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Genomic characterisation of mycoviruses associated with members of Ceratocystidaceae

by

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FABI

Table of Contents

Declaration

I, Bianca Hough, declare that the declaration, which I hereby submit for the degree Magister Scientiae at the University of Pretoria, is my own and has not previously been submitted by me for the degree at this or any other tertiary institution.

Signature:

Date: 20 October 2023

Ethics statement

The author, whose name appears on the title page of this dissertation, has obtained, for the research described in this work, the applicable research ethics approval.

The author declares that she has observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the policy guidelines for responsible research.

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Preface

Mycoviruses have been attracting considerable attention recently, for the remarkable ability of some to attenuate the pathogenicity of their fungal hosts. Such viruses have shown great promise as biocontrol agents against certain plant pathogenic fungi. However, despite their immense potential, numerous fungal taxonomic groups remain under-explored in this context. This study primarily focused on the identification and characterization of mycoviruses within Ceratocystidaceae, a fungal family hosting economically important fungi which devastate crops and forests worldwide. Limited information is available on mycoviral diversity within this family. Consequently, the results of this research provide invaluable insights into the mycoviral landscape within Ceratocystidaceae. By shedding light on mycoviral diversity within Ceratocystidaceae, this research not only enriches our comprehension of mycoviruses in this family, but also lays the foundation for future advancements in biocontrol strategies against some of the destructive fungal members. Moreover, this study highlights the role of utilizing transcriptomic data as a powerful tool for mycovirus discovery.

Chapter 1 serves as a comprehensive review on mycoviruses, encompassing their host range, taxonomic classification, effects on their fungal counterparts, and the techniques employed in their discovery. The potential application of mycoviruses as biocontrol agents against plant pathogenic fungi is also discussed. While this chapter provides a comprehensive overview of mycoviruses in general, a dedicated section specifically addressing mycoviruses within the Ceratocystidaceae family is also included. Chapter 2 delves into the assessment of mycoviral diversity in transcriptomes obtained from various members of the Ceratocystidaceae family. These transcriptomes, the majority of which underwent poly-A selection during library preparation, are publicly available and were sourced from the NCBI sequencing reads archive (SRA). Chapter 3 focuses on the identification and characterization of mycoviruses in fungal cultures from *Ceratocystis*, a fungal genus within Ceratocystidaceae. This was achieved by generating transcriptomes with a ribo-depletion step during library preparation, thereby mitigating biases associated with poly-A selection. Some of the transcriptomes used in this chapter was generously provided by Preston Shaw from the Forestry and Agricultural Biotechnology Institute (FABI), while others were obtained from manual sequencing of cultures obtained from the FABI CMW culture collection.

In both chapters, the genomes of putative mycoviruses were meticulously characterized, and their relationships to other cognate viruses were elucidated. Chapter 3 goes a step further by completing 3' and 5' Rapid Amplification of cDNA ends (RACE) PCRs for three selected

viruses, to generate complete genomes which are required for the official taxonomic placement of these viruses by the International Committee on the Taxonomy of Viruses (ICTV). Conformation PCRs were then performed on these viruses, to confirm their presence in the original fungal culture.

The research for this dissertation was conducted at the Forestry and Agricultural Biotechnology Institute (FABI), within the Department of Biochemistry, Genetics, and Microbiology at the University of Pretoria. This research was performed under the guidance of Dr. David Read and Prof. Brenda Wingfield. This dissertation is comprised of three independent units, and as a result, some information and references may appear duplicated. Chapter 1, chapter 2, and chapter 3 were written in the format required for submission to scientific journals.

It's important to note that Chapter 1 has recently been accepted for publication by MDPI Viruses. Consequently, the majority of the material in Chapter 1 will be consistent with the content published on MDPI, accessible through this link: https://doi.org/10.3390/v15051202.

Chapter 1: Fungal Viruses Unveiled: A Comprehensive Review of Mycoviruses.

Abstract

Mycoviruses are ubiquitous throughout the fungal kingdom and are currently classified into 23 viral families and the genus *Botybirnavirus* by the ICTV. The primary focus of mycoviral research has been on mycoviruses that infect plant pathogenic fungi, due to the ability of some to reduce the virulence of their host and thus act as potential biocontrol against these fungi. However, mycoviruses lack extracellular transmission mechanisms and rely on intercellular transmission through the hyphal anastomosis, which impedes successful transmission between different fungal strains. This review provides a comprehensive overview of mycoviruses, including their origins, host range, taxonomic classification into families, effects on their fungal counterparts, and the techniques employed in their discovery. The application of mycoviruses as biocontrol agents of plant pathogenic fungi is also discussed. This review also delves into the less-explored realm of mycoviruses associated with Ceratocystidaceae, shedding light on their characteristics and potential implications.

Introduction

Mycoviruses (viruses of fungi) are ubiquitous throughout the fungal kingdom [1]. They are known to associate with most of the major fungal taxonomic groups, including Ascomycota, Basidiomycota, Chytridiomycota, Zygomycota, and Neocallimastigomycota [2, 3]. The International Committee on the Taxonomy of Viruses (ICTV) currently classifies mycoviruses as 23 families and 1 unclassified genus, and this is based on the type of genome [4-8]. The majority of mycoviruses have double-stranded RNA (dsRNA) or positive sense single-stranded RNA (+ssRNA) genomes [5]. Mycoviruses with dsRNA genomes are classified into the viral families *Chrysoviridae*, *Amalgaviridae*, *Megabirnaviridae*, *Quadriviridae*, *Partitiviridae*, *Polymycoviridae*, *Reoviridae*, *Totiviridae*, and the genus *Botybirnavirus* (unclassified) [4, 6]. Those with +ssRNA genomes are grouped into the families *Endornaviridae*, *Alphaflexiviridae*, *Barnaviridae*, *Deltaflexiviridae*, *Gammaflexiviridae*, *Hypoviridae*, *Narnaviridae*, *Mitoviridae*,

Hadakaviridae, *Yadokariviridae*, and the reverse transcribing (RT) families *Metaviridae* and *Pseudoviridae* [4, 6-8]. Mycoviruses with negative-sense single-stranded RNA (−ssRNA) genomes have also been discovered [9-11], and belong to the family *Mymonaviridae* [4, 6]. Recently a number of ssDNA mycoviruses have also been found; however, only members from two viral species belong to a recognized mycoviral family, namely *Gemycircularvirus sclero1* and *Gemytripvirus fugra1* [12]. Both belong to the family *Genomoviridae* [13, 14].

Mycoviruses that elicit hypovirulence (decreased virulence) have been identified in both human and plant pathogenic fungi. These viruses frequently cause several adverse effects in their fungal hosts, such as decreased virulence, irregular growth, abnormal pigmentation, and defects in sexual development [5, 15, 16]. The recent discovery of hypovirulence inducing mycoviruses in human pathogenic fungi presents an opportunity for the development of therapeutic interventions against fungal infections in humans. The majority of mycoviral research, however, has been concerned with hypovirulence-inducing mycoviruses of plant pathogenic fungi [12, 17, 18]. These mycoviruses have the potential to be used as biocontrol agents against their fungal hosts, thereby reducing the losses in agriculture and forestry due to fungal infections [19, 20]. Despite their potential, the use of mycoviruses as biocontrol agents has a number of challenges. Mycoviruses lack the extracellular transmission mechanisms of plant and animal viruses [3]. Instead, viral transmission occurs intercellularly, through hyphal anastomosis (fusion of fungal hyphae), cell division, and sporulation [3]. Hyphal anastomosis impedes the successful transmission of mycoviruses between different fungal strains since they need to be vegetatively compatible with hyphal fusion [21]. Nevertheless, research is ongoing to find solutions to this problem [22, 23], and mycovirus-based biocontrol has already been used successfully [19].

Advances in high throughput sequencing have led to a surge in mycovirus discoveries and new insights into their origins, diversity, and impact on fungal hosts. This review examines the latest developments in the field of mycovirology and discusses the potential for using mycoviruses as biocontrol agents against plant pathogenic fungi. Various methods that have been employed to facilitate the discovery process are also summarized. Furthermore, the presence and potential implications of mycoviruses in one under-explored fungal family, Ceratocystidaceae, are also reviewed.

The History and Origins of Mycoviruses

In 1948, a disease-causing dieback of the commercially produced mushroom, *Agaricus bisporus*, was reported and named the 'La France' disease [24]. It was not until nearly a decade later that mycoviruses were identified as the causal agents of this eco-nomically important disease [25]. This quickly led to the development of a new field of study: mycovirology. Not long after, mycoviruses were also identified in the ascomycete *Penicillium stoloniferum*, and were determined to cause interferon stimulation in mammals [26, 27]. It was not until the 1970s, however, that a significant breakthrough was made in the field of mycovirology. A mycovirus of the chestnut blight pathogen, *Cryphonectria parasitica*, was observed to reduce the virulence of its host and had potential as a biocontrol agent against this plant pathogenic fungus [28]. This led to in-creased interest in the discovery of mycoviruses in plant pathogenic fungi, as they could serve as prospective biocontrol agents of such fungi. Another development during this period was the discovery of mycoviruses inducing the 'killer yeast' phenotype in *Saccharomyces cerevisiae*, a fungus that is routinely employed in winemaking, brewing, and baking [29]. These mycoviruses confer a competitive advantage to their fungal hosts by producing extracellular toxins which eliminates competing strains [30]. While these toxins were initially associated with fermentation failure, their potential use as a means of reducing undesirable strains was soon realized [30].

