt = coleoptile length in control and in F. poae treatments.
(6) APindex = Petri-dish aggressiveness index

2.7 Protoplast preparation, viral particle purification, and transfection assays (ongoing)

The protocol mainly follows the one described before (Ramamoorthy *et al.,* 2015) with some modifications.

2.7.1 Protoplasting

Strain F.1398 and F.1085's conidia (1×10^7 conidia) were inoculated in 100 ml PDB in 500 ml flasks; each strain can make 3-4 flasks to obtain 1g mycelium. They were incubated for 18 h-48 h at 28℃, 150 rpm on a rotary shaker. Harvest young mycelium by filtration through one layer of autoclaved MiracIoth (Calbiochem) (1 g approximately).

Wash with washing buffer (1 M KCl, pH 5.8, and autoclave sterilized.) while filtering it through the MiracIoth paper. Dry out the mycelium by pressing on paper.

Resuspend in 20 ml of ice-cold enzyme solution in a 50 ml falcon, mix thoroughly by hand and transfer the mixture into a sterilized 100ml flask. Incubate on ice for 5 min. Then incubate 3 h at 30°C, 50 rpm (check and count the protoplast concentration at 2 h, stop incubation when it has enough).

Collect protoplasts by filtration with MiracIoth, save the flow through in 50 ml falcon.

Add 20 ml washing buffer and centrifuge at 4000 g, 4°C for 10 min. Discard the supernatant and resuspend the pellet in 100μl ice-cold STC buffer (1 M sorbitol, 50 mM tris with a pH value of 8.0, and 50 mM $CaCl_2{\cdot}2H_2O$). Take 10 μl and dilute till 100 μl, count the protoplasts, and adjust the concentration to $2x10^8$ protoplasts/ml.

Protoplasts can be saved in the STC buffer at a concentration of 10^8. Add 5% DMSO before putting in -80℃.

2.7.2 Transfection

Purification of viral particles was described in Chapter 2 (2.6.1 and 2.6.2) and observed the particles under TEM. Save the particles for transfection use.

Mix 10 μl purified virus particles with 10 μl of protoplasts at a concentration of $2x10^8$ protoplasts/ml and added 85 μl of STC buffer. Mix gently by inversion in a falcon. Incubate on ice for 30 min.

1 ml of STC buffer containing 40% PEG 4000 was dropwise added. Mix gently for 15 s and incubate at room temperature for 25 min.

Add 1.2 ml of ice-cold STC buffer and centrifuge 5 min/5000 rpm. Resuspend pellet in 2 ml of cold STC buffer.

Place 500 μl protoplasts on the center of a 9 cm Petri dish (cut the top of the tips before using Pipettes) and mix with 20 ml of 42 °C regeneration medium (the final formula is 1 mM NaNO3, 0.1% yeast extract, 1.5% agar, pH 6.5 and1.2 M Sucrose in 5 mM TRIS buffer. Because

the sucrose cannot be autoclaved, they need to be prepared separately and mix before use), poured into Petri dishes, and incubated overnight at 24°C in the dark.

2.7.3 Detection of the virus presence

The regenerated fungi mycelia were collected and used for total RNA extraction using the Spectrum plant total RNA kit (Sigma-Aldrich, St. Louis, MO, USA).

By using a qPCR master mix kit with specific primers, the viral presence can be detected. This experiment conducted RT and RT-qPCR in one reaction in a 20 µl mixture (10 µl qPCR master mix, 1.8/1.8 µl primer F/R, 0.17 µl CXR, 1 µl M_MLV (1:100 dilution), 3.23 µl H2O, 2 µl RNA).

2.8 Statistic analysis

Multiple linear regression (Stepwise method, Criteria: Probability-of-F-to-enter ≤0,050, Probability-of-F-to-remove≥0,100) was conducted to assess which virus presence contributed more to the total variance change of growth rate of *F. poae* which showed in figure 4.6 (A).

Differences among treatments were evaluated by analysis of variance (ANOVA) and Duncan method (P<0,05) for post hoc multiple comparisons.

ANOVA and multiple linear regression were done with SPSS 19.0 (IBM, USA). Principal components analysis (PCA) was performed by CANOCO 4.5 for windows. We focus scaling on inter-species correlations and center by virus species.

