CAUÊ NEVES OLIVEIRA

Exploring RNA virus diversity in *Metarhizium* spp.

Dissertation submitted to the Agriculture Microbiology Pos Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae.*

Adviser: Poliane Alfenas-Zerbini

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ABSTRACT

OLIVEIRA, Cauê Neves. M. Sc., Universidade Federal de Viçosa, July, 2023. **Exploring RNA virus diversity in Metarhizium spp.** Adviser: Poliane AlfenasZerbini. Co-adviser: Flávia de Oliveira Souza.

Viruses are ubiquitous acellular organisms since there is evidence of them hosting any living cell in all kingdoms. Mycoviruses are characterized by infecting fungi. The International Committee on Taxonomy of Virus (ICTV) recently recognized 29 families with representatives of mycoviruses. Most of these families have an RNA genome, and only one has a circular ssDNA genome. The non-retroviral RNA viruses are sorted into three Baltimore groups. Group III is characterized by having a dsRNA genome, while groups IV and V correspond to a ssRNA positive and negative sense, respectively. The unique hallmark gene in these three groups is RNA-dependent RNA polymerase (RdRp). This protein is responsible for viral genome replication and is conserved in all groups. Moreover, mycovirus infection can change the host phenotype. Most of the time, these changes are related to modification of their host pathogenic trait. For example, hypovirulent-associated mycoviruses are characterized by reducing the aggressiveness of phytopathogenic fungi by slowing down their mycelial growth and sporulation rate, as well as downregulating pathogenic genes. On the other hand, hypervirulent-associated mycoviruses are characterized by promoting mycelial growth and sporulation rate and upregulating host pathogenic genes. Therefore, mycoviruses are a more eco-friendly alternative to pest-management strategies than the use of chemical compounds related to ecosystem harm. Hypovirulent-associated mycovirus can be used to control the incidence of phytopathogenic fungi, and hypervirulent-associated mycovirus can increase the efficacy of mycopesticides. Studies already demonstrate that hypervirulent-associated mycoviruses hosting entomopathogenic fungi (EPF) can increase their potential to control insect pests. This work used two approaches to identify novel mycoviruses infecting the EPF in the genus *Metarhizium*. The first strategy was carried out by the total nucleic acid extraction of a *Metarhizium robertsii* isolate SCJAN-21.11 followed by identifying and sequencing the dsRNA viral genome. As we know, we report the first case of a polymycovirus hosting a *Metarhizium* in Brazil. This polymycovirus has at least three dsRNA genome segments. Each segment encodes only one ORF

representing the RdRp, a hypothetical protein, and a methyltransferase. The RdRp aminoacid phylogenetic analyses revealed that this mycovirus represents a new species, and we attempted to call it *Polymycovirus mineiro*. In the second strategy, we used a database with 77 viral family-level profile Hidden Markov Models (pHMM) to look for RdRp-like sequences in the public RNAseq data available on NCBI tagged as "*Metarhizium*." The pHMM is a statistical model to identify sequence patterns. As a result, we could identify 42 virus-like sequences in approximately 20% of the analyzed data. In conclusion, this study helps elucidate the virosphere in Metarhizium genera and investigate the possibility of using mycovirus as a biocontrol agent.

Keywords: Mycovirus; Biocontrol; Entomopathogenic fungi; *Metarhizium.*

RESUMO

OLIVEIRA, Cauê Neves. M. Sc., Universidade Federal de Viçosa, julho de 2023. **Explorando a diversidade de vírus de RNA em *Metarhizium* spp.** Orientadora: Poliane Alfenas-Zerbini. Coorientadora: Flávia de Oliveira Souza.

Os vírus são organismos acelulares, presentes em todos os ambientes, infectando organismos celulares. Os micovírus, são conhecidos como vírus que infectam fungos. Atualmente, o Comitê Internacional de Taxonomia de Virus (ICTV) reconhece 29 famílias com representantes de micovírus. A maioria dessas famílias tem um genoma de RNA, e apenas uma família é caracterizada contendo genoma ssDNA. A infecção por alguns micovírus tem a capacidade alterar o fenótipo do hospedeiro, e em grande parte das vezes essas modificações estão relacionadas com mudanças no perfil de agressividade do fungo hospedeiro. Por exemplo, alguns micovírus são caracterizados por causar hipovirulência em fungos fitopatogênicos, diminuem o crescimento micelial, a taxa de esporulação e regulam negativamente genes de patogenicidade. Entretanto, micovírus também são capazes de promover o fenótipo de hipervirulência no seu hospedeiro. Nesse caso, a infecção está relacionada com o aumento do crescimento micelial, taxa de esporulação, além de regular positivamente genes relacionados com a patogenicidade do fungo. Dessa forma, a identificação e caracterização de micovírus que induzem a hipovirulência é extremamente relevante, pois podem ser uma alternativa mais sustentável para o manejo de doenças do que o uso de compostos químicos associados a danos ao ecossistema. Por outro lado, micovírus associados com a hipervirulência do hospedeiro surgem como uma estratégia para aumentar a eficácia de micoinseticidas. Estudos demonstram que o aumento da patogenicidade causado pela infecção viral em fungos entomopatogênicos (EPF) auxilia no seu potencial de controle de pragas. Neste trabalho utilizamos duas abordagens para identificar novos micovírus infectando EPF do gênero *Metarhizium*. A primeira foi realizada por meio da identificação de perfis de dsRNA viral em uma coleção de isolados de *Metarhizium robertsii*, seguida da identificação e sequenciamento do genoma viral. Dessa forma, relatamos aqui o primeiro polymycovirus infectando um fungo do genero *Metarhizium* no Brasil. Este vírus tem ao menos três segmentos genomicos de dsRNA. Cada segmento possui apenas uma ORF, codificando a RdRp, uma proteína hipotética e

uma metiltransferase. As análises filogenéticas da sequência de aminoácidos da RdRp revelaram que este micovírus representa uma nova espécie e sugerimos o nome *Polymycovirus mineiro*. Na segunda abordagem, analisamos um conjunto de 287 *short read archive* (SRA) disponíveis de forma pública no NCBI. Para isso, utilizamos um banco de dados com 77 *profile Hidden Markov Models* (pHMM) representando diferentes famílias virais com o objetivo de identificar sequências similares à RdRp. Com isso, identificamos um total de 42 sequências como putativas RNA polimerase viral em aproximadamente 20% dos dados analisados. Em conclusão, este estudo amplia nosso entendimento sobre a diversidade viral em fungos *Metarhizium*, bem como na possibilidade da aplicação de micovírus como ferramenta de biocontrole.

Palavras-chave: Mycovirus; Biocontrole; Fungos entomopatogênicos; *Metarhizium*.

SUMMARY

1 - Introduction

Viruses are acellular organisms with diverse genome types and lifestyles (Kondo et al., 2022). It is known that viruses infect the majority, if not all, of cellular organisms. Therefore, they are present in all environments, influencing the ecology and evolution of their hosts, thus actively participating in various processes in the biosphere (Watanabe et al., 2019). Viruses able to host fungi are called mycoviruses. Most members of this group have a single-stranded (ssRNA) or double-stranded (dsRNA) RNA genome, with few reported cases of mycoviruses with a single-strand DNA (ssDNA) genome (Genomoviridae). Furthermore, no mycovirus with a double-stranded DNA (dsDNA) genome has been identified until now (Boulanouar et al., 2023; Du et al., 2014; Kondo et al., 2022).

Among the RNA mycoviruses, we found diversity in the genome types as they can be a dsRNA or a ssRNA, in a positive ((+)ssRNA) or negative sense ((-)ssRNA). Also, they can vary in the number of segments and sizes (Kondo et al., 2022). For example, viruses belonging to the *Hypoviridae* family have a non-segmented (+)ssRNA genome, their size varies typically between 9kb to 15kb, and they encode one or two poly proteins with the capacity for self-processing (Chun et al., 2022; Rigling et al., 2018). On the other hand, many mycoviruses have a segmented genome, with 2 to 12 segments with sizes ranging from approximately 2kb to 6 kb. Usually, each genomic segment has a single open reading frame (ORF). The 3’ and 5’ UTR (untranslated region) regions of the cognate RNA segments are conserved, as they are specific recognition sites for initiation of replication and/or genome encapsidation (Jia et al., 2021; H. Liu et al., 2021; Sutela et al., 2020; Urayama et al., 2014). Furthermore, mycoviruses members of the proposed family Splipalmivirus were characterized by having the conserved RNA-dependent RNA polymerase (RdRp) motifs split into two different segments (Chiba, Oiki, Yaguchi, et al., 2021; Chiba, Oiki, Zhao, et al., 2021).

Some mycoviruses, members of *Narnaviridae* and *Mitoviridae*, do not encode capsid proteins. Those viruses usually have only a single (+)ssRNA segment that encodes the RdRp. Studies have indicated that in the first family, the viral replication occurs in the host cytoplasm, while the second replicates in the host mitochondria (de Rezende et al., 2021; Kondo et al., 2022; Marais et al., 2021; Wu et al., 2016). The family *Polymycoviridae*, in turn, are dsRNA viruses that have between 4 to 8 segments

and do not encode a known capsid protein. However, they encode PASrp protein, rich in the amino acids proline, alanine, and serine, which are associated with the viral dsRNA as ribonucleoproteins (Kanhayuwa et al., 2015; Kotta-Loizou et al., 2015; Kotta-Loizou & Coutts, 2022). Other mycoviruses with unique characteristics are the Yadokariviruses (*Yadokariviridae*), which have a (+)ssRNA genome of approximately 6kb and do not encode any capsid protein. However, they are capable of encapsidate their genome using the capsid of an unrelated dsRNA virus-helper (Yadonushivirus) when co-infected with the host (Das et al., 2021; Kondo et al., 2022; Sato et al., 2022, 2023).

Characterization of new mycoviruses usually occurs in isolates of phytopathogenic fungi with phenotypic changes and in which dsRNAs were detected as indicative of the viral genome and/or replication intermediate (Kondo et al., 2022). However, new studies using high-throughput sequencing techniques and analysis of public metatranscriptome and metagenome data have identified an underdetermined diversity of mycoviruses (Boulanouar et al., 2023; Chiba, Oiki, Yaguchi, et al., 2021; Dolja & Koonin, 2018; Edgar et al., 2022; Kondo et al., 2022; Olendraite et al., 2023).