Recent advances in high throughput technologies, specifically RNA sequencing, have facilitated the rapid discovery of mycoviruses. This has not only increased our understanding of mycoviral diversity within the fungal kingdom but has shed some light on the evolution and origins of these viruses. Two theories have been proposed. The 'ancient coevolution theory' posits that the relationship between mycoviruses and fungi is ancient and mirrors long-term coevolution [3, 5]. This is supported by the fact that mycoviruses lack an extracellular infection route, which complicates their trans-mission to other fungal species, and thus limits individual viruses to a single host [31]. Research by Neupane, et al. [32] also shows support for this theory, since the phylogenetic analysis of the RNA-dependent RNA polymerase (RdRp) sequences of mitoviruses from non-pathogenic arbuscular mycorrhizal fungi has revealed that they are highly conserved, and do not cluster with mitoviruses from other plant pathogenic fungi. The 'plant virus theory' on the other hand, suggests that mycoviruses originate from plant viruses, where they moved from the plant host to a fungus [3]. In support of this theory, studies have shown that mycoviruses often cluster with plant viruses on phylogenetic trees [33, 34]. For

example, members of the species *Alphahypovirus cryphonectriae*, *Alphahypovirus americanum*, *Betahypovirus cryphonectriae*, *Betahypovirus americanum*, *Botrytis virus X* and the unclassified virus Fusarium graminearum virus-DK21 (FgV-DK21), are all related to plant potyviruses or potex-like viruses [5, 35]. In addition, most mycoviruses lack a movement protein, and in some cases even a coat protein, which indicates that these viruses may have shed their non-essential genes to better adapt to their fungal hosts [31].

Natural cross-kingdom transfer of certain viruses between plants and fungi has also been demonstrated. Andika, et al. [36] provided evidence of this phenomenon by demonstrating the transmission of certain strains of *Cucumber mosaic virus* (CMV) from potato plants to *Rhizoctonia solani* through natural means. The transfer of plant viruses to fungi has also been shown by Cao et al. [37], where 11 different plant viruses were shown to transiently infect several plant pathogenic fungal genera, including *Alternaria*, *Lecanicillium* and *Sarocladium*. Cross-kingdom transmission has been proposed as a significant driver in the evolution of mycoviruses, given that the majority of mycovirus lineages have an ancestral link to plant viruses [38]. The opposite of this theory may also be true, where plant viruses may have originated from mycoviruses which moved from a fungus to a plant. For example, a recent study has demonstrated that mitoviral sequences can cross-transfer between *Botrytis cinerea* and cucumber plants [39]. Similarly, CMV has also been shown to move from *R. solani* to the plant host [36]. An alternative pathway for mycovirus transfer between fungi and plants, through the intermediary of a plant virus, has also been suggested. Bian, et al. [40] showed that Cryphonectria hypovirus 1 (CHV-1) was able to infect *Nicotiana tabacum* when co-inoculated with tobacco mosaic virus (TMV), a plant virus. Moreover, the findings suggest that CHV-1 may facilitate the accumulation of TMV in *Fusarium graminearum*, indicating a potential interaction between these viruses in a plant-fungal system. Due to a dearth of solid evidence and data, however, the exact origins of mycoviruses remains uncertain.

Diversity and Taxonomy

Fungal Host Range

Mycoviruses have been found across most of the major fungal taxonomic groups [5, 41]. The majority of these mycoviruses associate with hosts from Ascomycota and Basidiomycota [2] (collectively called Dikarya), as depicted in Tables 1 and 2. However, this is most likely due to disproportionate sampling across the fungal kingdom. Recent studies have thus focused on the discovery of mycoviruses within the early diverging lineages of fungi and has led to the

discovery of mycoviruses in Chytridiomycota, Zygomycota, Blastocladiomycota, and Neocallimastigomycota (Table 3) [2, 42]. The eco-logical impacts of these viruses on their fungal counterparts, however, remains unknown and requires further investigation.

Table 1. Mycoviruses associated with Ascomycota.

Ascomycota				
Class	**Order**	**Family**	**Genera**	**Mycoviruses**
Arthoniomycetes	**Arthoniales**	***Chrysotrichaceae***	*Chrysothrix*	dsRNA [43]
Dothideomycetes	**Botryosphaeriales**	***Aplosporellaceae***	*Aplosporella*	dsRNA [44]
		Botryosphaeriaceae	*Botryosphaeria*	dsRNA and +ssRNA [45-49]
			Diplodia	dsRNA and +ssRNA [50-52]
			Eutiarosporella	+ssRNA [53]
			Macrophomina	dsRNA, +ssRNA, −ssRNA and ssDNA [54-57]
			Neofusicoccum	dsRNA and +ssRNA [58-60]
			Phaeobotryon	dsRNA [61]
		Phyllostictaceae	*Phyllosticta*	dsRNA [62]
	Capnodiales	***Cladosporiaceae***	*Cladosporium*	+ssRNA, −ssRNA and RT ssRNA [63, 64]
		Mycosphaerellaceae	*Cercospora*	+ssRNA and −ssRNA [65]
			Dothistroma	dsRNA [66]
			Mycosphaerella	+ssRNA [53, 67]
			Pseudocercospora	dsRNA [67]
			Zymoseptoria	dsRNA and +ssRNA [62, 65, 68]
	Dothideales	***Saccotheciaceae***	*Aureobasidium*	+ssRNA [53]
	Mycosphaerellales	***Teratosphaeriaceae***	*Acidomyces*	+ssRNA [62]
			Hortaea	dsRNA [62]
	Pleosporales	***Coniothyriaceae***	*Coniothyrium*	dsRNA and −ssRNA [64, 69]
		Corynesporascaceae	*Corynespora*	dsRNA [68, 70]
		Cucurbitariaceae	*Cucurbitaria*	dsRNA [70, 71]`
		Delitschiaceae	*Delitschia*	dsRNA [62]
		Didymellaceae	*Didymella*	dsRNA [72]
			Epicoccum	+ssRNA [64]
			Leptosphaerulina	+ssRNA [64]
			Phoma	dsRNA and +ssRNA [72-76]
			Stagonosporopsis	dsRNA [48, 73-76]
		Leptosphaeriaceae	*Leptosphaeria*	dsRNA and + ssRNA [77-79]
		Lindgomycetaceae	*Clohesyomyces*	dsRNA [48, 62]
		Massarinaceae	*Helminthosporium*	dsRNA [80]
		Periconiaceae	*Periconia*	dsRNA and +ssRNA [62, 81]
		Pleosporaceae	*Alternaria*	dsRNA, +ssRNA, and −ssRNA [64, 81-89]
			Bipolaris	dsRNA and +ssRNA [90-93]
			Cochliobolus	dsRNA [94]
			Curvularia	dsRNA [95]
			Drechslera	dsRNA [62, 96]
			Exserohilum	dsRNA [96-98]
			Pleospora	dsRNA [97-99]
			Setosphaeria	dsRNA and +ssRNA [98, 100, 101]
			Stemphylium	+ssRNA [102]
Eurotiomycetes	**Chaetothyriales**	***Herpotrichiellaceae***	*Phialophora*	dsRNA [82-89, 103, 104]
	Eurotiales	***Aspergillaceae***	*Aspergillus*	dsRNA and +ssRNA [94, 105-108]
			Penicillium	dsRNA, +ssRNA and −ssRNA [15, 64, 90-93, 109, 110]
		Trichocomaceae	*Talaromyces*	dsRNA [95, 111-113]

	Onygenales	***Ajellomycetaceae***	*Blastomyces*	dsRNA [99, 114]
		Ascosphaeraceae	*Ascosphaera*	+ssRNA [98, 100, 101, 115, 116]
		Onygenales incertae sedis	*Myriodontium*	dsRNA [117]
Lecanoromycetes	**Lecanorales**	***Stereocaulaceae***	*Lepraria*	dsRNA [43, 102]
Sordariomycetes	**Diaporthales**	***Cryphonectriaceae***	*Cryphonectria*	dsRNA, +ssRNA and −ssRNA [118-122]
			Endothia	dsRNA [123]
		Diaporthaceae	*Diaporthe*	dsRNA and +ssRNA [57, 124, 125]
		Melanconiellaceae	*Melanconiella*	+ssRNA [126]
		Valsaceae	*Cytospora*	dsRNA [127]
			Phomopsis	dsRNA and +ssRNA [128-131]
			Valsa	+ssRNA [132]
	Glomerellales	***Glomerellaceae***	*Colletotrichum*	dsRNA and +ssRNA [133-139]
		Plectosphaerellaceae	*Sodiomyces*	dsRNA and +ssRNA [140]
			Verticillium	dsRNA and +ssRNA [141-144]
	Hypocreales	***Bionectriaceae***	*Clonostachys*	dsRNA [145]
		Clavicipitaceae	*Atkinsonella*	dsRNA [146]
			Epichloë	dsRNA [147, 148]
			Metarhizium	dsRNA [149-152]
			Ustilaginoidea	dsRNA [153-157]
		Cordycipitaceae	*Beauveria*	dsRNA [62, 158-161]
			Cordyceps	dsRNA [162, 163]
			Isaria	dsRNA [164]
		Hypocreaceae	*Hypomyces*	dsRNA [165]
			Trichoderma	dsRNA and +ssRNA [62, 166-169]
			Fusarium	dsRNA, +ssRNA, −ssRNA, and ssDNA [170-176]
			Ilyonectria	dsRNA [177, 178]
			Nectria	dsRNA [179]
			Rugonectria	dsRNA and +ssRNA [180]
			Thelonectria	dsRNA [181]
		Hypocreales incertae sedis	*Acremonium*	+ssRNA [64]
		Ophiocordycipitaceae	*Ophiocordyceps*	+ssRNA [62]
			Tolypocladium	dsRNA [182, 183]
	Magnaporthales	***Magnaporthaceae***	*Buergenerula*	+ssRNA [184]
			Gaeumannomyces	dsRNA and +ssRNA [62, 185]
			Magnaporthe	dsRNA and +ssRNA [186-189]
	Microascales	***Ceratocystidaceae***	*Berkeleyomyces*	dsRNA and +ssRNA [190, 191]
			Ceratocystis	dsRNA and +ssRNA [192]
			Endoconidiophora	dsRNA [193]
		Pyriculariaceae	*Pyriculariaceae*	dsRNA [194, 195]
	Ophiostomatales	***Ophiostomataceae***	*Grosmannia*	dsRNA and +ssRNA [62]
			Ophiostoma	dsRNA and +ssRNA [196-198]
	Sordariales	***Sordariaceae***	*Neurospora*	dsRNA and +ssRNA [199]
	Togniniales	***Togniniaceae***	*Phaeoacremonium*	dsRNA and +ssRNA [64]
	Xylariales	***Apiosporaceae***	*Nigrospora*	dsRNA and +ssRNA [200-203]
		Diatrypaceae	*Monosporascus*	dsRNA [204]
		Hypoxylaceae	*Annulohypoxylon*	+ssRNA [205]
		Microdochiaceae	*Microdochium*	+ssRNA [206]
		Sporocadaceae	*Pestalotiopsis*	dsRNA, +ssRNA and −ssRNA [207]
			Pseudopestalotiopsis	dsRNA [208]