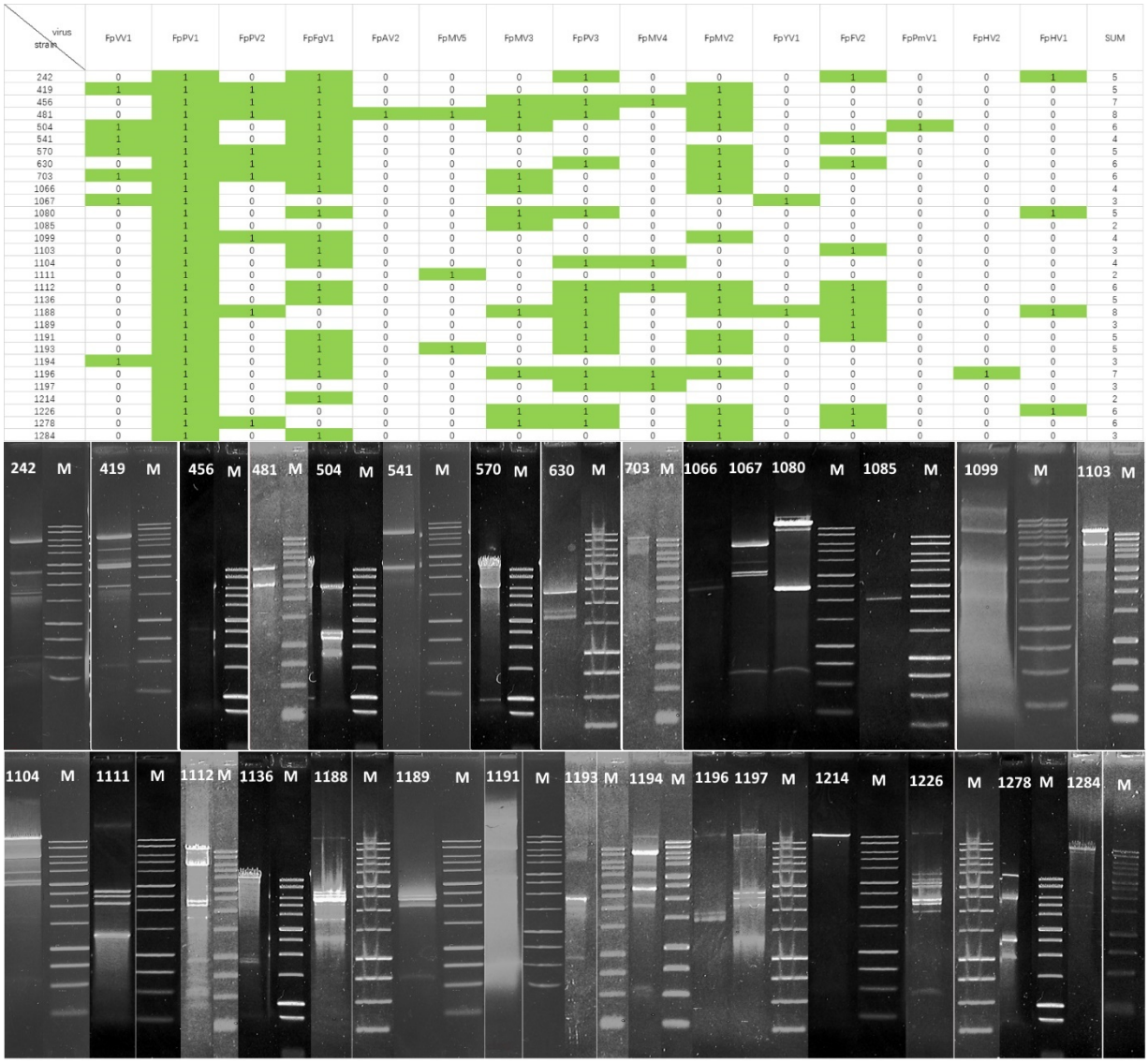
3. Results and Discussion

3.1 The virome of selected fungi

From the Chapter 3 data analysis, contigs that are associated with the viral genomes are used to designe specific primers.

30 *F. poae* and 9 *F. proliferatum* fungi mycelia were collected and used for dsRNA or total RNA extraction using the method described in Chapter 2 (2.2.3) or the Spectrum plant total RNA kit (Sigma-Aldrich, St. Louis, MO, USA).

By RT-PCR, specific bands with the expected size were observed and purified, then sent for sanger sequencing, and the virome composition in *F. poae* and *F. proliferatum isolates* are shown in figure 4.4 and figure 4.5, respectively.



virus / strain	FpVV1	FpPV1	FpPV2	FpFgV1	FpAV2	FpMV5	FpMV3	FpPV3	FpMV4	FpMV2	FpYV1	FpFV2	FpPmV1	FpHV2	FpHV1	SUM
242	0	1	0	1	0	0	0	1	0	0	0	1	0	0	1	5
419	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	5
456	0	1	1	1	0	0	1	1	1	1	0	0	0	0	0	7
481	0	1	1	1	1	1	1	1	0	1	0	0	0	0	0	8
504	1	1	0	1	0	0	1	0	0	1	0	0	1	0	0	6
541	1	1	0	1	0	0	0	0	0	0	0	1	0	0	0	4
570	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	5
630	0	1	1	1	0	0	0	1	0	1	0	1	0	0	0	6
703	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	6
1066	0	1	0	1	0	0	1	0	0	1	0	0	0	0	0	4
1067	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	3
1080	0	1	0	1	0	0	1	1	0	0	0	0	0	0	1	5
1085	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	2
1099	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	4
1103	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	3
1104	0	1	0	1	0	0	0	1	1	0	0	0	0	0	0	4
1111	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	2
1112	0	1	0	1	0	0	0	1	1	1	0	1	0	0	0	6
1136	0	1	0	1	0	0	0	1	0	1	0	1	0	0	0	5
1188	0	1	1	0	0	0	1	1	0	1	1	1	0	0	1	8
1189	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	3
1191	0	1	0	1	0	0	0	1	0	1	0	1	0	0	0	5
1193	0	1	0	1	0	1	0	1	0	1	0	0	0	0	0	5
1194	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	3
1196	0	1	0	1	0	0	1	1	1	1	0	0	0	1	0	7
1197	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	3
1214	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	2
1226	0	1	0	0	0	0	1	1	0	1	0	1	0	0	1	6
1278	0	1	1	0	0	0	1	1	0	1	0	1	0	0	0	6
1284	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	3

Figure 4.4 virome composition in each *F. poae* strain and its dsRNA gel profile

virus / strain	FprMV1	FprMV2	FprMV3	FprMnV1	SUM
CREA-17	0	0	0	0	0
1129	0	0	0	1	1
1304	0	0	0	0	0
1320	1	1	1	0	3
1398	0	0	0	0	0
1452	1	0	1	0	2
1504	0	0	0	1	1
R.96	0	0	0	0	0
R.422	0	0	0	0	0

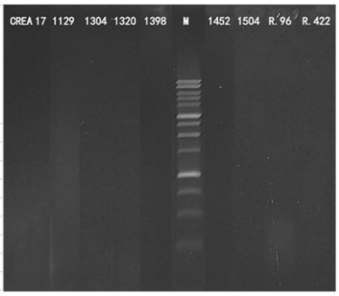


Figure 4.5 virome composition in each *F. proliferatum* strain and its dsRNA gel profile

All the *F. poae isolates* are infected by 2 to 8 viruses, and FpPV1 (infect 30 *F. poae isolates*)

is present in all the strains; next is FpFgV1 (22), followed by FpMV2 (18) and FpPV3 (16), FpMV3 and FpMV2 are tied for fifth (11). While in *F. proliferatum*, the situation is different: each isolate is infected by 0 to 3 viruses, and there is no significant dominant virus among these nine strains collection.

3.2 Assessing the impact of virome composition on the host characteristics

The growth rate and anastomosis number of 30 *F. poae* and 21 *F. proliferatum* were analyzed and shown in Figure 4.6-A&B, separately.