Mycoviruses transmission can occur through several processes that will determine the ability of the virus to spread in nature (García-Pedrajas et al., 2019; Kondo et al., 2022; Xie & Jiang, 2014). Some viruses, such as genomoviruses, can replicate in mycophagous insects using them as a transmission vector (S. Liu et al., 2016). However, many mycoviruses do not have an extracellular phase, so their transmission can occur vertically through asexual spore formation, which is infected by the virus in the cytoplasm of the host fungus. This method of transmission can be very efficient for several mycoviruses and can occur in up to 100% of cases, such as Alternaria alternata partitivirus 1 (Lemus-Minor et al., 2019; Sato et al., 2020; Xavier et al., 2018). Mycoviruses can also be transmitted to new hosts horizontally. In this case, an infected fungus (donor) transmits the mycovirus to another uninfected fungus (recipient) through hyphal anastomosis (Khalifa & Macdiarmid, 2021; Lemus-Minor et al., 2019; Yimjenjang Longkumer & Abbas Ahmad, 2020). This method of transmission is less efficient because the donor and the recipient need to be able to fuse their hyphae and have vegetative compatibility. (Fleißner et al., 2022; García-Pedrajas et al., 2019; Gonçalves & Glass, 2020).

Some mycoviruses have a cryptic infection and do not visually affect the host phenotype. However, other mycoviruses can cause phenotypic changes in their host.

These changes are often related to the reduction (hypovirulence) or the increase (hypervirulence) in the aggressiveness of pathogenic fungi (García-Pedrajas et al., 2019; Kotta-Loizou, 2021; Kotta-Loizou & Coutts, 2017; Özkan & Coutts, 2015; Y. Wang et al., 2022; Xavier et al., 2018; Zhang et al., 2020). Furthermore, mycoviruses have been described as able to convert a phytopathogen phenotype into an endophytic phenotype. For example, the mycovirus Pestalotiopsis theae Chrysovirus-1 (PtCV-1) is an Alphacrysovirus identified in the endophytic isolates of *Pestalotiopsis theae*. This fungus is usually the pathogenic agent of Gray Tea Blight disease in *Camellia sinensis*, generating up to 10% loss in annual tea production. However, this mycovirus not only eliminated the virulence of this fungus but also reduced the incidence of the disease when the plant was co-inoculated with strains of *P. theae* infected and virus-free (Zhou et al., 2021).

Hypovirulent-associated mycoviruses usually decrease the growth and sporulation rates, as well as reduce the pigmentation and mycotoxin production of the host (H. Liu et al., 2022; M. Sharma et al., 2021; Y. Wang et al., 2022). In addition, different studies that evaluated the interaction between mycoviruses and their host showed changes in the gene expression levels (Ćurković-Perica et al., 2022; García-Pedrajas et al., 2019; Kotta-Loizou, 2021). For example, the soybean leaf-associated gemygorvirus-1 (SlaGemV-1), a hypovirulent-associated mycovirus hosting *Sclerotinia sclerotiorum*, can upregulate genes related to DNA replication, protein synthesis and virulence repressors, as well as downregulate genes related to pathogenicity, carbon metabolism, and sclerotial formation (Pedersen & Marzano, 2022).

In contrast, hypervirulent-associated mycoviruses increase the aggressiveness of the host by promoting growth and sporulation rates, as well as upregulating pathogenic genes (Ahn & Lee, 2007; Kotta-Loizou, 2021; Olivé & Campo, 2021; Shah et al., 2018). The hypervirulence trait is not an undesirable characteristic. Recently, the mycovirus Beauveria bassiana Polymycovirus-4 (BbPmV-4) was characterized as hosting an important entomopathogenic fungus, *Beauveria bassiana*, used as a biological pesticide. Comparison between infected and virus-free isogenic isolates demonstrated an increase in mycelial growth, virulence, and mortality rate of corn borer larvae, *Ostrina furnacalis*, infected by the fungus (Kang et al., 2022). Therefore, hypervirulent-associated mycoviruses can increase the efficacy of entomopathogenic fungi used as mycopesticides (Kang et al., 2022; Kondo et al., 2022; Kotta-Loizou, 2021; Kotta-Loizou & Coutts, 2017; Wagemans et al., 2022).

Biocontrol consists of using a natural enemy to control the incidence of a pest or pathogen instead of chemical compounds that often negatively affect the environment and human health (Ćurković-Perica et al., 2022). The most successful example of mycovirus used in biocontrol is Cryphonectria hypovirus 1 (CHV1). This mycovirus was identified in a hypovirulent strain of *Chryphonectria parasitica*, the pathogenic agent of chestnut blight disease in *Castanea* trees. CHV1 was able to control the incidence and lethality of the phytopathogen in forests, mainly in Europe and the United States, where it is estimated that the disease is responsible for over four billion tree deaths (Anagnostakis, 1982; Ćurković-Perica et al., 2022; García-Pedrajas et al., 2019; Van Alfen et al., 1975).

Studies of mycoviruses as a biocontrol agent have mainly focused on hypovirulent-associated mycoviruses that infect important economic phytopathogenic fungi that cause significant economic loss in agriculture, such as *Botryosphaeria*, *Sclerotinia*, *Fusarium*, and *Pestalotiopsis* (Kotta-Loizou, 2021; H. Liu et al., 2021; Paudel et al., 2022; Pedersen & Marzano, 2022; Y. Wang et al., 2022; Zhou et al., 2021). However, the hypervirulence trait can also have a great interest in agriculture. Hypervirulent-associated mycoviruses can be used in association of entomopathogenic fungi commonly used as mycopesticides, such as members of the genera *Metarhizium*, *Beauveria*, and *Verticillium* (Carla et al., 2022; Islam et al., 2021; A. Sharma et al., 2023). This strategy can enhance the ability of mycopesticides to control many insects that are responsible for large economic loss in plantations (Kang et al., 2022; Kotta-Loizou, 2021; Ning et al., 2022; P. Wang et al., 2022). In addition, the increase in growth and sporulation rate by hypervirulent-associated mycoviruses may increase the infected fungi fitness, promoting their spread in the field and having a persistent infection, which reduces the chances of the fungus spontaneously curing itself of the virus (Bocos-Asenjo et al., 2022; Khan et al., 2023; Kotta-Loizou, 2021; Lemus-Minor et al., 2019; P. Wang et al., 2023).

As you can see, mycoviruses have a significant potential to be used in biocontrol. Because of this, there is an extensive effort to study the mycovirus diversity and their interaction with the host. This work aims to exploit the virosphere in an important entomopathogenic fungi genus, *Metarhizium*. For this purpose, we used two approaches. In the first one, we look for dsRNA patterns indicative of mycovirus infection in *Metarhizium* spp. isolate and then sequence the RNA genome. In the second strategy, we used bioinformatics tools to investigate contigs with RdRp

signature in public *Metarhizium* spp. metatranscriptome data available on GenBank (NCBI).

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2 - Chapter 1: Identification of a novel polymycovirus infecting an entomopathogenic fungus *Metarhizium rosberts*

Identification of a novel polymycovirus infecting an entomopathogenic fungus *Metarhizium rosbertsii*

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Abstract

Insects are responsible for 18% of the total losses in agriculture around the world. Because of this, chemical pesticides are currently used for pest management. However, these compounds are hazardous to the ecosystem and human health. The biocontrol strategy using entomopathogenic fungi (EPF) is an eco-friendly alternative for pest management to replace the classic pesticides. Mycoviruses are viruses that infect fungi. Some mycovirus are associated with host hypervirulence. In this case, the virus usually increases the aggressiveness of pathogenic fungi and can be used to development of new efficient mycopesticides. In this work, we report a novel polymycovirus, infecting *Metarhizium robertsii*.

The herbivorous lifestyle of insects represents a severe threat to agriculture. Because of this, they are responsible for 18% of economic loss worldwide (Sharma et al., 2023). Furthermore, insects are vectors for virus transmission associated with plant disease. (Bhattacharjee & Hallan, 2022; F. Li et al., 2022). The most common strategy for pest management is using chemical pesticides. However, these pesticides can also affect non-target organisms and harm the ecosystem (Islam et al., 2021; A. Sharma et al., 2023). On the other hand, biocontrol is an alternative eco-friendly. This strategy involves using natural enemies, e.g., predators or parasites, to control a pest or disease incidence (Ćurković-Perica et al., 2022a; Islam et al., 2021). Entomopathogenic fungi (EPF) exhibit great potential as biocontrol agents (Carla et al., 2022; Islam et al., 2021; A. Sharma et al., 2023). EPF is the majority cause of insect disease and usually has a narrow host range (Carla et al., 2022; Hegde et al., 2023; Islam et al., 2021; A. Sharma et al., 2023). *Metarhizium* is an EPF genus used in biocontrol, and some mycopesticides, like Met52®, Tick-Ex®, and Bio-Cane, are currently on the market. In addition, members of this genus can infect over 200 insect species for which pest management techniques are still required, like ticks, termites, cockroaches, and grasshoppers (Hegde et al., 2023; Islam et al., 2021; A. Sharma et al., 2023).

The International Committee on Taxonomy of Viruses (ICTV) recognizes 29 families with mycovirus representant, most of them have dsRNA (double-strand RNA) or ssRNA (single-strand RNA), and only the family *Genomoviridae* have a ssDNA circular genome (single-strand DNA) (Kondo et al., 2022; Villan Larios et al., 2023). Some mycoviruses are associated with the increase or reduction of mycelial growth

and sporulation rate, as well as up or downregulate pathogenic gene expression (García-Pedrajas et al., 2019; Kang et al., 2022; Kotta-Loizou, 2021; M. Sharma et al., 2021; Villan Larios et al., 2023). Hypovirulent-associated mycoviruses usually attenuate the aggressiveness of pathogenic fungi. Otherwise, hypervirulent-associated mycoviruses increase the aggressiveness of their host (Kotta-Loizou, 2021). Recent studies have demonstrated that hypervirulent-associated mycoviruses infecting EPF enhance their ability to be used as a biocontrol agent (Kang et al., 2022; Kotta-Loizou, 2021; Kotta-Loizou & Coutts, 2017; Ning et al., 2022; P. Wang et al., 2022a).

Polymycoviruses are a large group of mycoviruses. They usually have 4 up to 8 dsRNA segments and encode at least four proteins: an RNA-dependent RNA polymerase (RdRp) in the first segment, a hypothetical protein in the second segment, a methyltransferase protein in the third segment, and a PASrp (proline-alanine-serine rich protein) protein in the fourth segment. The other segments, when present, encode proteins of unknown function and with no homology among other viruses. (Hough et al., 2023; Kondo et al., 2022; Kotta-Loizou et al., 2015; Kotta-Loizou & Coutts, 2022a). Some polymycoviruses are already characterized by promoting hypervirulence in *B. bassiana* isolates, such as Beauveria bassiana polymycovirus 4 (BbPmV-4) and the co-infection of Beauveria bassiana polymycovirus 1 (BbPmV1) and Beauveria bassiana non-segmented virus (BbNV)-1. For example, isolates of *B. bassiana* infected by these polymycoviruses enhance the mortality of *Ostrina fucalis* and *Galleria mellonella*, respectively, when compared with their isogenic virus-free isolates showing a large biocontrol potential (Kang et al., 2022; Kotta-Loizou & Coutts, 2017).