		Xylariaceae	*Entoleuca*	dsRNA and +ssRNA [209-211]
			Rosellinia	dsRNA and +ssRNA [18, 209, 212-218]
Leotiomycetes	**Leotiomycetes incertae sedis**	***Myxotrichaceae***	*Oidiodendron*	+ssRNA [71, 219]
		Pseudeurotiaceae	*Pseudogymnoascus*	dsRNA [220]
	Erysiphales	***Erysiphaceae***	*Erysiphe*	dsRNA and +ssRNA [69, 221, 222]
			Podosphaera	dsRNA and +ssRNA [78]
	Helotiales	***Godroniaceae***	*Gremmeniella*	dsRNA and +ssRNA [52, 62, 223, 224]
		Helotiaceae	*Hymenoscyphus*	dsRNA, +ssRNA and −ssRNA [122, 225, 226]
		Mollisiaceae	*Loramyces*	+ssRNA [62]
		Rutstroemiaceae	*Rutstroemia*	+ssRNA [62]
		Sclerotiniaceae	*Botrytis*	dsRNA and +ssRNA [227-232]
			Monilinia	dsRNA, +ssRNA and ssDNA [233, 234]
			Sclerotinia	dsRNA, +ssRNA, −ssRNA and dsRNA [16, 176, 235-239]
			Sclerotium	dsRNA and +ssRNA [240, 241]
	Thelebolales	***Thelebolaceae***	*Thelebolus*	dsRNA [62]
Pezizomycetes	**Pezizales**	***Caloscyphaceae***	*Caloscypha*	dsRNA [242]
		Discinaceae	*Gyromitra*	dsRNA [62, 243]
		Morchellaceae	*Morchella*	dsRNA and +ssRNA [62, 244]
		Pezizaceae	*Peziza*	+ssRNA [205]
			Sarcosphaera	dsRNA [245]
			Terfezia	dsRNA [246]
		Pyronemataceae	*Geopora*	dsRNA and +ssRNA [122, 247]
			Picoa	dsRNA and +ssRNA [248]
		Tuberaceae	*Tuber*	dsRNA, +ssRNA and RT-ssRNA [122, 249-251]
Saccharomycetes	**Saccharomycetales**	***Debaryomycetaceae***	*Scheffersomyces*	dsRNA [252]
			Wickerhamia	dsRNA [253]
		Dipodascaceae	*Geotrichum*	dsRNA [254, 255]
			Magnusiomyces	dsRNA [256]
			Yarrowia	dsRNA [257]
		Phaffomycetaceae	*Wickerhamomyces*	dsRNA [258]
		Pichiaceae	*Pichia*	dsRNA [259]
		Saccharomycetaceae	*Candida*	RT-ssRNA [260]
			Saccharomyces	dsRNA, +ssRNA and RT-ssRNA [261-264]
			Torulaspora	dsRNA [258]
			Zygosaccharomyces	dsRNA [265, 266]
		Saccharomycodaceae	*Hanseniaspora*	dsRNA [265, 267]
		Saccharomycetales incertae sedis	*Ambrosiozyma*	dsRNA [259]
			Nadsonia	dsRNA [268]
			Starmerella	dsRNA [269]
			Magnaporthe	dsRNA and +ssRNA [186-189]

Table 2. Mycoviruses associated with Basidiomycota.

Basidiomycota				
Class	**Order**	**Family**	**Genera**	**Mycoviruses**
Agaricomycetes	**Agaricales**	***Agaricaceae***	*Agaricus*	dsRNA and +ssRNA [270-272]
			Leucocoprinus	+ssRNA [53]
		Clitocybaceae	*Clitocybe*	+ssRNA [273]
		Cyphellaceae	*Chondrostereum*	dsRNA [205, 274]
		Hydnangiaceae	*Laccaria*	+ssRNA [205]
		Hygrophoraceae	*Hygrophorus*	dsRNA [122, 275, 276]
		Hymenogastraceae	*Hebeloma*	dsRNA [277]
		Lyophyllaceae	*Leucocybe*	dsRNA [278]
		Marasmiaceae	*Moniliophthora*	+ssRNA [279]
		Nidulariaceae	*Cyathus*	+ssRNA [53]
		Omphalotaceae	*Collybiopsis*	+ssRNA [205]
			Lentinula	dsRNA and +ssRNA [205]
		Physalacriaceae	*Armillaria*	+ssRNA and −ssRNA [122, 280, 281]
			Flammulina	dsRNA [282, 283]
		Pleurotaceae	*Pleurotus*	dsRNA and +ssRNA [271, 284-286]
		Pluteaceae	*Volvariella*	dsRNA [205, 287]
		Porotheleaceae	*Megacollybia*	dsRNA [205]
		Psathyrellaceae	*Coprinopsi*	dsRNA and +ssRNA [205, 282]
		Schizophyllaceae	*Schizophyllum*	+ssRNA [205]
		Strophariaceae	*Agrocybe*	dsRNA [282, 288]
		Squamanitaceae	*Phaeolepiota*	+ssRNA [205]
	Auriculariales	***Auriculariaceae***	*Auricularia*	dsRNA, +ssRNA and −ssRNA [289-291]
	Boletales	***Boletaceae***	*Boletus*	+ssRNA [205]
		Pisolithaceae	*Pisolithus*	dsRNA [292]
	Cantharellales	***Cantharellaceae***	*Craterellus*	+ssRNA [205]
		Ceratobasidiaceae	*Ceratobasidium*	+ssRNA [293, 294]
			Rhizoctonia	dsRNA, +ssRNA and −ssRNA [295-298]
			Thanatephorus	+ssRNA [299]
		Tulasnellaceae	*Tulasnella*	−ssRNA [219]
	Gloeophyllales	***Gloeophyllaceae***	*Neolentinus*	+ssRNA [205]
	Hymenochaetales	***Hymenochaetaceae***	*Fomitiporia*	+ssRNA [64]
	Polyporales	***Grifolaceae***	*Grifola*	dsRNA [300]
		Phanerochaetaceae	*Phlebiopsis*	dsRNA and +ssRNA [301, 302]
	Russulales	***Albatrellaceae***	*Albatrellopsis*	+ssRNA [303]
		Bondarzewiaceae	*Bondarzewia*	dsRNA and −ssRNA [304]
			Heterobasidion	dsRNA and +ssRNA [305-307]
		Russulaceae	*Lactarius*	dsRNA [122]
	Thelephorales	***Thelephoraceae***	*Thelephora*	dsRNA [205, 308]
Exobasidiomycetes	**Exobasidiales**	***Exobasidiaceae***	*Exobasidium*	dsRNA [305, 309, 310]
	Tilletiales	***Tilletiaceae***	*Tilletia*	dsRNA [311]
Malasseziomycetes	**Malasseziales**	***Malasseziaceae***	*Malassezia*	dsRNA [312]
Microbotryomycetes	**Sporidiobolales**	***Sporidiobolaceae***	*Rhodosporidiobolus*	dsRNA [313]
	Pucciniales	***Coleosporiaceae***	*Cronartium*	dsRNA and +ssRNA [122, 314, 315]
		Melampsoraceae	*Melampsora*	dsRNA [316]

		Pucciniaceae	*Puccinia*	dsRNA and +ssRNA [317-320]
			Uromyces	dsRNA [53]
		Phakopsoraceae	*Phakopsora*	dsRNA [53]
Tremellomycetes	**Cystofilobasidiales**	***Mrakiaceae***	*Phaffia*	dsRNA [321, 322]
		Cystofilobasidiaceae	*Cystofilobasidium*	dsRNA [323]
	Tremellales	***Cryptococcaceae***	*Cryptococcus*	dsRNA [324]
	Trichosporonales	***Trichosporonaceae***	*Trichosporon*	dsRNA [325]
Wallemiomycetes	**Wallemiales**	***Wallemiaceae***	*Wallemia*	dsRNA [99]

Table 3. Mycoviruses associated with the early diverging lineages of fungi.