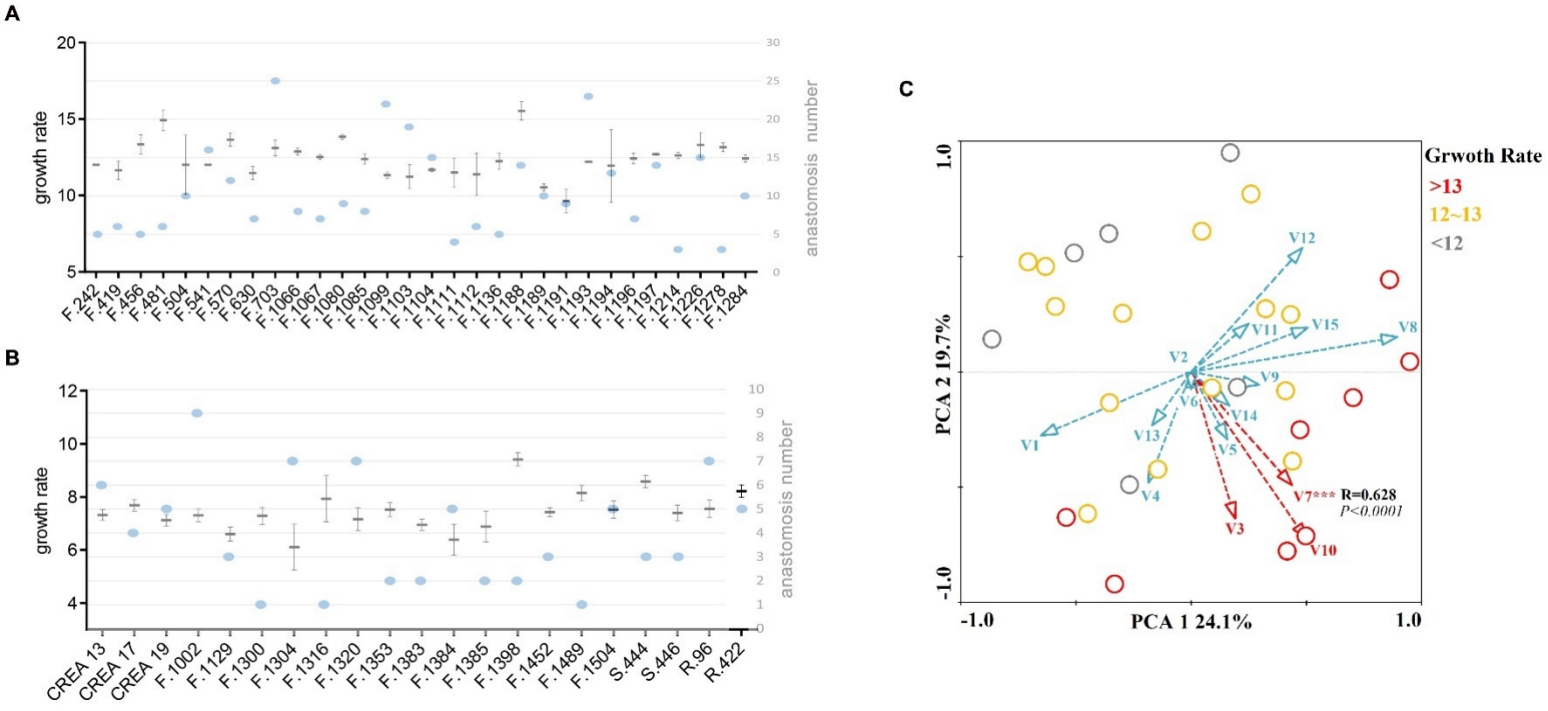


Figure 4.6 Assess the effects of virome composition on fungi characteristics. (A) The growth rate and anastomosis number of 30 *F. poae*. The bars refer to growth rate, and the blue circle refers to the number of anastomosis colonies of strains. **(B)** The growth rate and anastomosis number of 21 *F. proliferatum*. The bars refer to growth rate, and the blue circle refers to the number of anastomosis colonies of strains. **(C)** Principal component analysis (PCA) of viruses from 30 different species of *Fusarium poae*, which were in circle symbols. The red, yellow, and grey color showed the growth rate was higher than 13, between 12 to 13, and lower than 12, respectively. Dashed arrows represent viruses that we found in this experiment. The asterisk (*) is the significance when we use multiple linear regression（Method: Stepwise, Criteria: Probability-of-F-to-enter≤0.050, Probability-of-F-to-remove≥0.100）to predict the relationship between viruses present and growth rate of *Fusarium poae*. V1: FpVV1; V2: FpPV1; V3: FpPV2; V4: FpFgV1; V5: FpAV2; V6: FpMV5; V7: FpMV3; V8: FpPV3; V9: FpMV4; V10: FpMV2; V11: FpYV1; V12: FpFV2; V13: FpPmV1; V14: FpHV2; V15: FpHV1.

Principal component analysis (PCA) analyzed viruses from 30 *F. poae* (Figure 4.6-C). The first two components could explain 43.8 % of the cumulative percentage variance of species

data. Dashed arrows represent different viruses that we found in all 30 *F. poae* (fig. 4.4). Arrows pointing in the same direction correspond to viruses that are predicted to have a significant positive correlation, like the arrows of V3 (FpPV2), V7 (FpMV3), and V10 (FpMV2). According to the direction of those arrows, we found that the growth rate of different *F. poae* was increased. An interpretation derived from PCA statistics analysis was that poae with these three viruses grew faster than the species without them. Especially V7 (FpMV3), which significantly correlated with the growth rate (R=0,628, P<0.001), analyzed by multiple linear regression. These need to be further proved by experiments.

3.3 Pathogenicity of the cured fungi

After a series of cycloheximide treatments, 80 colonies were selected and tested of the virus presence for F.1226 and F.1080, respectively. After transfer and regrow the "cured" colonies on PDA for 3-pass, their virome composition were tested.

The original F.1226 contains six viruses: FpPV1, FpPV3, FpMV3, FpHV1, FpMV2, and FpFV2; and the original F.1080 contains six viruses: FpPV1, FpPV3, FpMV3, FpHV1, FpMV2, and FpFgV1. These two fungi are chosen because they harbor the top-five prevalent virus among the *F. poae* collection and an FpHV1 that belongs to *Hypoviridae*.

F.1226-5 and 12 were found to have eliminated the FpHV1, while F.1080-2 and 13 have lost FpPV3, and F.1080-5 and 23 have lost FpFgV1. Their pathogenicity was tested and showed in figure 4.7.

As we can see from the figure, the pathogenicity of F.1226-5 and 12 were significantly increased compared to the original strain, which indicated that FpHV1 might be associated with the host hypovirulence phenotype. The pathogenicity of the cured F.1080 strains was all decreased; the pathogenicity of strains that eliminated FpFgV1 has decreased more than the one losing FpPV3.

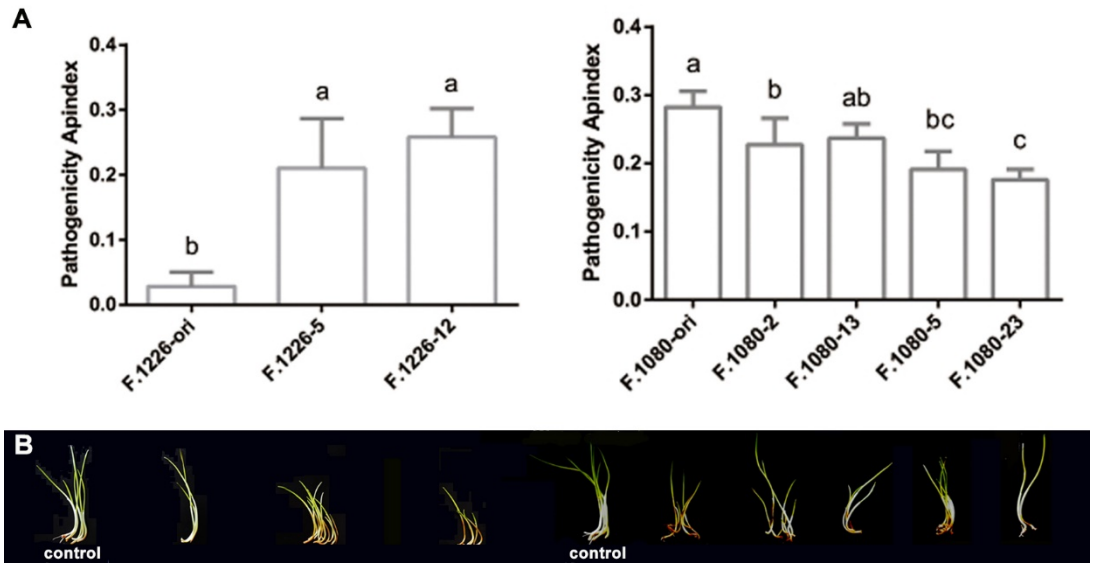


Figure 4.7 The pathogenicity of F.1226 and F.1080 strains and their cured colonies. **(A)** The pathogenicity apindex of the original F.1226 strain (F.1226-ori), cured F.1226-5, 12; and the original F.1080 strain (F.1080-ori), cured F.1080-2,13,5, and 23. Different small letters represent the significant difference at level P<0.05 (Duncan) among different treatments. **(B)** Coleoptiles were treated by different fungi. Their positions correspond to the treatment groups above.