Identification of a putative mycoviruses infecting Metarhizium robertsii

The isolate of *Metarhiziu robertsii* (SCJAN-21.11) (Figure 1A) used in this research was isolated from an organic coffee plantation in Patrocínio, Minas Gerais, Brazil, and provided by Professor Simon Luke Elliot and Professor Madelaine Venzon from the Laboratório de Controle Biológico da EPAMIG (Empresa de Pesquisa Agropecuária de Minas Gerais), The isolate SCJAN-21.11 was cultivated in potato dextrose agar medium (PDA Sigma) at 25 ºC for two weeks in darkness. We performed a total nucleic acid (TNA) extraction using approximately 100 mg of mycelium powder followed by the addition of 1 mL of extraction buffer (Guanidine Hydrochloride 4 M; Sodium Acetate 0,2 M; EDTA 25 mM pH 8,0; Potassium Acetate 1 M;

Polyvinylpyrrolidone - 40 2,5%) and 100 µL of SDS 10%. The solution was incubated at 70 ºC for 10 min and cooled in ice for 5 min. Then, cellular debris was eliminated by centrifugation at 13,000 RPM for 10 min. After that, the supernatant was pipetted to another microtube and was added 300 µL of wash buffer (Tris-HCl 10 mM pH 7,5; EDTA 0.5 mM pH 8,0; NaCl 50 mM), 600 µL of a 7 M NaI solution, and 50 µL of Silica (Sigma S5631) suspension. After 10 min at room temperature, the sample was centrifuged, and the upper solution was discarded. The silica pellet was then washed twice with 500 µL of wash buffer. Finally, 100 µL of DEPC-treated water was added to the dried silica pellet, incubated at 70 ºC for 5 min, and centrifuged for 10 min at 13,000 RPM. In the end, 80 µL of TNA was recovered. To identify the existence of dsRNA pattern, the TNA was treated separately with S1 nuclease (Promega), RQ1 DNAse (Promega), and RNAse A (Promega) according to the manufacturer's protocol. Respectively, each enzyme can digest any single-strand nucleotides, only single or double-strand DNA, and only single or double-strand RNA. The presence of four bands in S1 treated and DNAse treated samples as their absence in RNAse treated suggests the existence of four dsRNA segments in the isolate of *M. robertsii* SCJAN-21.11 (Figure 1B).

Analysis of the novel putative mycovirus sequence

The putative virus dsRNA genome was sequenced using MiSeq Ilumina. First the sample was treated simultaneously with S1 nuclease and RQ1 DNAse for one hour, and the reaction stopped with 10% (v/v) of EDTA 50 mM followed by heated 65 ºC for 30min. The cDNA reaction was carried out with 400 ng of dsRNA with Random Primer using Super Script III (ThermoFisher) as the manufacturer's protocol. The cDNA was submitted to an isothermal amplification using ϕ29 (Cellco) and random primer for 8h. Finally, we use the Illumina Nextera XT DNA Library Preparation Kit, and the dsRNA was sequenced in MiSeq Illumina platform. The raw reads were filtered with Trimmomatic v0.39, and contigs assembled with Trinity v2.5.1 using default parameters. Afterward, we queried the contigs using a BLASTx against the non-redundant protein NCBI database.

The first contig (dsRNA1) has 2501 nucleotides and encodes a single ORF with 585 amino acids. This protein shares 88.01% with the RNA-dependent RNA polymerase (RdRp) of Metarhizium anisopliae polymycovirus 1 (MaPmV1). The

second contig (dsRNA2) has 2,400 nucleotides and encodes a single ORF with 707 a.a. and 85.51% identity with a hypothetical protein encoded in the dsRNA2 of MaPmV1. The third dsRNA segment (dsRNA3) has 2200 nucleotides, encodes a single ORF with 700 a.a, and shares 82.55% identity with a Methyltransferase encoded by MaPmV1 (Figure 1C). We tried to investigate the presence of the last segment using the PASrp protein encoded by the dsRNA4 of the polymycovirus MaPmV1 as a query against our contigs. However, we couldn't find the fourth segment. In addition, the average sequence coverage was below 600-fold in all three segments, and we cannot prove the existence or absence of the fourth segment (Figure 1C).

The RdRp is the unique hallmark gene in all non-retroviral RNA viruses and is highly conserved (Hough et al., 2023; Kondo et al., 2022). We search for the characteristics RdRp motifs in our sequences (Jácome et al., 2022). For this, we multiple-alignment the amino acids sequence of the ORF encoded in dsRNA1 with other mycoviruses RdRp. We could find at least three conserved motifs IV, V, and VI, which have the GDNQ amino acids (figure 2). Most dsRNA mycoviruses and (+)ssRNA mycoviruses have a GDD triplet amino acid in the catalytic core of motif VI (Hough et al., 2023; Kondo et al., 2022). However, the negative-sense ssRNA viruses and the mycoviruses families *Polymycoviridae* (dsRNA) and *Hadakaviridae* (+ssRNA) have conserved amino acids GDNQ instead of a GDD in motif VI (Kondo et al., 2022; Kotta-Loizou & Coutts, 2022a; Sato, Turina, et al., 2023). To better understand the taxonomy of the novel mycovirus, we built a phylogenetic tree with the RdRp amino acid sequence of the top hits in BLASTp search, and we added some representative sequences of the phylum *Pisuviricota* and *Lenarviricota* (Figure 3). The novel mycovirus identified in this work grouped with high confidence with members of the *Polymycoviridae*. Therefore, as we know is the first report of a polymycovirus infecting a *Mertarhizium* spp. in Brazil, and we attempted to call as *Polymycovirus mineiro*.

According to ICTV taxonomy (https://ictv.global/taxonomy/) the *Polymycoviridae* is a family in *Riboviria* realm with unassigned phylum, class, and order (Kotta-Loizou & Coutts, 2022a). However, this family is close related to the *Hadakaviridae*, a family inside the phylum *Pisuviricota* without a stablish class and order (Sato, Turina, et al., 2023). Both families grouped in a clade with 100 bootstrap (Figure 3). The current taxon demarcation for *Pisuviricota* is a ssRNA or dsRNA viruses that encode his RdRp and do not infect prokaryotes (Koonin et al., 2019.). These two families appear to be more closely related to each other than other viruses in phylum *Pisuviricota*. They also

encode a Methyltransferase protein in one of their genomic segments. The differences in their biology are the genomic structure and capsid origin. The polymycoviruses are dsRNA viruses and encode the PASrp protein that protects their genome as a ribonucleoprotein, while hadakaviruses have (+)ssRNA genome and encode no capsid protein (Kotta-Loizou & Coutts, 2022a; Sato, Turina, et al., 2023). Because of this, we suggest that these two mycoviral families must be grouped in the same phylum and order. However, further analyses need to be made to confirm the actual taxon demarcation of this group.

The efforts to understand the mycovirus diversity have been characterized many novel polymycovirus, and the new ones, like *Polymycovirus mineiro*, show that we still have much to be exploited. We build a phylogenetic tree and a pairwise alignment with many recently characterized members of the families *Polymycoviridae* and *Hadakaviridae* (Figure 4). We could observe at least five clades inside the Polymycoviridae family with high confidence. Clade I and clade II appear to be more related to each other than clade III, clade IV, and clade V. Also, the bootstrap value of clade II is lower than the other ones. A pairwise alignment heatmap shows that the average identity between the sequences in each clade is above 50%. This analysis also suggests that clades I and II share identity values between 40 and 50%, while the other ones share less than 40% of identity among each other (Figure 4). Therefore, we suggest that these clades can be classified as new genera within *Polymycoviridae*, but more analyzes need to be done to establish the taxon criteria.

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Figures

Figure 1:

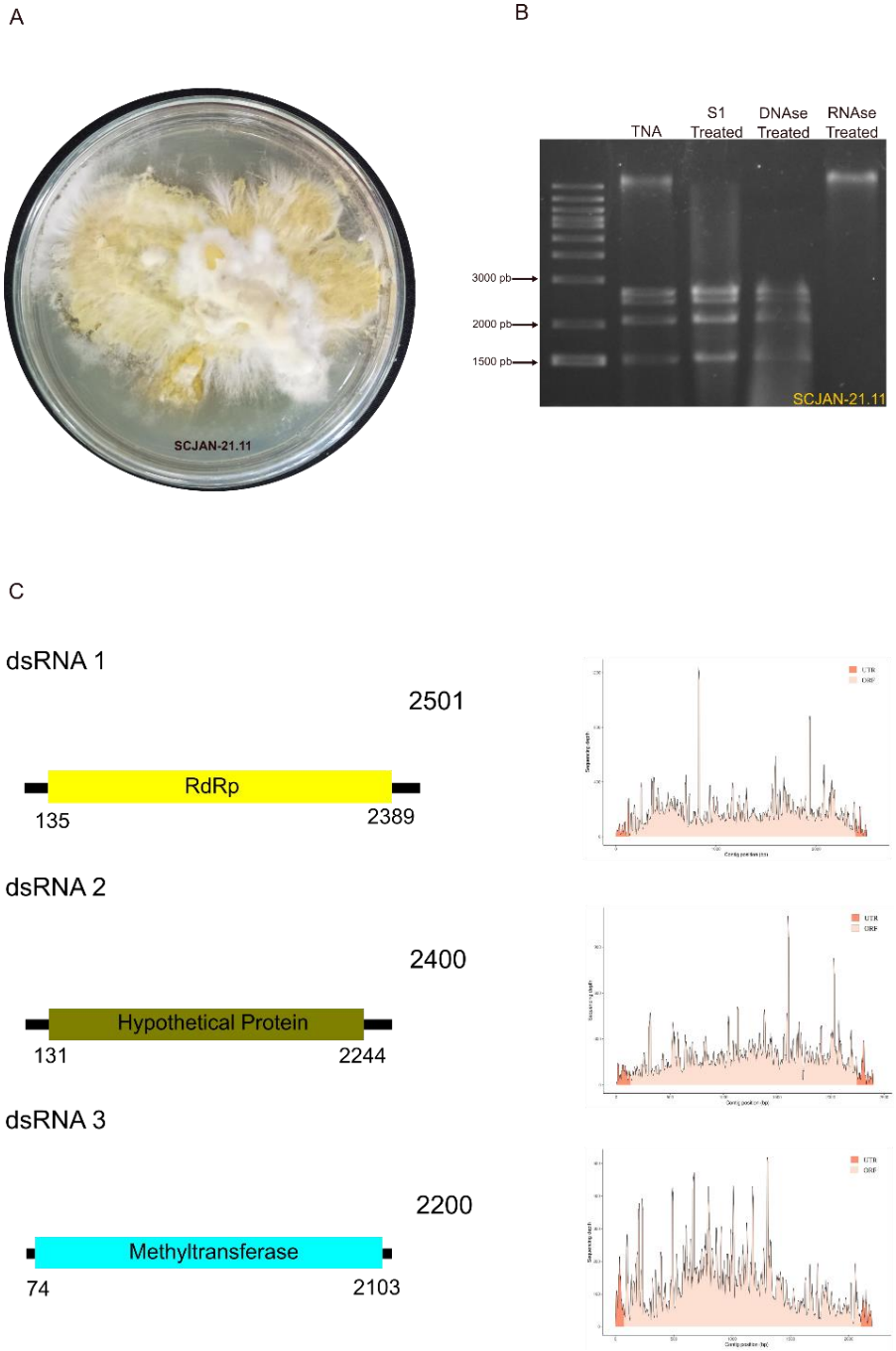

Figure 1: (A) a representative photo of the isolate *Metarhiziu robertsii* (SCJAN-21.11). (B) Electrophoresis on agarose gel 1% of the digest samples. TNA (Total nucleic acid) is the extraction without any treatment. S1, DNAse, and RNAse treated are the samples digested for 30 min with the respective nuclease. (C) Scheme of viral dsRNA sequence in our work (left) and the sequencing depth graph (right).