Blastocladiomycota				
Class	**Order**	**Family**	**Genera**	**Mycoviruses**
Blastocladiomycetes	**Blastocladiales**	***Blastocladiaceae***	*Allomyces*	dsRNA [2, 326, 327]
Chytridiomycota				
Class	Order	Family	Genera	Mycoviruses
Chytridiomycetes	**Chytridiales**	***Chytridiaceae***	*Zopfochytrium*	dsRNA [2]
	Cladochytriales	***Cladochytriaceae***	*Cladochytrium*	dsRNA [2]
	Rhizophydiales	***Rhizophydiales incertae sedis***	*Operculomyces*	dsRNA and +ssRNA [2]
		Rhizopodaceae	*Rhizopus*	dsRNA and +ssRNA [2, 328]
Mucoromycota				
Class	Order	Family	Genera	Mycoviruses
Glomeromycetes	**Archaeosporales**	***Geosiphonaceae***	*Geosiphon*	+ssRNA [2]
	Diversisporales	***Gigasporaceae***	*Gigaspora*	dsRNA, +ssRNA and ssDNA [2, 53, 329]
	Glomerales	***Glomeraceae***	*Glomus*	dsRNA [330]
			Racocetra	dsRNA and +ssRNA [53]
			Rhizophagus	+ssRNA [2, 32, 331]
	Paraglomerales	***Paraglomeraceae***	*Paraglomus*	+ ssRNA [53]
Mortierellomycetes	**Mortierellales**	***Mortierellaceae***	*Dissophora*	dsRNA [2]
			Lobosporangium	dsRNA [2]
			Mortierella	dsRNA, +ssRNA and −ssRNA [2]
Mucoromycetes	**Mucorales**	***Choanephoraceae***	*Blakeslea*	dsRNA [2]
			Choanephora	dsRNA and +ssRNA [2]
		Cunninghamellaceae	*Absidia*	dsRNA [2]
		Mucoraceae	*Mucor*	dsRNA, −ssRNA and ssDNA [53, 54, 332, 333]
		Phycomycetaceae	*Phycomyces*	[2]
		Syncephalastraceae	*Syncephalastrum*	dsRNA [108]
Umbelopsidomycetes	**Umbelopsidales**	***Umbelopsidaceae***	*Umbelopsis*	dsRNA [2, 42, 334]
Neocallimastigomycota				
Class	Order	Family	Genera	Mycoviruses
Neocallimastigomycetes	**Neocallimastigales**	***Neocallimastigaceae***	*Anaeromyces*	+ssRNA [2]
			Neocallimastix	dsRNA [2]
			Pecoramyces	ssDNA [53]
Zoopagomycota				
Class	Order	Family	Genera	Mycoviruses
Entomophthoromycetes	**Entomophthorales**	***Ancylistaceae***	*Conidiobolus*	dsRNA and +ssRNA [2, 335-337]
		Entomophthoraceae	*Entomophaga*	dsRNA [2]
			Entomophthora	+ssRNA [2, 53]
			Zoophthora	dsRNA and +ssRNA [2]
Kickxellomycetes	**Kickxellales**	***Kickxellaceae***	*Kickxella*	dsRNA and +ssRNA [2]
Zoopagomycetes	**Zoopagales**	***Piptocephalidaceae***	*Syncephalis*	+ssRNA [2]

Mycoviral Taxa

Mycoviruses are currently classified into 23 families and one unclassified genus (Fig. 1) by the ICTV according to their genome type and organization; https://talk.ictvonline.org/ (accessed on 27 March 2023). The majority of mycoviruses studied to date have dsRNA or ssRNA genomes, however, a few mycoviruses with ssDNA genomes have also been found [12, 53, 54, 176, 329].

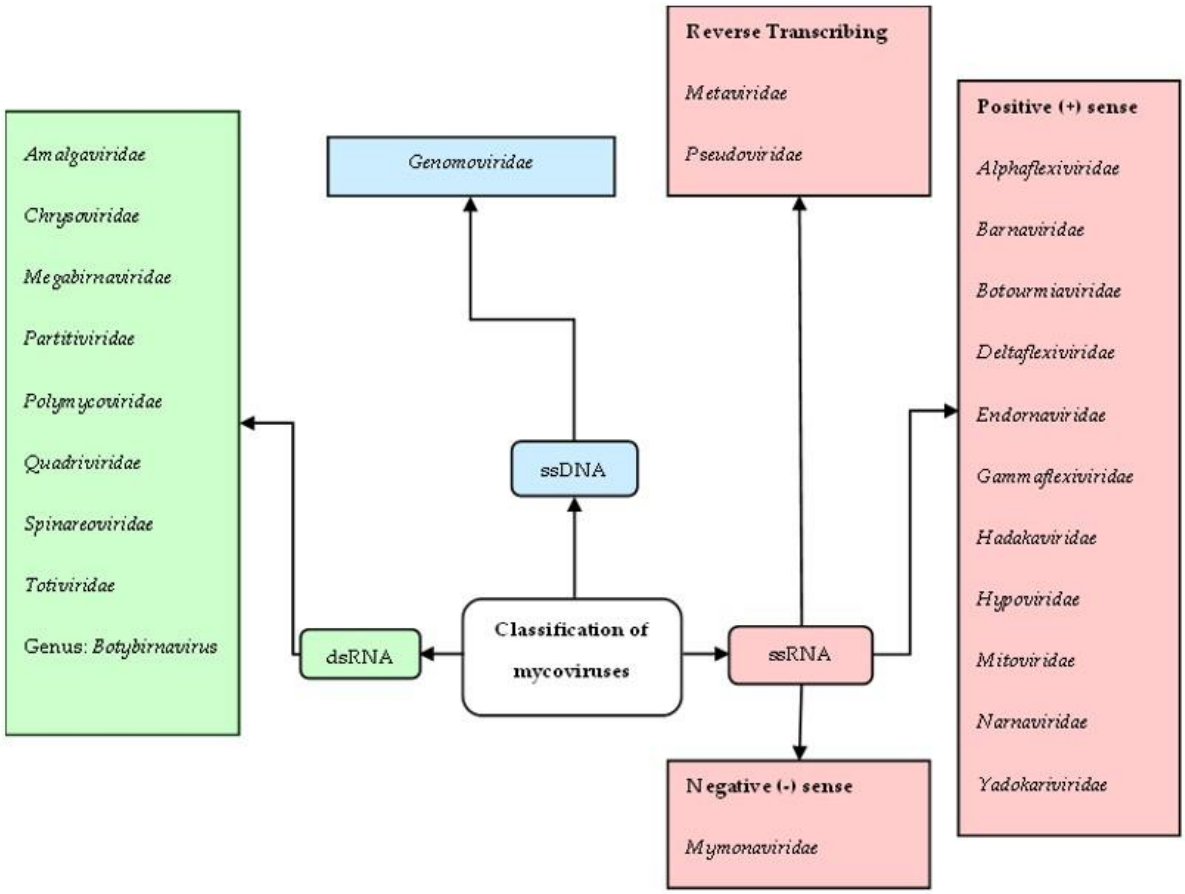

Fig. 1 Mycoviral taxa currently recognized by the ICTV. Compiled using information from the official ICTV website (https://talk.ictvonline.org/, (accessed on 27 March 2023)).

1. Double-Stranded RNA (dsRNA) Mycoviruses

The genome characteristics of mycoviruses, including size and segmentation, exhibit variation across families and genera. Specifically, the genomes of dsRNA mycoviruses can range from non-segmented, as observed in *Amalgaviridae* and *Totiviridae*, to bisegmented in *Megabirnaviridae*, *Partitiviridae*, and *Botybirnavirus*, quadripartite in *Quadriviridae*, or multisegmented in *Chrysoviridae*, *Polymycoviridae*, and *Spinareoviridae* [5, 338-344]. Furthermore, genome size among dsRNA mycoviruses is highly diverse, with some families

containing mycoviruses with genomes as small as 3.0 kb and others having members with genomes as large as 29 kb [339, 340]. Detailed information on genome length and segmentation of dsRNA mycoviruses is provided in Table 4.

In addition to differences in genome size and segmentation, the genome organization of mycoviruses also varies among families and genera. Although the presence of an RNA dependent RNA polymerase (RdRp) domain is ubiquitous among all mycoviruses, its location within the genome may differ [2]. For instance, the RdRp domain in multisegmented viruses of the *Chrysoviridae* family is encoded on the first genome segment, while in non-segmented viruses of the *Amalgaviridae* family it is located on the 3′ proximal open reading frame (ORF) [338, 343]. With the exception of members of the *Botybirnavirus* genus, all dsRNA mycoviruses encode a capsid protein (CP) [5, 338-343]. Additionally, some mycoviruses contain domains encoding proteins with unknown functions, as well as specific domains specific to certain families, such as the phytoreo S7 domain in the *Chrysoviridae*, a methyltransferase (Mtf) and proline-alanine-serine rich protein (PASrp) domain in *Polymycoviridae*, and the guanylyltransferase domain in *Spinareoviridae* [343-345]. Further details on the variation of genome organization among dsRNA mycovirus families can be found in Table 4.

2. Single Stranded RNA (ssRNA) Mycoviruses

2.1. Positive (+) sense

Mycoviruses belonging to families with positive-sense single-stranded RNA (+ssRNA) genomes display considerable diversity in terms of genome size, with genomes ranging from approximately 2.0 kb to 17.6 kb [173, 348]. The majority of +ssRNA mycoviruses have non-segmented genomes, however, the recently classified family *Hadakaviridae* consists of members with 10 to 11 genome segments [7]. While most mycoviruses replicate within the cytoplasm of their host, members of the *Mitoviridae* family demonstrate a unique replication pattern, taking place in the mitochondria of its fungal host [173]. More detail on the diversity of genome length and segmentation among +ssRNA mycovirus families and genera is presented in Table 5.

The presence of an RdRp domain is a characteristic feature of all +ssRNA mycoviruses. The genome of these viruses can include different protein domains, such as viral helicases (Hel), Mtfs, glycosyl transferases (Gtf), capsular polysaccharide synthases (CPS), phytoreo S7 domains, CPs, and proteases [348-352]. The location of the RdRp domain within the genome

can vary among different mycovirus families, as can the specific protein domains present in their genomes. Further information on the variation of genome organization among +ssRNA mycovirus families can be obtained from Table 5.

2.2. Negative (-) sense

Negative sense ssRNA (-ssRNA) mycoviruses belong to a single family, namely *Mymonaviridae*, which is characterized by filamentous, enveloped viruses with linear genomes that are around 6 to 10 kb [354]. The genomes of -ssRNA viruses encode for one or more proteins, two of which are known, namely an RdRp protein and a nucleoprotein (NP) which encloses the viral genome [231, 280, 354, 355]. The functions of other ORF encoded proteins are still uncertain [354]. Further details on genome organization and genera within *Mymonaviridae* is given in Table 6.