3.4 Transfection assay using fungal protoplast (ongoing)

Viral particles used in this study are FprMnV1 particle that purified from *F. proliferatum* F.1129; a mixture of particles purified from *F. poae* F.1080; and an ssDNA virus, Botrytis cinerea ssDNA virus 1 (BcssDV1, MN625247), belongs to the family *Genomoviridae*, which was given by Ana Ruiz-Padilla (Ruiz-Padilla et al. 2021).

FprMnV1 virions are filamentous, 10-20 nm in diameter, ~1500 nm in length (fig. 4.8).

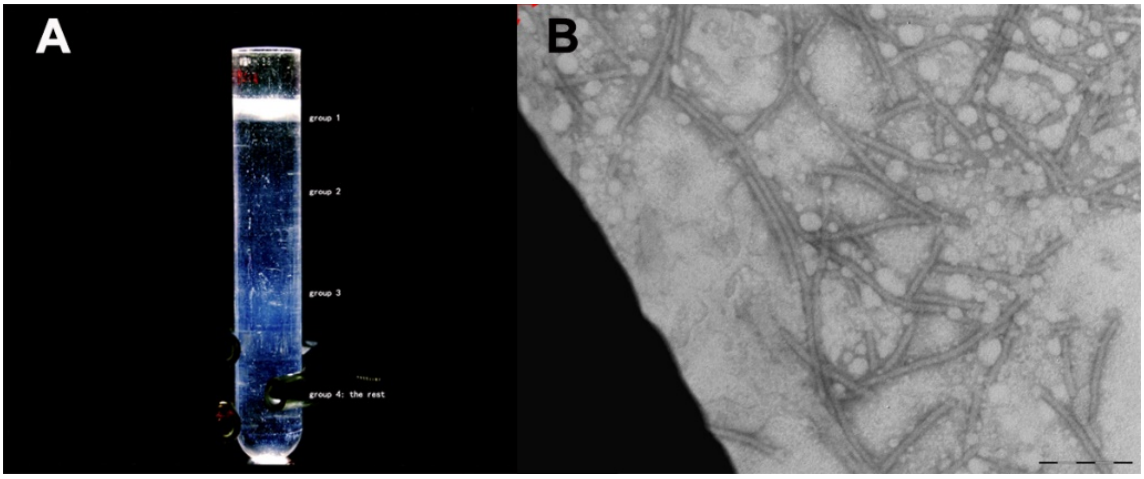


Figure 4.8 The FprMnV1 particle purification. **(A)** the sucrose gradient during the viral particle purification. **(B)** Morphology of FprMnV1 particle, the bar refers to 200nm.

Protoplasts were generated from *F. poae* strain F.1085, which contains two viruses, and *F. proliferatum* strain F.1398, which is free of mycovirus (Fig. 4.9-A). After transfection with viral

particles, they were regenerated (Fig. 4.9-B).

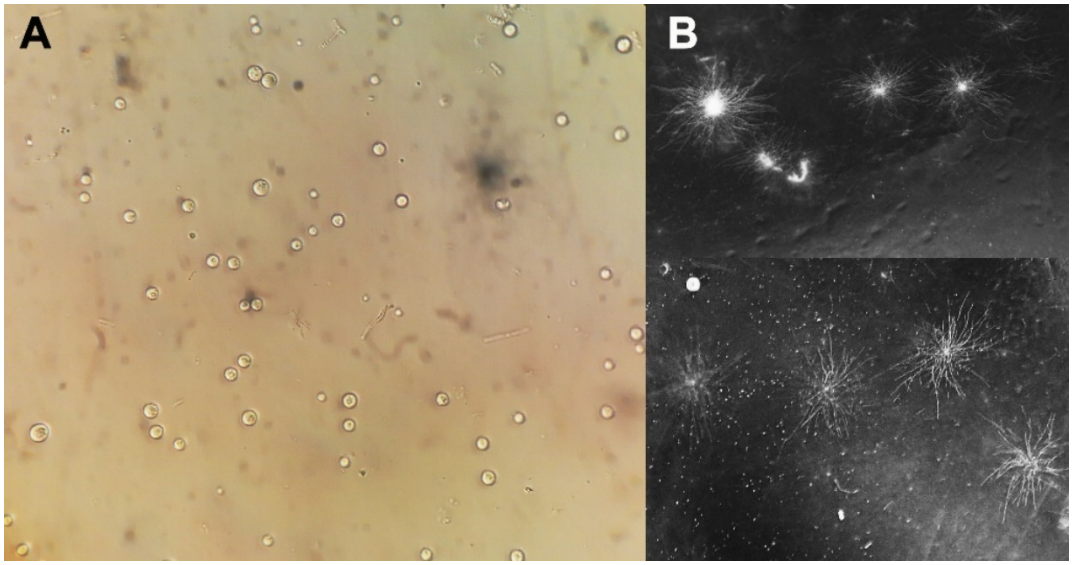


Figure 4.9 The protoplast observation and regeneration. **(A)** The protoplast was observed under 40×microscope; **(B)** Regenerated protoplast. The photo is processed in black and white.

There are 8 groups of treatment: (1) F.1398-control, (2) F.1398+FprMnV1, (3) F.1398+BcssDV1, (4) F.1398+viral mix; (5) F.1085-control, (6) F.1085+FprMnV1, (7) F.1085+BcssDV1, (8) F.1085+viral mix.

Sixty regenerated colonies were selected from each treatment and transferred on PDA, after 3 d, transferred to a new PDA plate to get the second pass. And viral detection using RT-qPCR was conducted with the mycelia collected from the second pass colonies.

Unfortunately, no transfected viral signal was detected in the regenerated colonies so far.

4. Conclusion and Future Perspective

F. poae collection has a much more diverse virome compared to *F. proliferatum* collection. All the *F. poae isolates* are multi-infected by 2 to 8 viruses, and its dominant virus is partitivirus FpPV1, which infects all 30 strains. Next is FpFgV1 (22), followed by FpMV2 (18) and FpPV3 (16), FpMV3 and FpMV2 are tied for fifth (11). While each *F. proliferatum* isolate is infected by 0 to 3 viruses, and there is no significant dominant virus among these nine strains collection. The reason behind this different virome diversity is worth exploring.

Since *F. poae* harbor such a diverse virome, we are curious to see the virome effect on the *F. poae* characteristics. Mycovirus transmission methods are mainly cytoplasmic exchange during anastomosis or by spores (Fig. 4.1) (Nuss 2005). Thus, the growth rate and the number of anastomosis strains were measured and examined. An interpretation derived from PCA

statistics analysis was that poae with FpMV3, FpPV2, and FpMV2 grew faster than the species without them. And FpMV3 has a significant correlation with the *F. poae* growth rate (R=0,628, P<0.001), analyzed by multiple linear regression. These need to be further proved by experiments.