Figure 2:

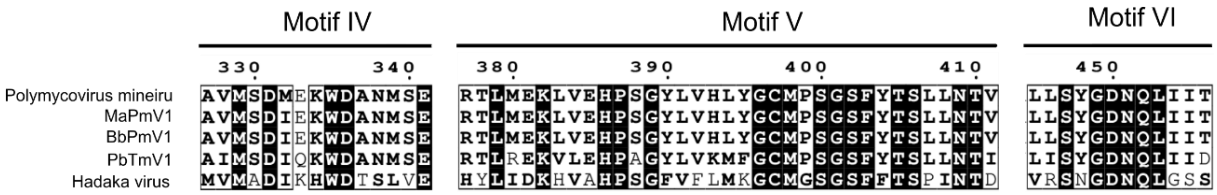

Figure 2: Multiple alignment of the mycoviral RdRp motifs IV, V, and VI. MaPmV1 - Metarhizium anisopliae polymycovirus 1; BbPmV1 - Beauveria bassiana polymycovirus 1; PbTmV1 - Penicillium brevicompactum tetramycovirus.

Figure 3:

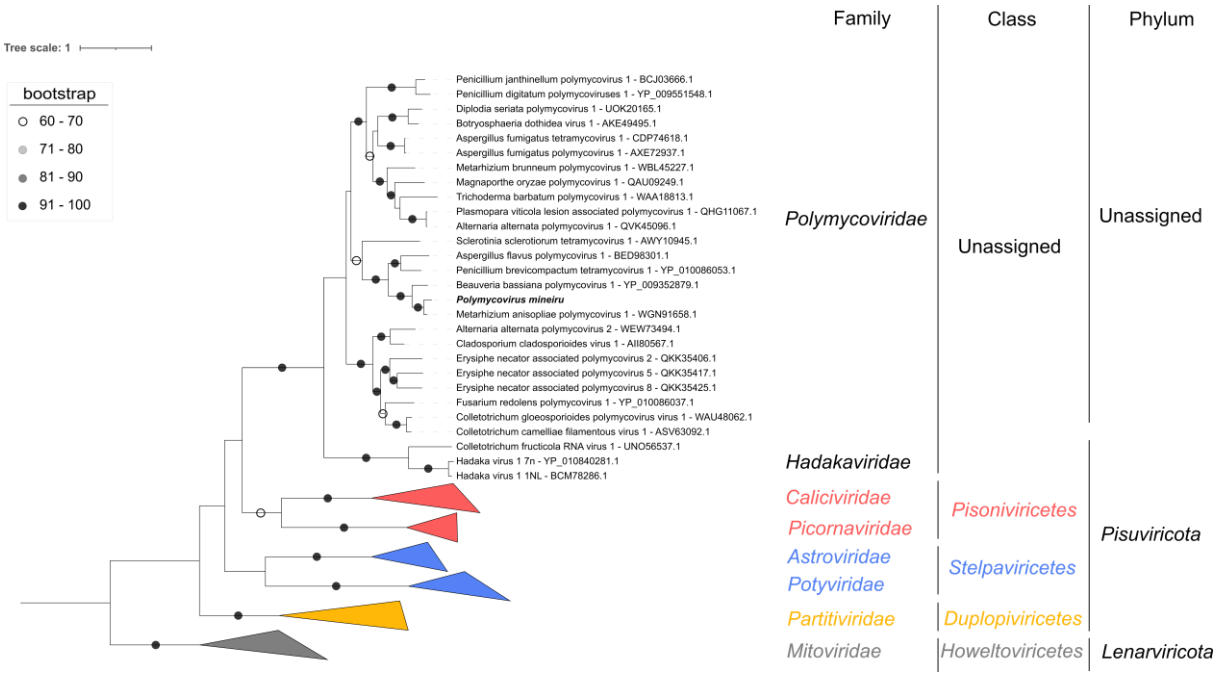

Figure 3: Phylogenetic tree built with the RdRp amino acid sequence of *Polymycoviridae*, *Hadakaviridae*, and other viruses in phylum *Pisuviricota* and *Lenarviricota*. The tree was constructed using RAxML, applying 1000 bootstrap replicates, and the best evolutive model LG+I+G4+F. The NCBI accession number of each sequence is displayed at the end of their names.

Figure 4:

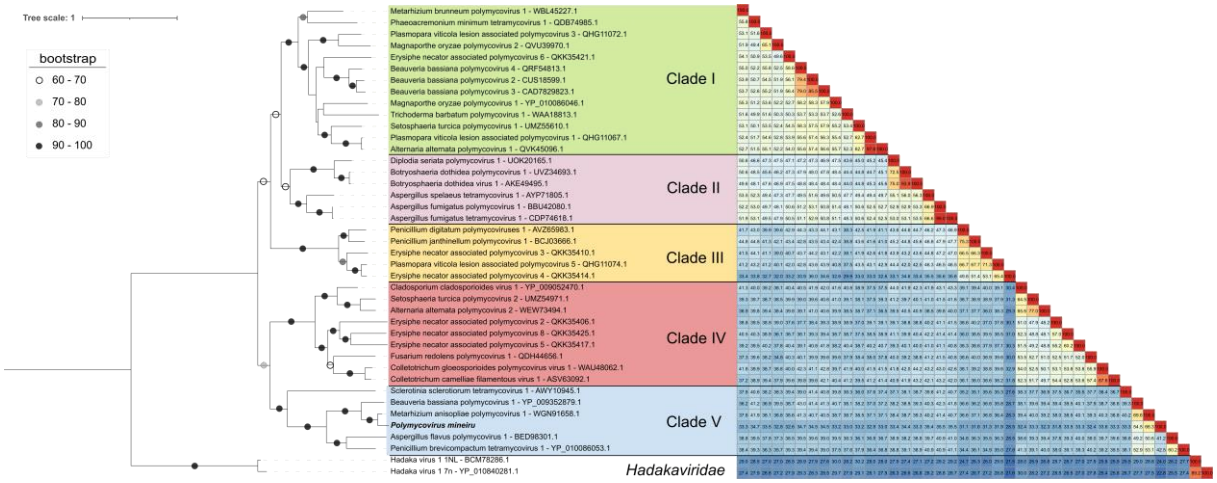

Figure 4: Phylogeny based on the RdRp sequence of a variety number of *Polymycoviridae* members and *Hadakaviridae*. This tree was build using RAxML, applying 1000 bootstrap replicates and the best evolutive model LG+I+G4+F. The heatmap represent the pairwise alignment of each sequence in the phylogenetic tree, inside each box there is the identity value of each alignment. The NCBI accession number of each sequence is displayed at the end of their names.

3 - Chapter 2: Analyses of the presence of mycovirus RdRp in Metarhiziumi public RNAseq data

Analyses of the presence of mycovirus RdRp in *Metarhiziumi* public RNAseq data

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Keywords: Mycoviruses; Virosphere; Entomopathogenic fungi; pHMM; RdRp; Mycopesticides.

Number of figures: 4

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Abstract

Fungal viruses (mycoviruses) are classified into 29 families by ICTV, and most of them have dsRNA or ssRNA genomes. All non-retrovirus RNA are members of the *Riboviria* realm and have a unique hallmark gene, the RNA-depended RNA polymerase (RdRp), necessary for genome replication. In this work, we used profile Hidden Markov Models (pHMM), a robust statistical model to identify conserved sequence patterns, to search viral RdRp-like ORFs in public *Metarhizium* RNAseq data. We found 42 virus-like sequences in 20% of the data analyzed. Each sequence was classified based on its similarity with the viral family-level pHMM in our database. We could classify these sequences into 12 families, and only 23,8% had an ambiguous classification. Our data also suggest a high abundance of ssRNA; only 1/42 was associated with dsRNA viruses. Phylogenetic analysis of the sequences initially identified as members of the families *Narnaviridae* and *Partitivirdae* was built to evaluate the pHMM taxonomy classification. We could identify mitovirus-like, botoumiavirus-like, and unirnavirus-like viruses associated with *Metarhizium* RNAseq data.

1 – Introduction

Mycoviruses represent a large group of viruses that infect fungi, yeast, and Oomycetes. Currently, mycoviruses are classified into 29 viral families recognized by the International Committee on Taxonomy of Viruses (ICTV) (Hough et al., 2023). Most of them have dsRNA (double-strand RNA) or ssRNA (single-strand RNA) genomes (Hough et al., 2023; Kondo et al., 2022), and there are only a few reports of a ssDNA (single-strand DNA) mycovirus hosting *Sclerotinia sclerotiorum* (Kondo et al., 2022; Varsani & Krupovic, 2021).

The mycoviruses infection can be asymptomatic, not visually affecting their host (Kotta-Loizou, 2021). On the other hand, some mycoviruses infections are characterized by increased or reduced host aggressiveness (Aulia et al., 2019; Kotta-Loizou, 2021). Hypovirulent-associated mycoviruses usually slow down mycelium growth and sporulation rate, as well as downregulate pathogenic genes (Du et al., 2014; Kotta-Loizou, 2021; Rigling et al., 2018; M. Sharma et al., 2021). Otherwise, hypervirulent-associated mycoviruses promote mycelium growth and sporulation rate,

as well as upregulate pathogenic genes (Kang et al., 2022; Kotta-Loizou & Coutts, 2017; Y. Li et al., 2022; Özkan & Coutts, 2015). Thus, mycoviruses are a potential biocontrol agent since hypovirulence trait can be used in the control of a fungus disease, and hypervirulent trait can increase the efficacy of entomopathogenic fungus used as mycopesticides (Ćurković-Perica et al., 2022b; Kang et al., 2022; Kotta-Loizou, 2021; Özkan & Coutts, 2015; A. Sharma et al., 2023; Van Alfen et al., 1975).