2.3. Reverse Transcribing (RT)

Concerning reverse transcribing ssRNA (RT ssRNA) mycoviruses, two families are currently recognized: *Metaviridae* and *Pseudoviridae*. Mycoviruses from the *Metaviridae* family are characterized by genomes ranging in length from 3 to 15 kb, while members of the *Pseudoviridae* family are generally shorter, ranging from 4 to 9 kb in length [356, 357]. The replication process of these viruses involves reverse transcription within intracellular virus like particles (VLP) to generate complementary DNA (cDNA), which is then integrated into the host chromosome through the action of an integrase protein [356, 357].

The genomes of RT ssRNA mycoviruses typically encodes for a capsid (CP) or nucleocapsid protein (NC), which is located on the gag gene. In addition, a protease (PR), reverse transcriptase (RT), integrase (INT), and ribonuclease H (RH), is also normally encoded on the pol gene [356-358]. The key difference between the two families lies in the fact that members of the *Pseudoviridae* family may encode the gag and pol proteins on separate ORFs, while members of the *Metaviridae* family encode these genes on a single ORF [356, 357]. Further information on the different genera and characteristics of RT ssRNA viral families can be found in Table 7.

3. Single-Stranded DNA (ssDNA) Mycoviruses

In comparison to RNA viruses, mycoviruses with DNA genomes are rare. There is currently only one recognized family containing ssDNA mycoviruses, namely *Genomoviridae*. This family encompasses two genera: *Gemycircularvirus* (represented by members of the species

Gemycircularvirus sclero1) and *Gemytripvirus* (represented by members of the species *Gemytripvirus fugra1*) [12, 176]. These mycoviruses exhibit small genome sizes, which range from 1.3 kb to 2.4 kb [14, 176]. The genomes of members from *Gemycircularvirus sclero1* and *Gemytripvirus fugra1* encode for a replicase protein (REP) and a CP, with the former encoded on a single circular ssDNA genome, and the latter encoded on three circular ssDNA components (DNA-A, DNA-B, and DNA-C). Not all ssDNA mycoviruses belong to *Genomoviridae*, however. For example, geminivirus-like ssDNA has also been discovered in *Macrophomina phaseolina* (M. phaseolina DNA virus; MpDV) and *Mucor racemosus* (M. racemosus DNA virus; MrDV) [54]. Additionally, a novel ssDNA mycovirus, Gigaspora circovirus A has also been discovered and belongs to the *Circoviridae* family but has not yet been assigned to a genus [53]. Unlike other circoviruses which encode for both a REP and CP, Gigaspora circovirus A encodes for a REP only [53]. Although not officially recognised by the ICTV, Gigaspora circovirus A might represent the first member of *Circoviridae* which infects a fungal host [14, 176]. Table 8 summarizes the properties of ssDNA mycoviruses which are currently recognized by ICTV.

Table 4. Genomic features and characteristics of dsRNA mycoviruses.

Families and Genera	Genome Size and Segmentation	Genome Organization	Exemplar Species
Family *Amalgaviridae* **Mycoviral associated genera** *Zybavirus*	Non-segmented genomes around 3.5 kb in size.	5′ proximal ORF encodes for a CP, followed by ribosomal frameshift signal and a 3′ proximal ORF encoding for an RdRp protein.	*Zybavirus bailii*
Family *Chrysoviridae* **Mycoviral associated genera** *Alphachrysovirus* *Betachrysovirus*	Multisegmented genomes around 8.9 to 16.0 kb in size. ***Alphachrysovirus***: 3–4 separately encapsidated segments. ***Betachrysovirus***: 4–7 separately encapsidated genomes.	**dsRNA 1**: Encodes for an RdRp. **dsRNA 2**: Encodes for a CP. **dsRNA 3**: Encodes for phytoreo S7 domain in *Alphachrysovirus*, and an unknown protein in *Betachrysovirus.* **dsRNA 4–7**: Encodes for a hypothetical protein with unknown function.	***Alphachrysovirus***: *Alphachrysovirus penicillii* ***Betachrysovirus***: *Betachrysovirus botryosphaeriae*
Family *Megabirnaviridae* **Mycoviral associated genera** *Megabirnavirus*	Bisegmented genomes around 16 kb in size.	**dsRNA 1**: 5′ proximal ORF encodes for a CP, followed by a ribosomal frameshift signal and a 3′ proximal ORF encoding for an RdRp. **dsRNA 2**: Encodes for hypothetical protein with unknown function.	*Rosellinia necatrix megabirnavirus 1*
Family *Partitiviridae* **Mycoviral associated genera** *Alphapartitivirus* *Betapartitivirus* *Gammapartivirus*	Bisegmented genomes around 3 to 4.8 kb in size.	**dsRNA 1**: Encodes for an RdRp. **dsRNA 2**: Encodes for a CP.	***Alphapartitivirus*:** *Rosellinia necatrix partitivirus 2* ***Betapartitivirus*:** *Ceratocystis resinifera virus 1* ***Gammapartitivirus*:** *Aspergillus ochraceous virus*
Family *Polymycoviridae* **Mycoviral associated genera** *Polymycovirus*	Multisegmented genomes around 7.5 to 12.5 kb in size.	**dsRNA 1**: Encodes for an RdRp. **dsRNA 2**: Encodes for hypothetical protein with unknown function. **dsRNA 3**: Encodes for a Mtf. **dsRNA 4**: Encodes for a PASrp. **dsRNA 5–8**: Encodes for hypothetical proteins with unknown functions.	*Aspergillus fumigatus polymycovirus 1*

Family *Quadriviridae* **Mycoviral associated genera** *Quadrivirus*	Quadripartite genomes around 3.5 to 5.0 kb in size.	**dsRNA 1:** Encodes for a hypothetical protein with unknown function. **dsRNA 2 and 4:** Encode for a CP. **dsRNA 3:** Encodes for an RdRp.	*Rosellinia necatrix quadrivirus 1*
Family *Spinareoviridae* **Mycoviral associated genera** *Mycoreovirus*	Multisegmented genomes (11–12 segments) around 23 to 29 kb in size.	Segments encodes for a single viral protein (VP1 to VP12). **VP 1**: Encodes for an RdRp. **VP 2**: Encodes for CP. **VP 3** or **VP10**: Encodes for a capping enzyme, (guanylyltransferase). **VP4** to **VP12**: Encodes for hypothetical proteins with unknown function.	*Mycoreovirus 1*
Family *Totiviridae* **Mycoviral associated genera** *Totivirus* *Victorivirus*	Non-segmented genomes around 4.6–7.0 kb in size.	5′ proximal ORF encodes for CP and the 3′ proximal ORF encodes for an RdRp. Totiviruses additionally encode for a ribosomal frameshift signal between the CP and RdRp. Some totiviruses may additionally produce satellite dsRNA which encode for killer proteins.	***Totivirus*:** *Saccharomyces cerevisiae virus L-A* ***Victorivirus*:** *Helminthosporium victoriae virus 190S*
Family Unclassified **Mycoviral associated genera** *Botybirnavirus*	Bisegmented genomes around 5.7 to 6.3 kb in size.	**dsRNA 1:** Encodes for an RdRp. **dsRNA 2:** Encodes for a hypothetical protein with unknown function.	*Bipolaris porri botybirnavirus 1*

Compiled using data from the International Committee on Taxonomy of Viruses (ICTV): https://ictv.global/taxonomy/ (accessed on 20 March 2023), Nibert, Ghabrial, Maiss, Lesker, Vainio, Jiang and Suzuki [33], Krupovic, Dolja and Koonin [338], Ghabrial, Castón, Jiang, Nibert and Suzuki [5], Lin, et al. [346], and Li, et al. [347].

Table 5. Genomic features and characteristics of + ssRNA mycoviruses.

Families and Genera	Genome Size and Segmentation	Genome Organization	Exemplar Species
Family *Alphaflexiviridae* **Mycovirus associated genera** *Botrexvirus* *Sclerodarnavirus*	Non-segmented genomes around 5.5 to 9 kb in size.	***Botrexvirus*:** Genome consists of up to 5 ORFs. **ORF1:** Encodes for an RdRp, Hel, and Mtf. **ORF3:** Encodes for a CP. All remaining ORFs encode for putative proteins with unknown function. ***Sclerodarnavirus*:** Genome consists of a single ORF encoding for an RdRp, Mtf, and Hel.	***Botrexvirus*:** *Botrytis virus X* ***Sclerodarnavirus*:** *Sclerotinia sclerotiorum debilitation-associated RNA virus*
Family *Barnaviridae* **Mycovirus associated genera** *Barnavirus*	Non-segmented with genomes around 4.0 kb in size.	Genome consists of 4 ORFs. **ORF1:** Encodes for a hypothetical protein with an unknown function. **ORF2:** Encodes for a putative serine protease. **ORF3:** Encodes for an RdRp. **ORF4:** Encodes for a CP.	*Mushroom bacilliform virus*
Family *Botourmiaviridae* **Mycovirus associated genera** *Botoulivirus* *Betabotoulivirus* *Magoulivirus* *Scleroulivirus* *Betascleroulivirus* *Deltascleroulivirus* *Penoulivirus* *Rhizoulivirus* *Betarhizoulivirus*	Non-segmented genomes around 3 to 5.3 kbp in size.	Genome consists of a single ORF encoding for an RdRp.	***Botoulivirus:*** *Botoulivirus alphabotrytidis* ***Betabotoulivirus:*** *Betabotoulivirus entoleucae* ***Magoulivirus:*** *Magoulivirus alphabotrytidis* ***Scleroulivirus:*** *Scleroulivirus oidiodendronae* ***Betascleroulivirus:*** *Betascleroulivirus botrytidis* ***Deltascleroulivirus:*** *Deltascleroulivirus botrytidis* ***Penoulivirus*:** *Sclerotinia penoulivirus* ***Rhizoulivirus*:** *Rhizoulivirus alpharhizoctoniae* ***Betarhizoulivirus*:** *Betarhizoulivirus solani*