FpMV3 seems to have positive effect on *F. poae*, while some mitovirus infection may bring negative effect on their host, it has been reported that mitovirus infection is often associated with reduced virulence of phytopathogenic fungi (Xiao *et al.,* 2014; Wu *et al.,* 2007; Khalifa and Pearson 2013).

The analysis of anastomosis number and virome showed that they do not have a significant correlation; it confirms that fungi vegetative compatibility is more affected by their genome.

The curing experiment results indicate that FpHV1 could reduce the pathogenicity of *F. poae* using the Petri-dish test. Moreover, dsRNA viruses such as FpFgV1 and FpPV3 could increase the fungi's aggressiveness. Similarly, previous studies also showed that FgHV2 is associated with its host hypovirulence phenotypes (Li *et al.,* 2015), and partitives infection is symptomless, and even some are clearly beneficial to their plant host (Roossinck 2019).

However, in our case, which virus influenced more on the hypovirulence phenotypes is still a question. By the single virus? Or by the changes of virome and other viruses present in the fungi. Although obtaining a virus-free isolate from a multi-infected strain is difficult, as in this study, the top-five dominant viruses are difficult to eliminate, but it is worth trying. Because mycoviruses are common in nature, and if we want to make a biocontrol mycovirus agent with long-lasting effects, the mycoviral community must be considered.

Finally, we know little about the relationship between virus-virus, virus-fungi. Trying to understand these ecosystem-level interactions is a fascinating work. Metagenomics, bioinformatic studies, and molecular experiments are all likely to play an important role.

Maybe in the near future, an invincible combination of another top-five dominant virus will be used in controlling plant disease.

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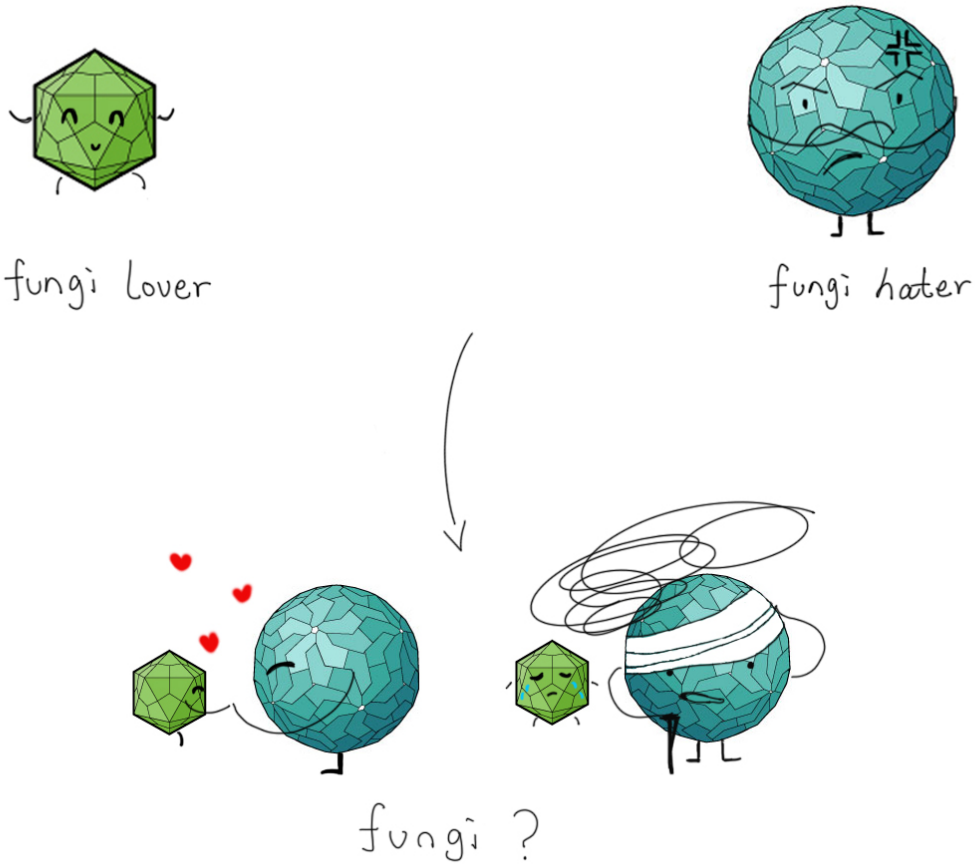
CHAPTER 5

Conclusion and Future Perspectives



We know little about viruses than other lifeforms. Recently, metagenomics has helped us to gain a better understanding of the virosphere. Using the metagenomic method can indeed accelerate the searching for potential biocontrol mycoviruses, at the same time exploring the virosphere.

Fusarium spp. contain plenty of mycoviruses in different genome types (Li *et al.,* 2019), and some genomes of *Fusarium* spp., like *F. graminearum* strain PH-1, are sequenced. Thus, it is suitable for studying mycovirus structure, virus-fungal interaction, virus-host evolution, etc. (Li *et al.,* 2019).

1. What We Learned from *F. poae*

Next-Generation Sequencing (NGS) analyses of 30 *F. poae isolates* revealed an extreme diversity of mycoviruses. Bioinformatic analysis shows that contigs associated with viral genomes belonging to the families: *Hypoviridae*, *Mitoviridae*, *Partitiviridae*, *Polymycoviridae*, proposed *Alternaviridae*, proposed *Fusagraviridae*, proposed *Fusariviridae*, proposed *Yadokariviridae*, and *Totiviridae*. Fifteen viruses were identified, and 12 viruses' complete genomes were obtained by assembling contigs and overlapping cloning sequences.

All the *F. poae isolates* analyzed are multi-infected. Fusarium poae partitivirus 1 appears in all the 30 strains, followed by Fusarium poae fusagravirus 1 (22), Fusarium poae mitovirus 2 (18), Fusarium poae partitivirus 3 (16), and Fusarium poae mitovirus 2 and 3 (11). They are the "top-five dominant virus".

Mycovirus transmission methods are mainly cytoplasmic exchange during anastomosis or by spores (Nuss 2005). Thus, the growth rate and the number of anastomosis strains were measured, the multiple linear regression result indicated that Fusarium poae mitovirus 3 (FpMV3) is significantly correlated with the fungal growth rate ($R=0{,}628$, $P<0.001$). Furthermore, the principal component analysis of the virome composition from 30 *F. poae* showed that the presence of FpMV3, Fusarium partitivirus 2 (FpPV2), and Fusarium mitovirus 2 (FpMV2) could increase the *F. poae* growth rate, but FpPV2 and FpMV2 effect is not as significant as the FpMV3.

The curing experiment and pathogenicity test indicated that Fusarium poae hypovirus 1 (FpHV1) might be associated with the host hypovirulence phenotype. Moreover, dsRNA

viruses such as FpFgV1 and FpPV3 could increase the fungi's aggressiveness. Similarly, previous studies also showed that FgHV2 is associated with its host hypovirulence phenotypes (Li *et al.,* 2015), and partitives infection is symptomless, and even some are clearly beneficial to their plant host (Roossinck 2019).