The characterization of a novel mycoviruses requires the isolation and sequencing of its genome (Y. Li et al., 2022; P. Wang et al., 2022a, 2022b; Y. Wang et al., 2022). However, the increase of metagenomic and metatranscriptomic data available in databases like the National Center for Biotechnology Information (NCBI) provides new strategies to identify an underestimated virus diversity (Charon et al., 2022; Dolja & Koonin, 2018; Hough et al., 2023; Koonin et al., 2022; Marzano & Domier, 2016; Nakagawa et al., 2023; Neri et al., 2022; Olendraite et al., 2023; Wright et al., 2020). For example, profile Hidden Markov Models (pHMM) is a statistical model for identifying conserved sequence patterns. In contrast to BLAST and structure-based approaches, pHMM is more sensitive to identifying distant homologous and requires less computational effort (Eddy, 2011; Olendraite et al., 2023).

As their genome organization, the exclusively RNA viruses are represented by Baltimore's groups III, IV, and V, and their genome corresponds to dsRNA viruses, positive-sense ssRNA viruses, and negative-sense ssRNA viruses, respectively (Baltimore, 1971). The unique hallmark gene in all non-retroviral members of the *Riboviria* realm (kingdom *Orthornavirae*) is the RNA-dependent RNA polymerase (RdRp). This protein is responsible for viral replication and is highly conserved. Consequently, the similarity of the RdRp amino acid sequence is commonly used for the taxonomy classification of these viruses (Hough et al., 2023; Jácome et al., 2022; Kondo et al., 2022; Koonin et al., 20019.; Mönttinen et al., 2021; Venkataraman et al., 2018). This protein has seven conserved motifs (A to G), especially motif C, which contains the GDD amino acid triplet essential for binding metal cofactor and necessary for the catalytic activity (Venkataraman et al., 2018). Nevertheless, great diversity can be found in mycoviruses' RdRp motifs. For example, members of *Polymycoviridae*, *Hadakaviridae*, and some viruses in the order *Mononegavirales* have GDNQ amino acids instead of the characteristic GDD triplet (Hough et al., 2023; Je et al., 2022; Kondo et al., 2022; Kotta-Loizou & Coutts, 2022b; Sato, Turina, et al., 2023). In addition, two new types of divided RdRp were discovered in segmented RNA

narnavirus-like. In Type I divided-RdRp, the first genome segment encodes RdRp motifs F, A, and B, while motifs C and D are encoded in the second segment. Likewise, in Type II divided-RdRp, the motifs F, A and B, C, D are split into the first and second segments, respectively (Chiba, Oiki, Yaguchi, et al., 2021; Chiba, Oiki, Zhao, et al., 2021; Je et al., 2022).

This work explores the mycoviral diversity in an important entomopathogenic fungus genus, *Metarhizium*. Members of this genera are known to infect many insects and are currently used as mycopesticides against ticks, cockroaches, and termites (A. Sharma et al., 2023; Sullivan et al., 2022). We download the short-read archive (SRA) from the NCBI database related with a public *Metarhizium* spp. Illumina RNAseq data. Then, we used a database with RdRp pHMM models from 77 different viral families, created by Olendraite et al., 2023, to identify viral RdRp-like sequences previously undetected.

2 – Materials and Methods

2.1 Download of fastq files from Short-Read Archive (SRA/NCBI) and transcriptome assembly

First, we searched for the public *Mietarhizium* spp. Illumina RNAseq data available on NCBI until February 27th, 2023. Then, we used the ncbi-sra-toolkit v.2.10.8 to download 148 pair-end and 139 single-end SRA data sets in fastq format. Then, we used Trimmomatic v0.39 for read quality filtering (parameters: LEAGIND:3 TRAILING:3 SLIDEWINDOW:4:15 MINLEN:40), and contigs were assembled using Trinity v2.5.1 using default parameters.

The getorf algorithm from EMBOSS package v6.6.0 was used to identify the open reading frames (ORFs) in each contig using the genetic code tables 1, 3, 4, 5, 6, 11, and 16 (parameters: -minsize 600 -find 0). Afterward, we use cd-hit v4.8.1 to remove redundant amino acid sequences over 98%.

2.2 Identification of viral RdRp by pHMMsearch

To search for viral RdRp sequences, we used the same protocol described by Olendraite et al., 2023. Briefly, the 77 pHMMs representing RdRp from diverse RNA

virus families (https://github.com/ingridole/ViralRdRp_pHMMs/) were queried against the non-redundant protein set using HMMsearch (parameters: -E 10^{-6}). Then, the hits were filtered, and only alignment with a score over 50 was kept. Next, the IDscore for each virus family was calculated by dividing the alignment score by the alignment size. The RdRp protein sequences identified were then assigned to the “Classified group” if they had only one family pHMM match with IDscore ≥ 0.25 or if the second-best IDscore was 20% lower than the first one, “ambiguous” if the second-best IDscore was higher than 20%, and “unclassified” if there was no pHMM hit with IDscore ≥ 0.25. Then, we used cd-hit v4.8.1 to remove the redundancy between SRAs in the same Bioproject. For this, we clustered the virus-like sequences into clusters above 90% identity, and the most extended sequence was maintained.

2.3 Phylogenetic analyses and tree building

Each viral RdRp sequence was manually confirmed with a BLASTp search in the NCBI non-redundant (nr) protein database, and the top hits were downloaded. Then, sequences initially classified into one recognized mycoviral family were chosen to build a phylogenetic tree based on their RdRp amino acids sequence.

First, we used mafft v7.471 to create a multiple alignment of the viral RdRp sequence inside each family and their best BLASTp matches. Then, the best-fit evolution model was calculated with modeltest-ng v0.1.7. Finally, a maximum-likelihood phylogenetic tree was created for each selected viral family using RAxML-ng v1.0.3, applying 1000 bootstrap replicates. The trees were edited by iTOL v6, and graphics were generated using GraphPad Prism v8.4.3.

3 – Results

3.1 Mycovirus prospection in public Metarhizium RNAseq database

In total, 287 SRA data, single and pair-ended, were analyzed. We notice that 20.21% of SRA data have at least one RdRp-like sequence (Figure 1A). After filtering the redundant sequences, 42 contigs were identified with a putative viral RdRp signature. Curiously, 39 contigs came from pair-ended SRA data, and only three were identified in single-ended SRA data: SRR1536286, SRR14339744, and

SRR14339752. The RdRp amino acid sequence of all virus-like contigs was extracted and then queried with BLASTp against an NCBI non-reduntant protein database. All sequences matched with E-value below e^{-40} to known RNA viruses RdRp. Some sequences also matched with hypothetical proteins, which may indicate an erroneous protein annotation in the NCBI database. In addition, the genome structure of each virus-like contig was predicted based on their identity with the family-levels pHMM. We estimate that 97,6 % of the sequences were associated with ssRNA viruses, of which 73,8% were closer to positive-sense and 23,8% to negative-sense ssRNA viruses. Moreover, only one contig was predicted to be a dsRNA virus (Figure 1B).

The length of the viral RdRp core region ranges from 350 to 600 amino acids (Olendraite et al., 2023). However, the full ORF length varies between the viral families because some viruses encode the RdRp into a polyprotein (Hough et al., 2023; Kondo et al., 2022). Our analyses revealed a diversity in RdRp lengths (Figure 1C). We expected that the proteins lower than 300 a.a. were incomplete sequences. Also, RdRp-like ORFs higher than 1000 a.a. were predicted to be polyproteins. This idea is supported by the fact that most of these sequences were afterward classified into families characterized by encoding their RdRP in a polyprotein. For example, sequences identified in the families *Iflaviridae*, *Dicistroviridae*, and *Chuviridae* had two, two, and four ORFs over 1000 a.a., respectively (Hu et al., 2023; Valles, et al., 2017a; Valles, et al., 2017b).

We attempt to classify the virus-like sequence based on its IDscore with each family-level pHMM in Olendraite et al., 2023 database. If the sequence has no IDscore ≥ 0.25 with any family-level pHMM, this sequence was designated as taxonomy unclassified. Otherwise, sequences with only one IDscore above 0.25 or the second-best IDscore is 20% lower than the first were classified into the respective pHMM family. The sequences could also have ambiguous classification if the first and second higher IDscore values were closer to each other. To our surprise, none of the sequences detected were set in an unclassified group, and only 23,8% had an ambiguous classification (Figure 2A). The remaining sequences were classified into twelve family taxa corresponding to their family-level pHMM best hit (Figure 2B). The most present families were *Dicistroviridae,* eight virus-like sequences; *Orthomyxoviridae*, five virus-like sequences; *Chuviridae* and *Iflaviridae*, four virus-like sequences. These families are usually known to infect insects, and until this day, there is no evidence of members of these families infecting fungi (Ren et al., 2022; Takemae

et al., 2023; Valles, Chen, Firth, Guérin, Hashimoto, Herrero, De Miranda, et al., 2017). Curiously, 4/5 sequences identified as orthomyxovirus have more than 85% identity in BLASTp with influenza A viruses and may indicate contamination of the *Metarhizium* RNAseq data with other kingdom taxa. The only exception shares 68,5% identity with an atrhopod virus, Neuropteran orthomyxo-related virus (Käfer et al., 2019; Ma, 2022).

Other virus-like sequences were classified into families characterized by infect insects (*Dicistroviridae, Flaviridae*, *Chuviridae*, *Iflaviridae*, *Polycipiviridae*) and plants (*Betaflexiviridae*, *Tymoviridae*). A BLASTp search of the sequences revealed E-value < 10^{-25} to known insects and plant viruses, respectively (Canto et al., 1997; Chang & Chen, 2018; Katsarou et al., 2023; Meng & Rowhani, 2017; Zhang et al., 2023). A BLASTp analysis of the virus-like sequences classified in *Tombusviridae* shows E-values < 10^{-30} with Providence virus (PrV), although they share only 35% of identity. PrV is an insect virus known to infect members of the lepidopteran. This virus can also replicate in plant cells and produce infectious particles. This virus's ability to infect plants and insects may be correlated with their close biological interaction (Jiwaji et al., 2019). One contig was classified in the family *Phenuiviridae*. This family is known to infect vertebrates and invertebrates, and recently, a new genus, Mycobunyavirus, was proposed inside this family to accommodate all mycovirus (H. Huang et al., 2023; Sasaya et al., 2023; Velasco et al., 2019). Despite that, the phenuivirus-like sequence identified in our work shares more than 40% identity with the insect virus, Mothra virus (X. Wang et al., 2021). The best hit in BLASTp search of all virus-like sequences identified in this work can be visualized in Table S1.