Family *Deltaflexiviridae* **Mycovirus associated genera** *Deltaflexivirus*	Non-segmented with genomes around 6 to 8 kbp in size.	Genome consists of 1–5 ORFs. **ORF 1**: Encodes for polyprotein with Mt, Gtf, Hel and RdRp domains. **ORF 2–5**: Encode for hypothetical proteins with unknown functions.	*Sclerotinia deltaflexivirus 1*
Family *Endornaviridae* **Mycovirus associated genera** *Alphaendornavirus* *Betaendornavirus*	Non segmented genomes around 9.5 to 17.6 kb in size.	Genome consists of a single ORF encoding for a large polyprotein. The polyprotein always contains an RdRp domain, but may also include domains for Hel, Mt, Gtf, CPS, and phytoreo S7 domains.	***Alphaendornavirus*:** *Oryza sativa alphaendornavirus* ***Betaendornavirus*:** *Sclerotinia sclerotiorum betaendornavirus 1*
Family *Gammaflexiviridae* **Mycovirus associated genera** *Mycoflexivirus*	Non-segmented genomes around 6.8 to 9.2 kb in size.	Consists of 2–3 ORFs. **ORF 1:** Encodes for a replicase (REP) with an upstream Mtf domain, a Hel domain, and a downstream RdRp domain. **ORF 2:** Encodes for either a CP or proteins which resemble movement proteins. **ORF 3:** Encodes for either a CP or a hypothetical protein with an unknown function.	*Botrytis virus F*
Family *Hadakaviridae* **Mycovirus associated genera** *Hadakavirus*	Multisegmented genomes around 14 to 15 kb in total size.	**RNA 1:** Encodes for an RdRp. **RNA3:** Encodes for a Mtf. **RNA 2, 4–7, 9 and 10:** Encode for hypothetical proteins with an unknown function. **RNA8**: May encode for a C_2H_2-type zinc finger protein.	*Hadakavirus nanga*
Family *Hypoviridae* **Mycovirus associated genera** *Alphahypovirus* *Betahypovirus* *Epsilonhypovirus* *Etahypovirus* *Thetahypovirus*	Non-segmented genomes around 9.1 to 12.7 kb in size.	Consist of 1–2 ORFs. Encodes for a polyprotein containing RdRp and sometimes protease, Hel, and Gtf domains. Some hypoviruses also have short, internally deleted, defective interfering replicative forms of dsRNA molecules, while others have replicative forms of satellite like RNAs.	***Alphahypovirus*** *Alphahypovirus americanum* ***Betahypovirus*:** *Betahypovirus americanum* ***Epsilonhypovirus*:** *Epsilonhypovirus agarici* ***Etahypovirus*:** *Etahypovirus sclerotii* ***Thetahypovirus*:** *Thetahypovirus botrytidis*

Family *Mitoviridae* **Mycovirus associated genera** *Unuamitovirus* *Duamitovirus* *Triamitovirus* *Kvaramitovirus*	Non-segmented genomes around 2.0 kbp to 4.5 kb in size.	Consists of 1 ORF encoding for an RdRp.	***Unuamitovirus*:** *Unuamitovirus boci2* ***Duamitovirus*:** *Duamitovirus alal1* ***Triamitovirus*:** *Triamitovirus rhso30* ***Kvaramitovirus*:** *Kvaramitovirus opno7*
Family *Narnaviridae* **Mycovirus associated genera** *Narnavirus*	Non-segmented genomes around 2.3 to 3.6 kb in size.	Consists of 1 ORF encoding for an RdRp.	*Saccharomyces 20S RNA narnavirus*
Family *Yadokariviridae* **Mycovirus associated genera** *Alphayadokarivirus* *Betayadokarivirus*	Non-segmented genomes around 3.6 to 6.3 kb in size.	Genome consists of a 1–2 ORFs encoding for a polyprotein. ***Alphayadokarivirus*** Consist of 1 ORF which encodes for a polyprotein with an RdRp domain, 2A-like self-cleaving peptide and a hypothetical protein domain with an unknown function. ***Betayadokarivirus*** Consist of 1 or 2 ORFs encoding for a polyprotein, which may or may not include a 2A-like self-cleaving peptide. The polyprotein contains an RdRp domain (5′ proximal ORF), and a hypothetical protein domain (3′ proximal ORF) with an unknown function.	**Alphayadokarivirus:** *Alphayadokarivirus ichibani* ***Betayadokarivirus*:** *Betayadokarivirus nibani*

Compiled using data from the International Committee on Taxonomy of Viruses (ICTV): https://ictv.global/taxonomy/ (accessed on 27 March 2023). Hamid, et al. [353], Li, Zheng, Cheng, Chen, Fu, Jiang and Xie [236], Ma, Zhang, Qi, Zhang, Ma, Jiang, Qin and Qi [173], Li, Sun, Yu, Chen, Liu, Yin, Guang, Yang and Mo [29].

Table 6. Genomic features and characteristics of -ssRNA mycoviruses.

Families and Genera	Genome Size and Segmentation	Genome Organization	Exemplar Species
Family *Mymonaviridae* **Mycovirus associated genera** *Auricularimonavirus* *Botrytimonavirus* *Lentimonavirus* *Penicillimonavirus* *Sclerotimonavirus*	Non-segmented genomes around 6 to 10 kb in size.	Consists of 1–7 ORFs. All mymonaviruses encode for an RdRp near the C-terminus, and some may encode for a NP near the 5′ terminus. Some also encode for hypothetical proteins with unknown functions.	***Auricularimonavirus*:** *Auricularimonavirus armillariae* ***Botrytimonavirus*:** *Botrytimonavirus alphabotrytidis* ***Lentimonavirus*:** *Lentimonavirus armillariae* ***Penicillimonavirus*:** *Penicillimonavirus alphapenicillii* ***Sclerotimonavirus*:** *Sclerotimonavirus botrytidis*

Compiled using data from the International Committee on Taxonomy of Viruses (ICTV): https://ictv.global/taxonomy/ (accessed on 20 March 2023).

Table 7. Genomic features and characteristics of RT ssRNA mycoviruses.

Families and Genera	Genome Size and Segmentation	Genome Organization	Exemplar Species
Family *Metaviridae* **Mycovirus associated genera** *Metavirus*	Non-segmented genomes around 3 to 15 kb in size.	Genome consists of an intragenic region which may code for up to 2 genes (gag and pol), which is flanked by long terminal repeat sequences (LTR). The gag gene usually encodes for the CP or NC protein while the pol gene encodes a polyprotein with PR, RT, INT and RH domains.	*Cladosporium fulvum T-1 virus*
Family *Pseudoviridae* **Mycovirus associated genera** *Hemivirus* *Pseudovirus*	Non-segmented genomes that range in length from 4 to 9 kb in size.	Genome consists of an internal region with 1 (gag-pol) or 2 ORFs (gag and pol), which are flanked by long terminal repeat sequences (LTR). The gag protein typically contains the CP and NC domains, while the pol protein contains PR, RT, INT, and RH domains.	***Hemivirus*:** *Candida albicans Tca2 virus* ***Pseudovirus*:** *Saccharomyces cerevisiae Ty1 virus*

Compiled using data from the International Committee on Taxonomy of Viruses (ICTV): https://ictv.global/taxonomy/ (accessed on 20 March 2023).

Table 8.. Genomic features and characteristics of ssDNA mycoviruses.

Families and Genera	Genome Size and Segmentation	Genome Organization	Exemplar Species
Family *Genomoviridae* **Mycovirus associated genera** *Gemycircularvirus* *Gemytripvirus*	***Gemycircularvirus*** Monopartite genomes around 1.8 to 2.4 kb in size. ***Gemytripvirus*** Tripartite genome with individual segments around 1.3 kb.	***Gemycircularvirus*** Genome encodes for a rolling circle REP protein as well as a CP in ambisense orientation. ***Gemytripvirus*** **DNA-A:** Encodes for the REP protein. **DNA-B**: Encodes for the CP. **DNA-C:** Encodes for p26, a protein with an unknown function.	***Gemycircularvirus*:** *Gemycircularvirus sclero1* ***Gemytripvirus*:** *Gemytripvirus fugra1*

Compiled using data from the International Committee on Taxonomy of Viruses (ICTV): https://ictv.global/taxonomy/ (accessed on 20 March 2023), Li, Wang, Zhang, Qiu, Zhou and Guo [176] and Varsani and Krupovic [14].

The Effect of Mycoviruses on Fungi

As obligate intracellular parasites, mycoviruses rely upon host genes and their associated gene products to facilitate their replication. Consequently, certain mycoviruses have the capacity to exert a discernible influence on the expression of the fungal phenotype [5,6]. These mycoviruses are capable of modulating the virulence of their fungal hosts by instigating changes in the transcriptome profiles, primarily achieved through the disruption of protein-protein interactions and the suppression of antiviral proteins [1, 6]. It should be noted, however, that these associations are sometimes complex. Several environmental factors, including the growth media [159], temperature [359], other mycoviruses [360], and the fungal plant or animal host [187] may also play a role in the outcome of the host phenotype.

Host–virus Arms Race

The study of mycoviral evasion strategies against host defences and the fundamental mechanisms underlying antiviral responses is often conducted employing *Cryphonectria parasitica*, the model organism for mycoviral research [21, 361, 362], and more recently *Neurospora crassa* [363]. The chestnut blight pathogen, *C. parasitica*, can defend against viral invaders by use of an RNA-mediated gene regulation mechanism known as RNA silencing or RNA interference (RNAi) [364-366]. The fungus achieves this by producing endoribonucleases known as Dicer-like proteins (DCLs), and Argonaute-like proteins (AGLs) [364-366]. Together, the DCL and AGL proteins forms part of the RNA-induced silencing complex (RISC), which recognizes and cleaves the dsRNA genomes, or replicative intermediates, of infecting mycoviruses [6]. An in-depth review of RNA silencing mechanisms is covered by Zhao and Guo [367].