2. What We Learned from *F. proliferatum*

The viruses in *F. proliferatum* collections resulted in lower diversity and abundance. The identified mycoviruses belong to the family *Mitoviridae* and *Mymonaviridae*. Interestingly, most *F. proliferatum isolates* are not multi-infected. The complete genomes of four viruses were obtained by assembling contigs and overlapping cloning sequences.

In contrast, 9 *F. proliferatum isolates* do not harbor dsRNA viruses consistent with its dsRNA gel profile result. Instead, *F. proliferatum* has (+)ssRNA mitovirus and (-)ssRNA mymonavirus. Moreover, it is the first time the *F. proliferatum* virome has been explored, and mitovirus and mymonavirus are found in this species.

3. Discussion and Future Perspective

Metagenomic technology sheds light on virus research. However, massive sequencing data also proposed new challenges in virus taxonomy and classification. For example, *Polymycoviridae* was reported to have an unprecedented dynamic nature in terms of genomic element number and sequence (Kotta-Loizou and Coutts 2017), which indicates that segmentation is a flexible process that is not strong enough to be a taxa-defining trait (Shi *et al.,* 2016; Ladner *et al.,* 2016). According to the above, maybe we should consider better taxa-defining traits for virus taxonomy.

The massive complex viral genome structures raise more questions about driving evolutionary selective pressures and how they can clarify interacting with the host. Furthermore, new protocols that enrich the viral materials for NGS should be explored, and the biases associated with different methods should be minimized (Callanan *et al.,* 2021).

The virome diversity of *F. poae* and *F. proliferatum* collections has a big difference; the virome of *F. proliferatum* is much simpler than *F. poae*, which might be related to *F. proliferatum* defense mechanisms or the different timescale when the two species appeared; both are assumptions and need future exploration.

FpMV3, FpPV2, and FpMV2 have a beneficial effect on *F. poae*, and this result is

consistent with some previous reports (Roossinck 2019). However, the mitovirus infection effect is controversial; it has been reported that it is often associated with reduced virulence of phytopathogenic fungi (Xiao *et al.,* 2014; Wu *et al.,* 2007; Khalifa and Pearson 2013), which is a harmful effect. So why do viruses from the same genus have the opposite effect on their host? One reason might be the host and their genome; and some viruses were found and evaluated in their original host, while others were not. The other hypothesis is that the fungal virome is a community, and viruses face environmental pressure from the other viral neighbors, making them behave variously.

As in our case, who influenced more on the growth rate and hypovirulence phenotypes is still a question. By the single virus? Or by the changes of virome and other viruses present in the fungal. Or the presence of other viruses might influence the changing level. Although obtaining a virus-free isolate from a multi-infected strain is difficult, as in this study, the top-five dominant virus are difficult to eliminate, but it is worth trying. Because mycoviruses are common in nature, and if we want to make a biocontrol mycovirus agent with long-lasting effects, the mycoviral community must be considered. Maybe in the near future, an invincible combination of another top-five dominant virus will be used in controlling plant disease.

Future studies should continue screening mycoviruses, identifying viral and host factors involved in the interactions and mycotoxin production pathway, exploring the virus-virus interaction in a single strain. It is also worth study the viruses that do not have a significant effect on the host. They may become a suitable candidate as a gene vector or a good material for fundamental mechanism studies.

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Appendix

Primers, Buffer and Medium Used in the Study

Table 1 Functional specific primers of the viruses in F. poae and F. proliferatum

Reference virus name	Primer name	Sequence (5'-3')
Fusarium poae dsRNA virus 3 isolate SX63	FpdsV3-529-R	CGTAGGGTGCTCAGTAGGAA
	FpdsV3-5725-F	CTAGTCTGGAATGCTGGCAAC
	FpdsV3-6150-R	GAATAGCGAGGTTCAAGGCGT
	FpdsV3-9020-F	CTCAAACCCTATGCACCGGT
	FpdsV3-9374 R	GTCATGTTCTGCGGTGGGTA
Fusarium poae partitivirus 2 genomic RNA, segment 1	FpPV2-s1-1438-R	ACGGGTCTGATCTTCTTGGG
	FpPV2-s1-1194-F	CCGCACTGAGTACCACCATA
	FpPV2-s1-2299-R	GATGGGACGTTCAGGCATAGA
	FpPV2-s1-2002-F	ACTGCGTCCTTGGTGATGAT
	FpPV2-s1-2456-R	CGGGCGGAACTTTTCGTATACC
	FpPV2-s1-2370-F	GCTAACTGTGGACAAGATGCTC
	FpPV2-s1-216-F	TGAACCTGGCATTCCCTTCC
	FpPV2-s1-622-R	TCGGTGAGGACGTGTTGTTT
Fusarium poae partitivirus 2 genomic RNA, segment 2	FpPV2-S2-1F	ACACAATGTCCCGTTTCG
	FpPV2-s2-365-F	ATCATCGCTTCCCCCTCCT
	FpPV2-s2-1577-R	GTGGGATTGAGCGACGTTTG
	FpPV2-s2-1223-F	CGTCAACCCCTACCTCCTTATG
	FpPV2-s2-2586-R	GGTTGTAGTGACGGTAGCTTGA
	FpPV2-s2-548-R-new race	TGCTTTGAGGGAGTGGGA
	FpPV2-s2-2492F-new race	CGACTCCACAAATGTCTTCC
	FpPV2-s2-160-F	CAAAATGTCTCGTTTCGGCC
	FpPV2-s2-545-R	TTTGAGGGAGTGGGACGTTG
	FpPV2-s2-2413R	CGGGGGTTGGTTTTTTTGG
Fusarium poae virus 1-240374 genomic RNA, segment 1	FpV1-s1-776-F	ACCCACCCGCATTAATCACG
	FpV1-s1-2328-R	ACTTTCGTCTAGATCGGCGC
	FpV1-s1-410-R	TTCGTGGGGAAAGCCTTGTT
	FpV1-s1-2085-F	AAACTCGTCGCACAGCTCTG
	FpV1-2369-F	CCTGGCTACCTCAAGATAGATG
	FpV1-2517-F	CCGAAATGGGATCTTGCTCA
	FpV1-S1-2870-R	CATCTATCCTTATCTGAGCGC
Fusarium poae virus 1-240374 genomic RNA, segment 2	FpV1-s1-285-F	ACTTCAATCGTTCCCCAGCT
	FpV1-s1-1001-R	TGGAGGTTCGTTTGGATTCG
	FpV1-s2-308-F	CTGTCCCACCATCCAATGCT
	FpV1-s2-1855-R	ACGGGAAAACAGGTTGTGGT
	FpV1-s2-1618-F	TGGTTCTGTCCTCTCCTCTCTA
	FpV1-s2-2608-R	GGCGGGGTTCTTCTTTGTGT
	FpV1-s2-599-R-new race	AGAGTGAGACAGTAGCCAG