3.2 Phylogenetic analysis of virus-like sequences in Narnaviridae

To investigate the accuracy of the taxonomy pHMM classification, we select two families, *Narnaviridae* and *Partitiviridae*, which currently have mycovirus representative members characterized and built a phylogenetic tree for the sequences classified into these families (Hough et al., 2023; Kondo et al., 2022). The three narnavirus-like sequences were queried with BLASTp against the nr NCBI protein database, and the best hits were selected to create a phylogenetic tree (figure 3). We could observe the presence of two consistent clades (bootstrap 100) representing the families *Mitoviridae* and *Botourmiaviridae*. The phylogenetic analyses revealed that one virus-like sequence grouped with other botourmiavirus. Because of this, they were

called Metarhizium-associated Botourmiavirus-like 1. In addition, the other two virus-like sequences were classified as members of *Mitoviridae* and called Metarhizium-associated mitovirus-like 1 and 2.

Both families are closely related to *Narnaviridae* since they belong to the same phylum, *Lenarviricota* (Hough et al., 2023; Kondo et al., 2022). Members of *Mitoviridae* are characterized by infecting their hosts' mitochondria. Consequently, these viruses have the same codon usage bias as mitochondrial genes and don't have an extracellular phase (de Rezende et al., 2021; X. Li et al., 2023; Villan Larios et al., 2023). The two mitovirus-like sequences identified were grouped in the same clade with other plants' mitovirus. We could also observe the presence of hypothetical plant proteins in this clade. These sequences were among the best BLASTp hits, and it is essential to note that these hypothetical proteins came from works unrelated to viruses, and maybe their wrong annotation in the NCBI database is associated with an undetected mitovirus RdRp. Likewise, *Botourmiaviridae* is a family with a broad host range and thus is characterized by hosting plants and fungi (Ayllón et al., 2020). Different from mitovirus, this family adapts to cytoplasmatic replication. Consequently, botourmiavirus shares the same codon usage bias as its host and is believed to have an extracellular phase (Liu et al., 2020; Song et al., 2023).

3.3 Phylogenetic analysis of virus-like sequences in Partitivirdae

Partitiviridae is a family into the order *Durnavirales,* as with *Amalgaviridae*, *Hypoviridae*, and *Fusariviridae*. All these families have evidence of viruses infecting fungi (Herrero, 2016; Kondo et al., 2022; Villan Larios et al., 2023; Yang et al., 2020). Therefore, the virus-like sequence classified into this family was queried with BLASTp against the nr protein NCBI database. The five top matches were selected and downloaded. In addition, we include other virus sequences in the *Durnavirales* order. The putative virus sequences identified were sorted into a consistent clade (bootstrap 100) with the proposed group Unirnavirus (Kotta-Loizou et al., 2015). Therefore, we called them Metarhizium-associated unirnavirus-like 1. Some unirnavirus-like viruses are characterized by hosting fungi, especially entomopathogenic fungi. For example, B. bassiana non-segmented virus (BbNV)-1 and Penicillium janczewskii Beauveria bassiana-like virus 1 (PjBlV1) (Y. Huang et al., 2023; Kotta-Loizou, 2021). Moreover, the members of this proposed group show a relative similarity to other virus families in

Durnavirales, like *Partitivirdae* and *Amalgaviridae* (Herrero, 2016; Y. Huang et al., 2023; Yang et al., 2020).

4 – Discussion

This work used a pHMM approach to identify virus-like sequences in *Metarhizium* public transcriptomic data. In total, we could find 42 RNA virus-like sequences in approximately 20% of the SRA data analyzed. We also tried to taxonomy classify this sequence based on their IDscore with each 77 family-level pHMM. Our analyses suggest the presence of at least twelve viral families. Since we were looking for RdRp pattern, all sequences identified were related to RNA viruses. The genomic structure analysis revealed a high abundance of ssRNA viruses and only 2% of dsRNA viruses. The mycovirome diversity revealed by Jo et al., 2022 also predicts a high percentage of ssRNA 47%, followed by dsRNA-related viruses 44,6%, and 4,5% of DNA-related viruses. Historically, mycoviruses were believed to be predominantly dsRNA viruses. However, the characterization of new mycoviruses usually requires the isolation and sequencing of the viral dsRNA genome or its replicative intermediate. New studies using different approaches, like metatranscriptome, to identify mycoviruses revealed a previously unknown diversity of ssRNA viruses infecting fungi (S. Li et al., 2024; Ye et al., 2023).

The increase in public metatranscriptomic and/or metagenomic data available on NCBI is an excellent opportunity to explore the virosphere in works unrelated to viruses. For example, studies have used sequence alignment or a structure-based approach (PalmScan) to identify virus-like sequences in all kingdom life taxa. However, these strategies require more computational effort and usually expend more time than HMMsearch (Charon et al., 2022; Dheilly et al., 2022; Eddy, 2011; Edgar et al., 2022; Jo et al., 2022; Nakagawa et al., 2023; Neri et al., 2022; Olendraite et al., 2023). Because of this, HMMsearch is a quick method to find RdRp-like sequences with no false positives and still identify sequences with low similarity to other known RNA viruses (Olendraite et al., 2023). Accordingly, our analyses didn't identify any false positives since all virus-like sequences matched with known viral RdRp (E-values below 10^{-30}), and we still could detect sequences with low identity to other known viruses (≤ 40%).

The HMMsearch strategy is sensitive to identifying divergent sequences (Olendraite et al., 2023). For example, sequences initially classified as narnavirus were subsequently allocated into other families, *Mitovirdae* and *Botourmiaviridae*. These families belong to the same phylum (*Lenarviricota*) and are phylogenetically related (Hough et al., 2023; Kondo et al., 2022; X. Li et al., 2023; Liu et al., 2020). Similarly, the virus-like sequence identified as a member of *Partitiviridae* grouped in a consistent clade with another mycovirus in the proposed genus Unirnavirus, which is also related to other families in the *Durnavirales* order (Herrero, 2016; Y. Huang et al., 2023; Kotta-Loizou et al., 2015). The initially wrong annotation in the family taxa of these sequences is due to the lack of a representative of these families in the 77 family-level pHMM used in this work. Building a pHMM model is not easy since it requires many accurate viral RdRp sequences. Moreover, an overrepresented genus in its construction may make it difficult to identify other genera in the same family (Olendraite et al., 2023). Consequently, our analyses may underestimate mycoviral families with no pHMM profile in the database or incorrectly classified in other phylogenetic correlated families. For example, there is no pHMM representative of the mycoviral families *Yadokariviridae*, *Hadakaviridae*, *Polymycoviridae*, *Mitoviridae*, and *Botourminarviridae* (Hough et al., 2023; Kotta-Loizou & Coutts, 2022b; Olendraite et al., 2023; Sato, Das, et al., 2023; Sato, Turina, et al., 2023). Because of this, the RdRp sequence of novel-characterized virus must be used to create new families' pHMMs and improve the efficacy of this strategy.

The expansion of high-throughput sequencing technology in recent years has created a large amount of data available for public analysis in databases like NCBI. Most came from works unrelated to viruses and are a wealthy environment to explore a new virus diversity (Edgar et al., 2022; Neri et al., 2022; Olendraite et al., 2023). In fact, recent works are using these data to expand our knowledge about the virosphere. A study by Edgar, et al., 2022 developed the Serratus structure, this cluster is optimized to create sequence alignment in a petabase-scale (over 10^{15} nucleotide bases) and search for the palm motif in viral RdRp. They could identify over 10^5 new viral RdRp-like sequences in all life kingdoms and improve our knowledge about the RNA virosphere. Unfortunately, we cannot infer the virus-host when analyzing public metatranscriptomic and metagenome data. This is because most data are associated with environmental sources, and even in cases where the data was associated with an isolated specie we have no control over the possibility of other kingdoms taxa

contamination (Cobbin et al., 2021; Mifsud et al., 2022; Neri et al., 2022; Olendraite et al., 2023). Accordingly, we identify four human-associated influenza A viruses in *Metarhizium* spp. RNAseq. Other virus-like sequences classified in the families *Betaflexiviridae, Dicistoviridae,* and *Iflaviridae* also share over 90% of their identity with known plants and arthropods viruses, respectively. These sequences could be an example of another life kingdom taxa contamination in the public *Metarhizium* SRA data. Otherwise, sequences in the families *Phenuiviridae*, *Polycipiviridae*, *Tombusviridae*, and *Tymoviridae* show less than 50% identity, with known viruses. These sequences may represent new groups of mycoviruses until now unknown. For example, a new genus, mycobunyavirus, was proposed in *Phenuiviridae* hosting fungi (Velasco et al., 2019), but deeper analyses need to be made to accurately investigate these sequences taxon.

In conclusion, we used a set of pHMM associated with 77 different viral families RdRp to identify virus-like sequences in public RNAseq data from *Metarhizium* spp. available on NCBI. Our analyses bring a little light into the diversity of mycovirus in entomopathogenic fungi and may help in the future characterization of new mycoviruses. In addition, searching for pHMM patterns is a costless strategy to investigate RNA virus diversity and evolution inside all kingdoms. Furthermore, while new virus taxons are characterized, we can build new pHMM associated with them, improving the HMMsearch strategy.

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Figures

Figure 1:

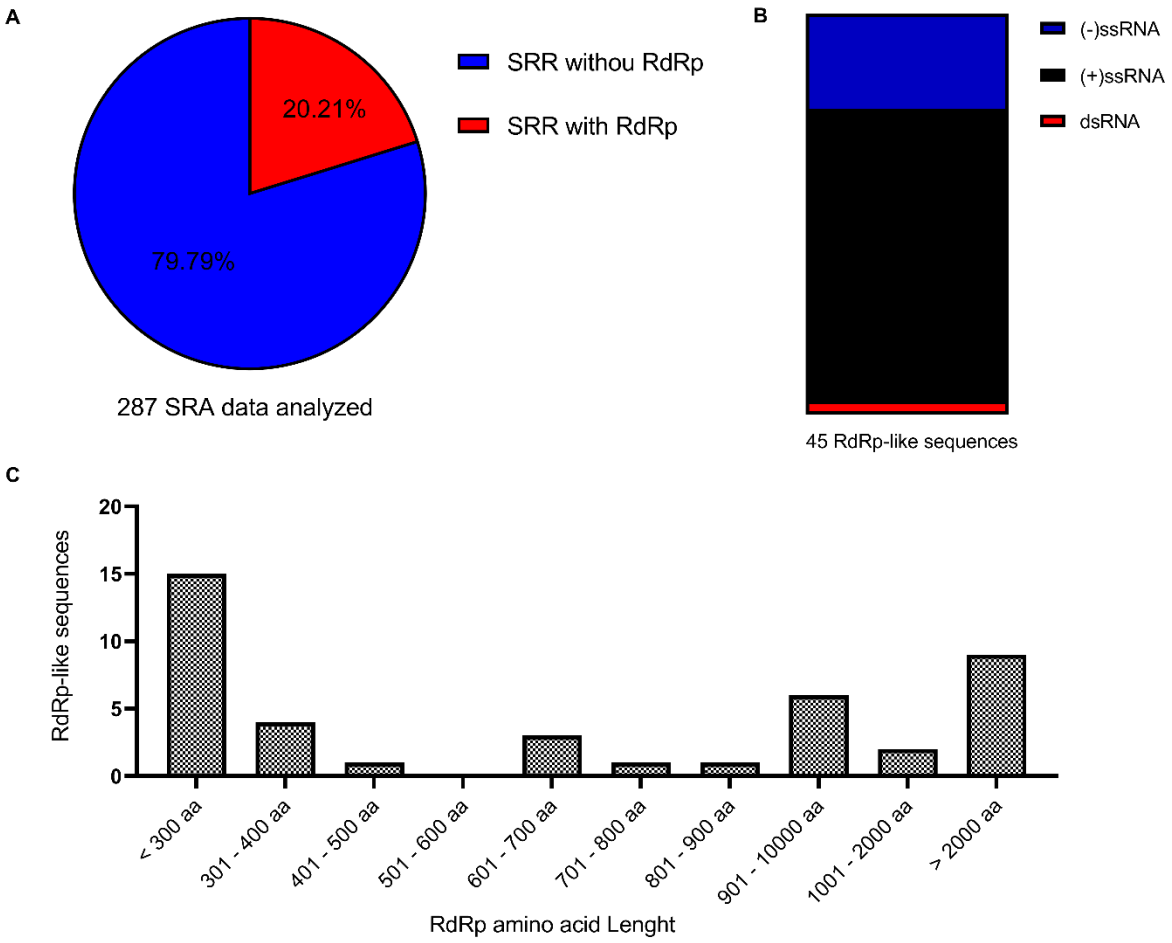

Figure 1: (A) Proportion of the presence of RdRp-like ORF in *Metarhizium* Ilumina RNAseq database. In blue is the total of *Metarhizium* SRA data without RdRp-like ORF, and in red is *Metarhizium* SRA data with RdRp-like ORF. (B) Genomic structure of the virus-like sequences identified. (C) Size distribution of the amino acid length of the RdRp-like ORFs identified. Sequences lower than 300 aa are considered incomplete, and sequences up to 1000 aa are predicted to be polyproteins.

Figure 2:

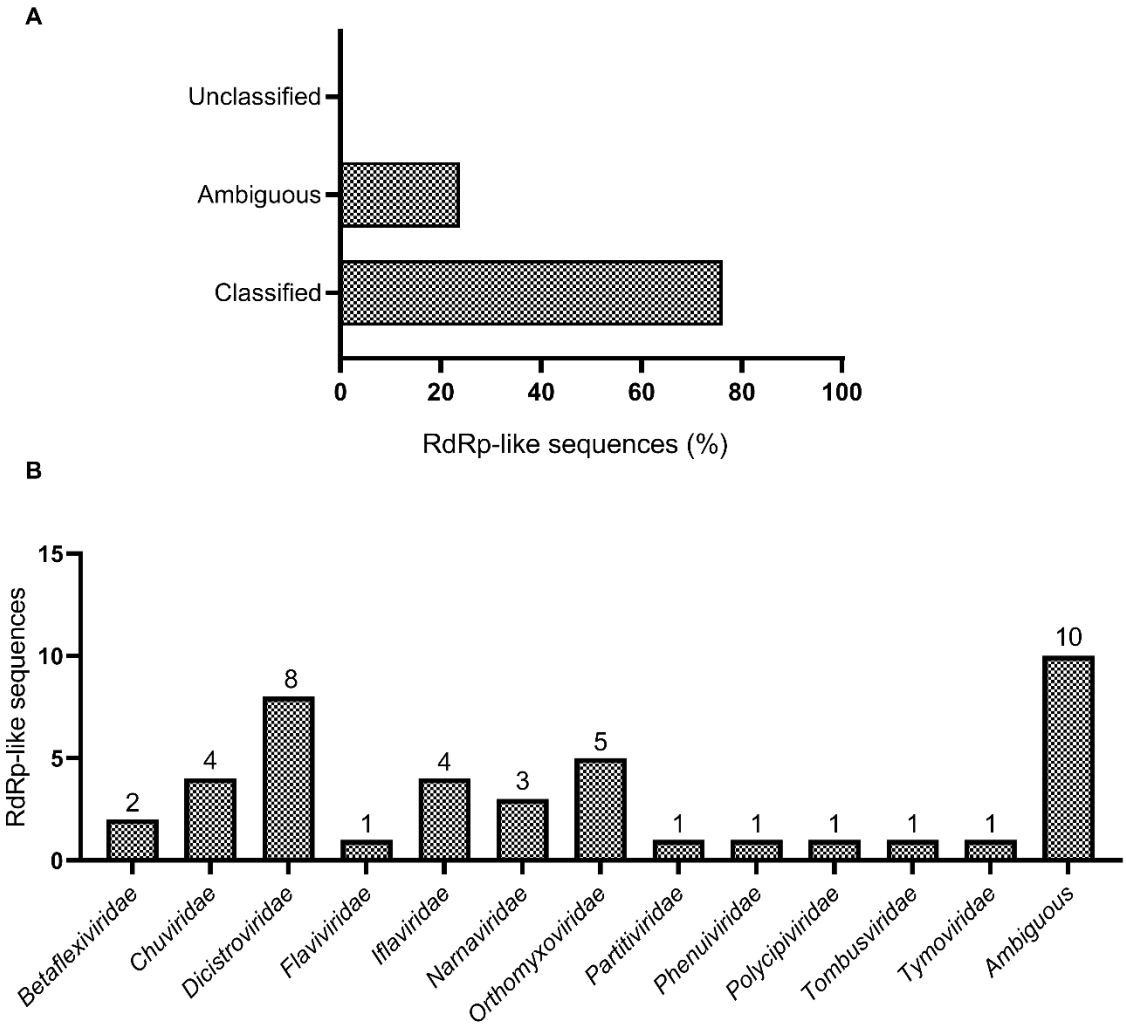

Figure 2: (A) Percentage of the RdRp-like ORFs sorted into the three groups “Classified,” “Ambiguous,” and “Unclassified.” (B) The number of virus-like sequences classified into each family based on their IDscore with the respective family-level pHMM in the database.

Figure 3:

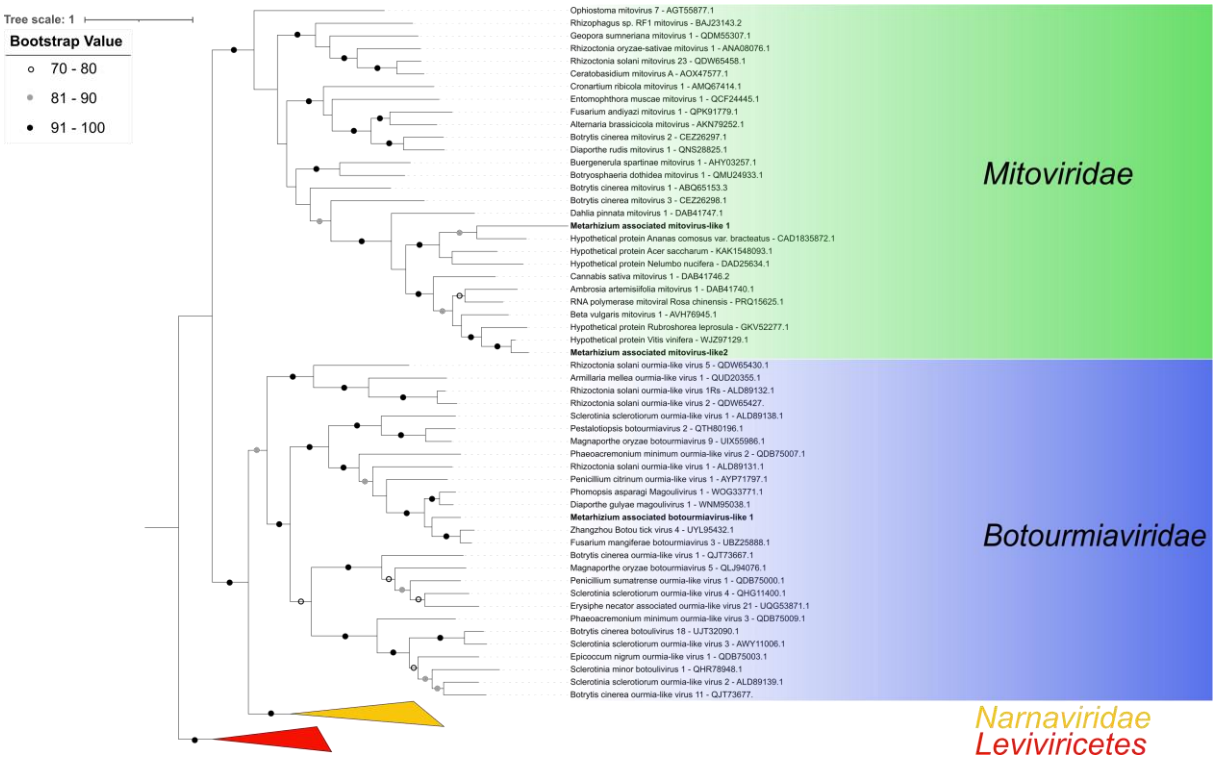

Figure 3: Phylogenetic tree built with the amino acid sequence of the RdRp-like ORFs classified into *Narnaviridae* by pHMM database. This tree was constructed with RAxML, applying 1000 bootstrap replicates, and the best evolutive model BLOSUM62+G4+F. The NCBI accession number of each sequence is displayed at the end of their names. Viruses in the class *Leviviricetes* was used as outrgroup.