Mycoviruses on the other hand, are often able to suppress RNA silencing. Through proteins such as p29 and p27, members of the species *Alphahypovirus cryphonectriae* (Cryphonectria hypovirus 1; CHV-1) and *Alphahypovirus americanum* (Cryphonectria hypovirus 2; CHV-2) suppress this pathway in *C. parasitica*, allowing enhanced replication and attenuated virulence of the host [368, 369]. Similarly, Rosellinia necatrix mycoreovirus 3 (RnMYRV-3) produces a protein known as S10, which aids in the suppression of antiviral silencing in *Rosellinia necatrix* [370]. Interestingly, some types of fungi do not possess an RNAi pathway, making them highly susceptible to mycovirus infection. For example, the persistence and replication of Malassezia sympodialis mycovirus 1 (MsMV-1), a mycovirus of *Malassezia sympodialis* (a yeast linked to common skin disorders, pancreatic cancer, and Chron's disease), is thought to be due to the absence of an RNAi pathway within this organism [312, 371-373].

Hypervirulence, Hypovirulence, and the Killer Yeast Phenotype

Mycoviruses that induce hypervirulence increase the virulence of the fungal host [6]. Despite its perceived negative effects, hypervirulence may also have desirable effects. For example, a mycovirus known as Leptosphaeria biglobosa quadrivirus 1 (LbQV-1) induces hypervirulence in *Leptosphaeria biglobosa* [78]. The infected strains of *L. biglobosa* confers systemic resistance and protects the host plant, *Brassica napus* (oilseed rape or canola), from a closely related and more aggressive fungus known as *Leptosphaeria maculans* [78]. Some mycoviruses also confer hypervirulence to entomopathogenic fungi from the genera *Metarhizium* and *Beauveria*, which are routinely used as biocontrol agents against some arthropod pests [374]. In these circumstances, hypervirulence is a more desirable trait as it may increase the effectiveness of these biocontrol agents.

Mycoviruses that induce hypovirulence on the other hand, adversely affect the virulence, morphology, sporulation, growth rate, and pigmentation of their fungal hosts [6, 15, 16]. Although the precise nature and molecular pathways by which these viruses cause hypovirulence are still largely unclear, they are known to exert some of their effects through virus–host protein–protein interactions [6]. Mycovirus-induced hypovirulence of *C. parasitica* can be attributed to the alteration of numerous signal transduction pathways, including those important for virulence [375-377]. For example, the reduction of pigmentation, sporulation, and laccase accumulation in *C. parasitica* is linked to the papain-like protease p27 produced by members of the species *Alphahypovirus cryphonectriae* [378]. Isolates of *C.parasitica* infected by members of this species additionally upregulates ATG8, a homolog of ubiquitin-like yeast autophagy protein, which is essential for the replication of these viruses [379]. In contrast, members of *Alphahypovirus cryphonectriae* differentially regulates proteins that prevent the expression of viral RNA, such as DNA methyltransferases, which in turn results in retarded growth and aberrant colony morphology of the host [380].

Mycoviruses that induce hypovirulence have been discovered in various plant pathogenic fungi, including several *Fusarium* spp. isolates [350, 381, 382], the white root rot fungus *R. necatrix* [214, 216, 383], white mold fungus *S. sclerotiorum* [12, 16, 384], rice blast fungus *Magnaporthe oryzae* [385], grey mold rot fungus *Botrytis cinerea* [39, 386, 387] and a few *Alternaria* spp. isolates [83, 388]. Hypovirulence-inducing mycoviruses are of immense interest to plant pathologists, as they are potential biocontrol agents of plant pathogenic fungi.

The precise impact of mycoviruses on pathogenic fungi affecting human health remains largely unknown, yet a handful of studies have shed some light on these relationships. For example,

infection with MsMV-1 appears to result in significant transcriptional rewiring in *M. sympodiali,* causing upregulation of transcriptional factors and ribosomal genes, while simultaneously repressing genes responsible for cellular metabolism [312]. Interestingly, the MsMV-1 putative mycoviral toxin also elicits an immune response in macrophages and augments the ability of infected isolates to colonize murine skin [312]. This suggests that the mycovirus may play a role in the pathogenicity of the host fungus. Similarly, recent investigations have discovered the presence of antibodies against a certain strain of mycovirus infected *Aspergillus flavus* (MCAF) in the plasma of patients that were in remission from acute lymphoblastic leukemia (ALL) [389]. In this study, the exposure of plasma from ALL patients in remission caused a notable re-emergence of cell surface and genetic markers that were consistent with this disease [389]. The authors, therefore, hypothesize that exposure to MCAF may contribute to the development of ALL [389]. These studies show that the role of mycoviruses in diseases caused by human pathogenic fungi, with and without their hosts, needs to be further explored.

Mycoviruses do not exclusively infect filamentous fungi but have also been associated with various species of yeast [265, 269]. Some induce the 'yeast killer phenotype', eliminating competing yeasts and providing a competitive advantage to the host [390]. The killer phenotype in the model organism *S. saccharomyces* is normally determined by two co-infecting totiviruses with a mutualistic relationship, namely the helper LA and satellite M virus [391]. *Saccharomyces cerevisiae virus* (ScV) -*L-A* encodes essential proteins for replication and transcription, while the satellite M virus encodes for a preprotoxin which provides immunity to the host and kills off non-infected cells when processed into toxin [390]. The toxins generated by this satellite virus vary among strains and can induce cell death through different mechanisms. These include the disruption of cytoplasmic membrane function and the formation of lethal ion channels (caused by K1 and K2 toxins), as well as cell cycle arrest in the G1 or early S phase (caused by K28 toxin) [392-394].

Industrial yeast strains with virally encoded killer systems are highly sought after for their ability to restrain spoilage microorganisms and preserve the quality of food products and beverages [395-397]. For example, *Ustilago maydis*, *Kluyveromyces wickerhamii*, *Pichia anomala*, and *Pichia membranifaciens* produce virally encoded toxins that have antifungal activity against the wine spoilage yeast, *Brettanomyces bruxellensis* [398, 399]. Mycoviruses have also been discovered in *Zygosaccharomyces bailii*, where they encode for the toxin zygocin, which has broad antifungal activity and shows potential as an antimycotic drug [400].

Mycoviruses as Biocontrol Agents

Among the best-known applications of a mycovirus in the field is members of the species *Alphahypovirus cryphonectriae* against the chestnut blight pathogen, *C. parasitica* [401, 402]. Under typical conditions, *C. parasitica* infections result in the appearance of cankers on the stems and branches of susceptible trees, which destroy the cambium tissue and ultimately result in tree death [403]. CHV-1 infected strains, on the other hand, display significantly reduced virulence, resulting in superficial cankers which eventually stop growing and become passive [403]. Mycovirus-based biocontrol of chestnut blight has proven to be a great success in Europe, largely due to the low genetic diversity among strains of *C. parasitica* [403]. In regions where naturally hypovirulent strains of this fungus were present, CHV-1 effectively spread without human intervention [404]. However, in areas with little to no natural hypovirulence, CHV-1 could also be artificially introduced by treating bark cankers with hypovirulent *C. parasitica* [405, 406]. Research into hypovirulence-associated mycoviruses in other plant pathogenic fungi has since been prompted by the success of hypovirus-mediated hypovirulence in *C. parasitica*.

Members of the species *Gemycircularvirus sclero1* (Sclerotinia sclerotiorum hypovirulence-associated DNA virus 1; SsHADV1), for example, is infectious as purified particles and can directly infect the hyphae of *S. sclerotiorum* [407]. Researchers have thus developed an aerial spray, composed of hyphal fragments from an infected fungus, capable of killing *S. sclerotiorum* on infected rapeseed plants in the field, [407]. Sclerotinia sclerotiorum partitivirus 1 (SsPV1), another mycovirus of *S. sclerotiorum*, appears to spread through hyphal contact to different strains regardless of vegetative incompatibility [20]. This suggests that SsPV1 also has potential as a biocontrol agent against *S. sclerotiorum* in the field [20]. While the focus of mycoviral research has predominantly centred around plant pathogenic fungi, an emerging area of investigation pertains to the search for mycoviruses with potential therapeutic benefits for human health [108, 359, 408].

Limitations of Biocontrol: The Role of Mycovirus Transmission

Despite their immense potential, the use of mycoviruses as biocontrol agents of plant pathogenic fungi is complicated by their mode of transmission. Mycoviruses lack an extracellular route of transmission [3]. Instead, they can only spread intracellularly, through hyphal anastomosis (horizontal transmission) or sporulation (vertical transmission) [5].

1. Horizontal Transmission

One of the greatest barriers to the successful spread of mycoviruses pertains to hyphal anastomosis. Hyphal anastomosis occurs when specialized hyphae from the same fungus, or

hyphae from different fungi fuse and exchange cytoplasmic content, which includes any associated mycoviruses [409]. However, for the hyphae from different fungal strains or species to fuse, they need to be vegetatively compatible [3]. Vegetative compatibility is determined by the fungal vegetative incompatibility genes (*vic* genes), which will trigger programmed cell death (PCD) when contact between incompatible fungi occurs [410]. Thus, mycoviruses cannot be transmitted from a hypovirulent fungal strain to a target fungal strain if they are vegetatively incompatible [21]. For example, CHV1 has been effective against *C. parasitica* in Europe, but not in America, where there is greater diversity of *vic* groups between fungal strains [404, 411]. Similarly, although *S. sclerotiorum* harbours a diverse range of mycoviruses, the use of these viruses in the field is restricted due to the high diversity of *vic* loci among different strains, which may also be very complicated under field conditions [412, 413]. However, research is underway to resolve the issue of vegetative incompatibility as it relates to mycovirus transmission.