	FpV1-s2-2471-F-new race	GAACTCCACCACGTTAGC
	FpV1-s2-893-F	TCTCGCTGGCTACATTCACA
Fusarium graminearum hypovirus 2 isolate FgHV2JS16, complete genome	FgHV2-614R-5'RACE	GCCTCACCTAACACTGTT
	FgHV2-12318F-3'RACE	CGGGGCATCTTCCACTTTA
	FgHV2-f-1249-F	GCGTTGGATGCATAGCTTGAGT
	FgHV2f-419 F	GCGTCTCGTCCAGTTTCGTA
	FgHV2f-438 R	TACGAAACTGGACGAGACGC
	FgHV2-3559-F	GGAGCCAAGCAGAAAAGGT
	FgHV2-4449-R	GCCTTAGACAAATCCTCGTC
	FgHV2-6408-F	GGGTAGACTGGAGCATTACA
	FgHV2-8033-R	GCTTGTGGTTTCCCTTGGTA
	FgHV2-4717-F	CCCACCAGTTCTTTGAGCCA
	FgHV2-5000-R	CGCCACTCATGAGCATTGTC
	FgHV2-5111-Rt	GGAAACCTAGGAACGAGTAG
Fusarium poae mitovirus 3 genomic RNA	FpMV3-402-F	CGTATCCGGGATAGACTAGT
	FpMV3-1087-R	CCGCATAGAACACTTAGTCTGG
	FpMV3-838-F	TCTGGCTCCTACTACTTTCC
	FpMV3-1498-F	GGGTTGTCTTTCTTCATGAGCAGG
	FpMV3-1218 F	TGCGGCATCTTCCCAATGAT
	FpMV3-1711 R	TCCCAATCACTCCATAACCTGC
	FpMV3-133-F	CGGACCGGAGCAAAACAGAA
	FpMV3-2396-R	GGTCAGTAGCGGCACTAGAT
	FpMV3-578-RACE-R	GGAAGTCCTGGTTCAATCTC
	FpMV3-2161-RACE-F	GGAAGGTCGTGAAGAGTGAT
	FpMV3-2,755-R	GTAATCCTGAAAGTCGTCAGCC
Fusarium poae hypovirus 1 genomic RNA, complete genome	FpHV1-9140-F	CTCAGACAGCACATCCAAGC
	FpHV1-9610-R	GGTTTTCCCTGAACTCCACATC
	FpHV1-9795-Rt	CCAATACAGGCACGAGACGA
Fusarium graminearum hypovirus 1 isolate HN10, complete genome	FgHV1-9376-F	CATGCATTACAAGAATCGGGGC
	FgHV1-9827-R	CGACGTACTTAGGGTTGTTGAG
	FgHV1-10159-Rt	CCTTTGAGCTCCTTGAGG
Botrytis cinerea partitivirus 2 strain QT5-19 RNA-dependent RNA polymerase gene，S1	BcPV2-S1-234-F	GCATTGTTCCCCAGCACTAG
	BcPV2-1531-R	TCGGGGTCTTTCAGGGTAGA
	BcPV2-1029-F	ACGATCAGCAACACCCATGA
	BcPV2-S1-859-F	CGTGGTGGATGGATGAAGCT
	BcPV2-S1-1202-R	GAGTTGGGTCTGTTGGAAGC
	BcPV2-RdRp-521-R	TGAGCAATACGGTTCTGCCA
	BcPV2-RdRp-1388-F	AACGCCGATTTGTCCACAGA
Botrytis cinerea partitivirus 2 strain QT5-19 capsid protein gene, complete cds, S2	BcPV2-S2-514R	GCTGAGAAGAGGGATTGACA
	BcPV2-CP-512-R	TGAGAAGAGGGATTGACA
	BcPV2-CP-1278-F	CGTTTCTTTCCCGACTCCGT
	BcPV2-S2-727-F	CCCTACGCCCTTCATTGGAA
	BcPV2-S2-1135-R	CTTGAGGGCGTAGGTGTA
Fusarium poae mycovirus 1 genomic RNA	FpMV1-442-F	CAGCGAGTATATGAAGCGGT
	FpMV1-1,070-R	CGGCTTCATTCATGGAACCT
	FpMV1-1,911-F	CTAACCACCCTAACGCTAG
	FpMV1-3430-F	CGCCCCTTCTACCGTTTCTT
	FpMV1-3809-R	GCCACGTTCTGCTCTAGGTT
	FpMV1-369-F	GCACCGCCATCTCTCAAAAC
	FpMV1-2063-R	GATTCGGTCTGTGATGGGCA

	FpMV1-2705-F	TCCTCCTAACATGGCCCAGA
	FpMV1-4511-R	TACGGGCAGCAGAATCTTGG
Aspergillus fumigatus polymycovirus 1	AfPV1-659-F	CCCGCGGTTCTTGAACTA
	AfPV1-1533-R	AGATTGTTGCCCTCGGTGTT
Fusarium graminearum alternavirus 1 isolate FgAV1,AH11 segment RNA1	FgAV1-214-F	CCAAAAACGTTCCGAGCCTT
	FgAV1-770-R	ACGCTTATGTTTGGTGCTCC
Fusarium graminearum alternavirus 1 isolate FgAV1 AH11 segment RNA2, complete sequence	FgAV1-RNA2-855-F	GCCCGTGCTCGTGATTGGTG
	FgAV1-RNA2-1496-R	CAACCCTTGCGATGAGTCTC
	FgAV1-RNA2-513-R	GCGACATCTCAAGCACAGT
	FgAV1-RNA2-1838-F	CTCGTTATCATAGGGTTCTGCC
	FgAV1-S2-1-F	GGCTGTGTGTTTGTTCTGGA
	FgAV1-S2-2470-R	GCCCCCAGTCCAATAAAACC
Fusarium poae victorivirus 1 genomic RNA, complete genome	FpVV1-5,132-F	CGACAAGATGCGGATTCACGT
	FpVV1-5,814-F	CCACCAGTCAACGAGTCGTT
	FpVV1-6,362-R	GGCCCGCTGTCTTAAACGTA
Fusarium poae fusarivirus 1 genomic RNA, complete genome	FpFV1-1-F	CTCACATAGAGAACCACGGGCT
	FpFV1-1,462-R	GTCTTCCTCCACAACTCCTT
	FpFV1-3,422-F	GTGTGGTATGTGACTGCTCA
	FpFV1-5,090-R	GTCCATCTCGTACCCAGCTT
	FpFV1-4,618-F	GCCCCGAAACTTGTTTAGGC
	FpFV1-6,395-R	CTTATCGGCTTCCCCCTCCATA
Fusarium poae mitovirus 4 genomic RNA, complete genome	31F2	ACACATTCATCAAAGAGGCTATGG
	31R	CTAGAGGCTGAGATAGGACGTAC
	FpMV4-1-F	AGCCTTTAAGCTTCAAGCGCTG
	FpMV4-1,334-R	GCCTTTGCAAACGATTCACCGA
	FpMV4-2,414-R	GTCTTTACGACCTGTGGGCT
Fusarium poae mycovirus 2 genomic RNA, complete genome	FpV2-3-F	CGGGGGCACACAACGATTTA
	FpV2-2,651-R	GACCCACTTGTCCACATCTC
	FpV2-2,001-F	GAACTTCGTGCTGGTGTTGA
	FpV2-4,242-R	ATGACTATAGCCAGCAGGTGTC
Fusarium poae mitovirus 2 genomic RNA, complete genome	FpMV2-134-F	GCTTATCACATACTGCGGCC
	FpMV2-1,591-R	GGGTACACTCGTAGGGCATT
	FpMV2-884-F	GATCCTGAGGGTAAGCGTAGAG
	FpMV2-2,418-R	CACCATGGGCAATTAGGGCATA
Fusarium poae partitivirus 1 strain FpPv12516 segment RNA1, complete sequence	FpV1-RP-615-F	ACCACTAACGCCACAGTATC
	FpV1-RP-1150-R	GCATAGTTGGACGTTGAAGG
Fusarium poae partitivirus 1 strain FpPv12516 segment RNA2, complete sequence	FpPV1-S2-645-F	CTATGACGTCCTTCTCTCCTCC
	FpPV1-S2-1,489-R	GGAGTACGGGAAAACAGGTTGT
	FpPV1-S2-1,874-F-RACE	CCACTGCTGCCGAAAATAG
Fusarium poae mitovirus 1 genomic RNA, complete genome	FpMV1-2-1,094-F	GTTCCGCAACTGATAGATTTCC
	FpMV1-2-2,402 R	CCTGAGGTGGTCTTCTGA
Bremia lactucae associated mitovirus 1 isolate DML-A_DN28798 RNA-dependent RNA polymerase gene, complete cds	BlaMV1-1,950-F	GGATCTATCACCAAGCCTAAGG
	BlaMV1-2,157 R	CTTGGAAAATCACGAGTGTAGC
Mitovirus sp. isolate H1_Bulk_30_scaffold_1302 RNA-dependent RNA polymerase	MVH1-1,286 F	TGGGAAATCTATCAGTAGCTGC
	MVH1-2,054-R	GAGACTACACTGCACACGTT