Figure 4:

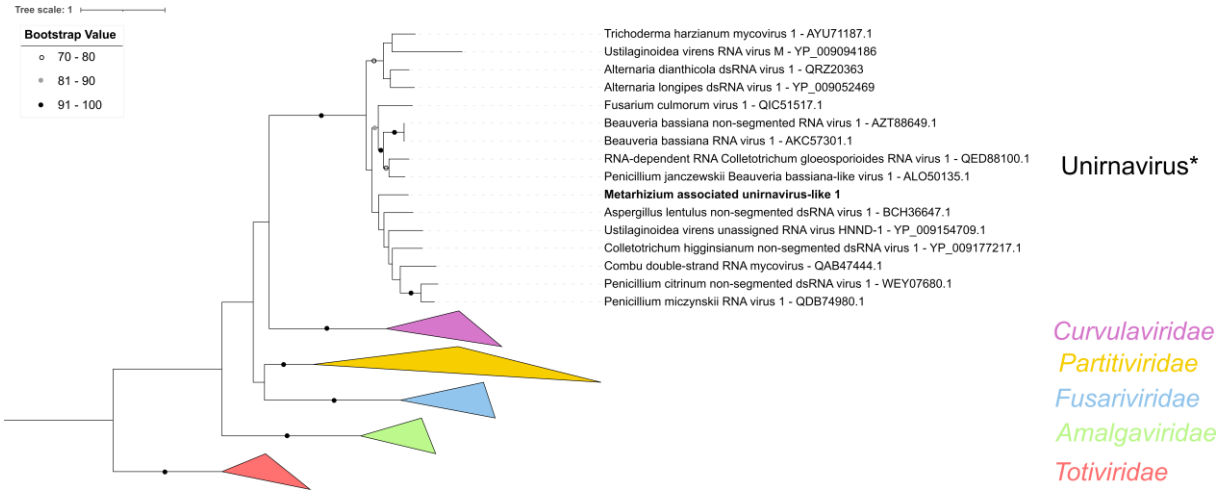

Figure 4: Phylogenetic tree with the amino acid sequence of the RdRp-like ORFs classified into *Partitivirdae* by our pHMM database. The tree was built with RAxML applying 1000 bootstrap replicates and the best evolutive model LG+I+G4+F. Family *Totiviridae* was used as an outgroup. The NCBI accession number of each sequence is displayed at the end of their names. * Proposed family by Kotta-Loizou et al., 2015.

Tabela S1: Best hit of RdRp BLASTp search of the virus-like sequences identified.

Contigs	pHMM classification	Predict genome type	Scientific Name	Query Cover	E value	Identity (%)	NCBI Accession
TRINITY_DN29691_c0_g2_i1_1	Betaflexiviridae	(+)ssRNA	Grapevine rupestris stem pitting-associated virus	100%	0.0	99,37	UVK69396.1
TRINITY_DN31778_c0_g1_i1_3	Betaflexiviridae	(+)ssRNA	Grapevine rupestris stem pitting-associated virus	86%	0.0	99,68	UTQ36120.1
TRINITY_DN8358_c0_g1_i1_4	Chuviridae	(-)ssRNA	Orthopteran chu-related virus OKIAV152	97%	0.0	54,07	QPL15312.1
TRINITY_DN29062_c0_g1_i1_2	Chuviridae	(-)ssRNA	Chuviridae sp.	96%	0.0	72,95	QXV86378.1
TRINITY_DN40209_c0_g5_i2_1	Chuviridae	(-)ssRNA	Chuviridae sp.	96%	0.0	70,66	QXV86378.1
TRINITY_DN40396_c1_g3_i1_4	Chuviridae	(-)ssRNA	Chuviridae sp.	96%	0.0	70,62	QXV86378.1
TRINITY_DN1233_c0_g1_i1_1	Dicistroviridae	(+)ssRNA	Bactrocera dorsalis cripavirus	100%	0.0	94,99	QKF95551.1
TRINITY_DN4664_c0_g3_i1_1	Dicistroviridae	(+)ssRNA	Atrato picorna-like virus 1	92%	0.0	52,68	QHA33681.1
TRINITY_DN7088_c0_g1_i1_2	Dicistroviridae	(+)ssRNA	Cripavirus sp.	97%	0.0	99,9	ULF99859.1
TRINITY_DN7233_c0_g2_i1_1	Dicistroviridae	(+)ssRNA	Dicistroviridae sp.	100%	0.0	74,47	AVA30705.1
TRINITY_DN13250_c0_g1_i1_1	Dicistroviridae	(+)ssRNA	Fushun dicistrovirus 1	98%	1,00E-177	95,9	UHM27697.1
TRINITY_DN20831_c0_g3_i1_1	Dicistroviridae	(+)ssRNA	Drosophila C virus	100%	0.0	98,93	QEQ50978.1
TRINITY_DN20831_c0_g4_i1_1	Dicistroviridae	(+)ssRNA	Drosophila C virus	100%	5,00E-135	98,53	QEQ50980.1
TRINITY_DN42914_c0_g1_i1_1	Dicistroviridae	(+)ssRNA	Aphid lethal paralysis virus	60%	4E-26	84,38	WAK72320.1
TRINITY_DN6738_c0_g1_i1_3	Flaviviridae	(+)ssRNA	Wuhan aphid virus 1	94%	0.0	52,01	YP_009179388.1
TRINITY_DN6206_c0_g1_i1_1	Iflaviridae	(+)ssRNA	Bombyx mori iflavirus	98%	0.0	99,73	YP_009162630.1
TRINITY_DN10727_c0_g1_i1_7	Iflaviridae	(+)ssRNA	Iflaviridae sp.	95%	7E-133	35,03	QKN89051.1
TRINITY_DN13739_c0_g1_i1_1	Iflaviridae	(+)ssRNA	Sacbrood virus	93%	0.0	97,93	AHL96303.1
TRINITY_DN26382_c1_g1_i1_3	Iflaviridae	(+)ssRNA	Lymantria dispar iflavirus 1	99%	0.0	74,44	YP_009047245.1
Metarhizium associated Botourmiavirus-like 1	Narnaviridae	(+)ssRNA	Botourmiaviridae sp.	98%	0.0	66,96	UJQ91955.1

Table S1 continued

Metarhizium associated Mitovirus-like 1	Narnaviridae	(+)ssRNA	Hypothetical Protein Nelumbo nucifera	46%	9,00E-34	65,66	DAD25634.1
Metarhizium associated Mitovirus-like 2	Narnaviridae	(+)ssRNA	Hypothetical Protein Shorea leprosula	88%	7,00E-81	60,44	GKV52277.1
TRINITY_DN1012_c0_g1_i1_1	Orthomyxoviridae	(-)ssRNA	Influenza A virus (A/pelican/Peru-MM24/2007(H4N5))	100%	4,00E-168	97,47	AKM14101.1
TRINITY_DN2852_c0_g2_i1_1	Orthomyxoviridae	(-)ssRNA	Influenza A virus (A/Wilson-Smith/1933(H1N1))	100%	0.0	100	P03430.1
TRINITY_DN4618_c0_g1_i4_1	Orthomyxoviridae	(-)ssRNA	Influenza A virus (A/Wilson-Smith/1933(H1N1))	97%	0.0	100	ABF21250.1
TRINITY_DN6984_c0_g1_i1_2	Orthomyxoviridae	(-)ssRNA	Influenza A virus (A/swine/Korea/S190/2004(H9N2))	63%	1,00E-86	85,99	AAV68020.1
TRINITY_DN31245_c0_g1_i1_1	Orthomyxoviridae	(-)ssRNA	Neuropteran orthomyxo-related virus OKIAV210	99%	0.0	68,49	QPL15346.1
Metarhizium associated unirnavirus-like 1	Partitiviridae	dsRNA	Penicillium janczewskii Beauveria bassiana-like virus 1	85%	0.0	63,23	ALO50135.1
TRINITY_DN27995_c0_g1_i1_2	Phenuiviridae	(-)ssRNA	Mothra virus	96%	0.0	42,82	YP_009666266.1
TRINITY_DN8852_c0_g1_i1_1	Polycipiviridae	(+)ssRNA	Guiyang polycipivirus 1	97%	2,00E-41	36,71	UHK03092.1
TRINITY_DN40388_c0_g1_i1_2	Tombusviridae	(+)ssRNA	Providence virus	70%	7,00E-125	33,95	AMQ67162.1
TRINITY_DN15231_c0_g1_i1_5	Tymoviridae	(+)ssRNA	Sinomenium acutum tymovirus 1	58%	2,00E-111	44,29	QQG34657.1
TRINITY_DN1265_c0_g1_i1_1	Closteroviridae/ Bromoviridae	(+)ssRNA	Hypothetical Protein Rhizopus arrhizus	46%	4,00E-106	34,25	KAG0780141.1
TRINITY_DN6814_c0_g1_i1_3	Closteroviridae/ Bromoviridae	(+)ssRNA	Hypothetical Protein Rhizopus arrhizus	31%	6,00E-87	35,43	KAG0804577.1
TRINITY_DN8323_c0_g1_i1_3	Closteroviridae/ Bromoviridae	(+)ssRNA	Hypothetical Protein Rhizopus arrhizus	31%	4,00E-87	35,68	KAG0804577.1
TRINITY_DN12994_c0_g1_i1_1	Closteroviridae /Virgaviridae	(+)ssRNA	Hypothetical Protein Rhizopus arrhizus	49%	6,00E-122	45,93	KAG0738144.1

Table S1 continued

TRINITY_DN22101_c0_g1_i1_2	Closteroviridae/ Virgaviridae	(+)ssRNA	Sanya virga-like virus 1	82%	3,00E-54	46,73	UHM27517.1
TRINITY_DN5860_c0_g1_i1_1	Dicistroviridae/ Picornaviridae	(+)ssRNA	Tetranychus urticae-associated picorna-like virus 1	100%	5,00E-151	99,12	QBS55248.1
TRINITY_DN5776_c0_g1_i1_1	Iflaviridae/ Dicistroviridae	(+)ssRNA	Tetranychus urticae-associated picorna-like virus 1	100%	7,00E-147	94,71	QIN54759.1
TRINITY_DN6100_c0_g1_i1_1	Iflaviridae/ Dicistroviridae	(+)ssRNA	Tetranychus urticae-associated picorna-like virus 1	85%	2,00E-131	94,74	QIN54759.1
TRINITY_DN5658_c0_g1_i1_1	Iflaviridae_4/ Iflaviridae	(+)ssRNA	Iflaviridae sp.	81%	1,00E-82	32,46	QKN89051.1
TRINITY_DN1224_c0_g1_i1_1	Polycipiviridae/ Dicistroviridae	(+)ssRNA	Guiyang polycipivirus 1	94%	4,00E-28	36,49	UHK03092.1

4 - Conclusion

Our work successfully used two approaches to investigate the RNA viral diversity in *Metarhizium* spp. The analyses of the dsRNA pattern present in *Metarhiziu robertsii* isolate SCJAN-21.11 revealed the existence of a new polymycovirus. These viruses are characterized by hosting fungi, and some are associated with host hypervirulent phenotype. As we know, it is the first case of a member of *Polymycoviridae* hosting entomopathogenic fungi in Brazil, and future studies will analyze if this mycovirus has biocontrol potential. We also exploit the existence of virus-like sequences in public RNAseq data available on NCBI. We search for SRA data originating from *Metarhizium* spp. metatranscriptomic and use a set of pHMM representative of 77 viral families RdRp. This analysis identifies at least 12 families and 138 virus-like sequences. However, the taxonomic classification of each family needs to be individually analyzed. In addition, we observe the contamination of another kingdom taxa virus in *Metarhizium* spp. metatranscriptomes since we could identify virus-like sequences with high identity to plants, arthropods, and even human viruses. In conclusion, this work helps to elucidate the diversity of EPF mycovirome and characterize a new member of *Polymycoviridae*, which we attempt to call *Polymycovirus mineiro*.