(H1Bulk301302_000001) gene, complete cds; and hypothetical protein (H1Bulk301302_000002) gene, partial cds		
Fusarium circinatum mitovirus 2-2 RNA-dependent RNA polymerase gene, partial cds	FcMV2-2-256-F	GGCATTTCAACCTGACCATT
	FcMV2-2-818-R	CCTTGGCAAAACTACTAACTGC
Fusarium graminearum negative-stranded RNA virus 1	FgnsV1-4,013-F	AGCTACAGACGACTACTCGA
	FgnsV1-4,474-R	GCGCTTGAGAGAATACTCCT
	FgnsV1-6,272-F	CCAATAGACAGACCGAACGAC
	FgnsV1-7,408-R	GGTACCTCGGATGCAAACAT
	FgnsV1-9,271-R	GGACGGCATTTCCTAAGTTT

Table 2 Primers of RLM-RACE (used as poly-A RACE's substitute experiment)

Primers' name	Sequence
3RACE-adaptor	[PH O] CAATACCTTCTGACCATGCAGTGAC
3RACE-1st	CATGCTGACTGTCACTGCAT
3RACE-2nd	TGCATGGTCAGAAGGTATTG

Table 3 Primers of qPCR (used in Chapter 4)

Primer's name	Sequence
FpV1-s1-285-F	ACTTCAATCGTTCCCCAGCT
FpV1-s1-410-R	TTCGTGGGGAAAGCCTTGTT
FpMV2-q-497 F	GACCCGTGGATTGAAACCCA
FpMV2-q-576 R	CCTTGTGGGGAGCTGTAATAGG
FpMV3-q-1,206 F	TTGCGGCATCTTCCCAATGA
FpMV3-q-1,285 R	CCTGCCTCACTCGCTTTCTT
FgnsV1-q-4,783 F	CGCAAATTCCATCGGGTGAAG
FgnsV1-q-4,922 R	GGAGACAACGGATACATGCCA
BcPV2-S1-q-1,013 F	TGAGCCAACGATCAGCAACA
BcPV2-S1-q-1,092 R	TCCAAGTCCAGAGTCGGTCA
FgHV2-q-9,377 F	CGATAAGGGACGCATTGGATATC
FgHV2-q-9,470 R	GATTGCCGCTCTTTCGCTCA
FpdsV3-q-6,108 F	CCAAGCTAGATGAGGCCTACC
FpdsV3-q-6,223 R	GAGAAGAGCGTACTGTCCCG

Table 4 Universal primers

Primer's name	sequence
dN6	GCCGGAGCTCTGCAGAATTCNNNNNN
dN6-Tag	GCCGGAGCTCTGCAGAATTC
dN12	CCTTCGGATCCTCCNNNNNNNNNNNN
dN12-Tag	ACGTCCTTCGGATCCTCC
M4T	GTTTTCCCAGTCACGACTTTTTTTTTTTTTT
M4	GTTTTCCCAGTCACGAC
M13 forward	GTAAAACGACGGCCAGTG
M13 reverse	GGAAACAGCTATGACCAT
OLIGOdT21 Hind III	AAAAAGCTTTTTTTTTTTTTTTTTTT

Table 5 Buffer and Medium

Name	Formula
V8 (for growing fungi mycelium)	1L tomato juice, centrifuge for 25min at 7000g, add water to 4L.
Water agar	8g agar in 400ml H2O.
PDA agar	156g in 400ml H2O.
Phosphate buffer for extract virus particles	0.5L of 1M K2HPO4(174.18): 87.09g (enlarge it to 1L is better) 0.5L of 1M KH2PO4(136.09): 68.04g Adjust to 1L to make 0.5 M buffer. Dilute to 0.25M or 0.01M before use. If want to make it pH=7.2, the volume of K2HPO4 is about 71.7ml, KH2PO4 is about 28.3ml.
dsRNA Extraction buffer (EB)	0.2M NaCl; 0.1M Tris-HCl; 0.004M EDTA pH 8.0; 2% SDS
STE buffer	0.1M NaCl; 0.05M tris; 0.001M EDTA pH 8.0, Adjust pH to 6.8 with concentrate HCl
Double digest buffer 10×	150µl 3M NaOAC; 30µl 5M NaCl ; 75µl 1M MgCl2; 150µl 0.1M ZnSO4; 95µl H2O
Ampicillin solution	The stock solution concentration is: 100mg/ml, 500mg Amp in 5ml of H_2O. When use, put 100µl in 100ml medium. To make the final concentration 0.1mg/ml.
Rifampicine solution	The stock solution concentration is: 50mg/ml, 250mg Rif in 5ml of DMSO When use, put 100µl in 100ml medium. To make the final concentration 0.05mg/ml.
S+N antibiotic	Streptomycin sulfate biochemical 6g, and Neomycin trisulfate salt hydrate 3g, dissolved in 30ml H_2O and filtered with 0.22-0.25µm film, stock in 2.0ml tubes.
LB plates for blue-white cloning	100ml LB with 500µl 0.1M iPTG, 100µl 100mg/ml AMP and 100µl 80mg/ml xgal. Xgal is resuspended in N,N-Dimethylformamide or DMSO.
sucrose gradient	make 50%, 40%, 30%, 20%, 10% solutions (0.5g, 1g, 1.5g, 2g, 2.5g in 5ml 0.25M potassium phosphate buffer), in each tube, load 2ml of each solution, from 50% to 10%. And leave it at 4℃ overnight.