Many methods and techniques have been developed to study vegetatively compatibility systems in fungi and to find ways to overcome the *vic* system. For example, *vic* genes related to five to six loci in *C. parasitica* have been linked to vegetative incompatibility and virus transmission in one study [21]. The disruption of these genes allowed for the development of a super donor strain, which could spread mycoviruses between incompatible strains [414]. This approach will not be feasible for all fungi, however, as some plant pathogenic fungi may have more complicated *vic* systems that result in a high *vic* diversity [16, 229]. Chemical compounds have also been used to enhance viral transmission and to prevent PCD [415]. In one study, when vegetatively incompatible strains of *R. necatrix* were cultured together on a medium supplemented with zinc, hyphal anastomosis improved and mycoviruses could be transmitted to isolates from different *vic* groups [416].

While vegetative incompatibility prevents transmission of mycoviruses in most cases, other factors also often play a role. In situ inoculation on chestnut wood increased transmission efficiency of CHV1 between vegetatively incompatible strains [417]. In another study, two vegetatively incompatible strains of *R. necatrix*, one of which contained a mycovirus, were inoculated on apple trees and later found to harbor the same mycovirus [418]. This may be due to several factors, including different environmental conditions, and a weakened vegetative incompatibility response due to environmental microorganisms or the host plant itself [410]. Research has also indicated that horizontal transmission of mycoviruses between fungal and plant hosts may occur in cases of co-infection with plant viruses [40]. In such instances, the replication of these viruses in both hosts is facilitated. This phenomenon has been linked to the

suppression of antiviral mechanisms in both the plant and fungus [40]. For example, members of the species *Alphahypovirus cryphonectriae* produce a protein called p29 that downregulates components of the antiviral RNAi system in fungal hosts, thereby promoting virus accumulation [368]. However, research by Bian et al. [39] has shown that this protein has limited functionality in certain plant hosts, such as *Nicotiana tabacum*. TMV however, encodes for a replicase that interferes with the antiviral response in the plant host, thereby enhancing CHV-1 accumulation in the plant [40]. Additionally, TMV produces a cellto-cell movement protein (MP) that is typically absent in mycoviruses, thus enabling the dissemination of CHV-1 throughout *N. tabacum* [368]. This may then enhance the ability of the mycovirus to access other fungi that may have established themselves in the same plant [40]. Conversely, CHV-1 inhibits the fungal antiviral defense mechanism, which typically acts to eliminate the TMV [40]. This, in turn, permits the accumulation of TMV within the fungal host, specifically *F. graminearum*, as demonstrated in this study [40]. Different viral strains also play a role in transmission efficiency, where researchers have found that strains with higher virulence have higher transmissibility [419].

Interestingly, several mycoviruses are able to either influence the host *vic* system to transmit between vegetatively incompatible strains, or to infect the host directly as infectious particles [189, 353, 407, 420]. As mentioned previously, hypoviruses from the species *Alphahypovirus cryphonectriae* are able to down-regulate genes that are involved in PCD, thus allowing for transmission between vegetatively incompatible strains [420, 421]. Co-infection of mycoviruses can also result in the transmission of viruses between vegetatively incompatible fungi. One study has demonstrated that Sclerotinia sclerotiorum mycoreovirus 4 (SsMYR4) downregulates cellular activities and pathways associated with vegetative incompatibility mediated PCD [422]. This in turn facilitates the horizontal transmission of other hypovirulent co-infecting viruses [422]. Mycoviruses with these traits hold immense potential as biocontrol agents, however, more research is required to fully understand the mechanisms behind these phenomena.

Regarding their clinical application, mycoviruses that induce hypovirulence would require administration through delivery methods such as injection or topical application to the target fungus of an infected patient [108]. This is because the fungal cell wall is impenetrable, and thus acts as a barrier against mycovirus uptake, limiting their potential as therapeutic agents against human pathogenic fungi [423]. A promising avenue for the development of therapeutic intervention for humans has emerged with the discovery of ssDNA mycoviruses that are capable of extracellular transmission in *S. sclerotiorum* [108, 407]. As such, ssDNA viruses may hold

promise as candidates for addressing human fungal infections. However, most mycoviruses lack an extracellular route of infection. Consequently, the transmission of mycoviruses between incompatible fungi in laboratory settings typically relies on transfection or transformation methods involving full-length viral cDNA clones, purified virus particles, and in vitro RNA transcripts [424-426]. The development of such clones is complex, however, especially in the case of multisegmented mycoviruses [410]. Nonetheless, research findings have shown that encapsulated mycoviruses can be successfully introduced into fungal protoplasts through the application of polyethylene glycol-mediated protocols [12, 427, 428]. This method has now become the prevailing standard for mycovirus transmission within laboratory environments. Mycoviruses can also be transmitted between incompatible fungal strains through protoplast fusion, which has the advantage of allowing transmission of both encapsulated and unencapsulated viruses [22]. Although these methods are traditionally used to investigate host and viral factors relevant to viral replication or symptom induction, they have also been employed to broaden the host range for certain mycoviruses [22].

Vectors, such as insects or parasites, can serve as vehicles for the transmission of mycoviruses between fungi [20]. This strategy can effectively surmount barriers related to vegetative compatibility and facilitate the rapid establishment of mycovirus populations in natural field environments. For example, the transmission of SsHADV-1 by frugivorous insects to other vegetatively incompatible strains has been observed under laboratory conditions [20]. However, producing and dispersing such vectors in the field is impractical [20]. Hence, it is more feasible to exploit a naturally occurring vector, such as mycoparasites, which can transmit the mycovirus via hyphal parasitisation [20]. In this context, a mycovirus associated with hypovirulence can be introduced into the mycoparasite through transfection techniques or dual culturing, which can then be employed to infect a host fungus [20].

While much of the mycoviral research in the context of biocontrol predominantly centres around hypovirulence-inducing viruses, some researchers have also exploited the "killer phenotypes" found in dimorphic fungi to provide resistance to plant hosts. For instance, a study has demonstrated that the transgenic expression of the viral KP4 killer toxin from *Ustilago maydis* in Swiss wheat confers protection against *U. maydis* and related pathogens, thus enhancing resistance in these crops [429]. It is worth noting that these killer systems are not commonly found in filamentous fungi, but analogous strategies may also be explored for plant pathogenic yeasts or other dimorphic fungi.

2. Vertical Transmission

For mycoviruses to be regarded as proficient biocontrol agents, they must possess several key attributes. Beyond inducing hypovirulence and having the capability to transmit to uninfected fungi via hyphal anastomosis, they must also exhibit a high degree of efficiency in transmitting to the fungal progeny. This requirement is crucial for the sustained effectiveness of mycovirusbased biocontrol strategies. The transmission of some mycoviruses to the fungal progeny occurs primarily through sporulation, which can be sexual or asexual [1, 3, 15]. Transmission rates, however, vary greatly between fungus-virus combinations and between different spore types (asexual vs. sexual) [15]. For example, one study has demonstrated that the transmission of CHV1-EP713 to the ascospore progeny of *C. parasitica* is ineffective, as infection results in a loss of female fertility [119]. In contrast, other studies have demonstrated the successful transmission of mycoviruses to the ascospore progeny of *C. parasitica* strains infected by reoviruses (Mycoreovirus 1-Cp9B21 and Mycovreovirus 2-CpC1) or a mitovirus (Cryphonectria mitovirus-1/CpNB631) [119, 430].

Vertical transmission through asexual spores or other asexual structures such as sclerotia are commonly observed in mycoviruses [229, 382, 431, 432]. This mode of transmission allows mycoviruses to spread within their host over longer distances than horizontal transmission alone and enables mycoviruses to persist within the fungal population [417]. However, mycovirus-based biocontrol strategies may encounter challenges with vertical transmission through asexual fragments. In some instances, hypovirulence has been linked to the disruption of conidia, resulting in lower transmission rates. For instance, members of the species *Alphahypovirus americanum* (Cryphonectria hypovirus 2; CHV-2) induces substantial hypovirulence in *C. parasitica* but has limited transmission to conidia with only a 2–5% transmission rate, leading to its restricted geographical distribution [433]. It is important to note that this scenario may not be universal. According to a study by Lee, et al. [434], four mycoviruses, which reduce the virulence of *Fusarium graminearum*, exhibit more efficient transmission to conidia than those that cause symptomless infections. Another issue related to the transmission of fungal pathogens through asexual spores is that some fungal species either do not produce conidia or the conidia play a minimal role in their life cycle [410].

In general, the transmission of mycoviruses to sexual spores (ascospores, basidiospores, etc.) is believed to be less prevalent than the transmission of mycoviruses to asexual spores (conidia) [20]. However, studies now indicate that this transmission mode may be more common than previously thought [119, 233, 435, 436]. The mechanisms behind mycovirus transmission via

sexual spores remain unclear, but it is believed that this could represent a potential pathway for exchanging mycoviruses among different *vic* groups [410]. Hence, an increased focus on investigating these mechanisms could provide new avenues for controlling mycovirus transmission and boosting the potential of biological control efforts.

The Detection of Mycoviruses

In vitro Based Detection

In the past, mycoviruses were primarily detected by culture-based approaches. The basis for culture-based detection is rooted in the observation that most mycoviruses have dsRNA genomes, or a dsRNA replicative intermediate, which are not generated by the host [5, 15]. The conventional technique for mycovirus detection involving RNA genomes entails the isolation of dsRNA from total fungal RNA extracts, typically accomplished through cellulose chromatography or other column-based methods [437, 438]. While these approaches are usually rapid and inexpensive, they suffer from several disadvantages [2]. Employing an in vitro-based approach can potentially result in the overestimation of dsRNA levels or the underestimation of ssRNA levels, as such methods often involve the application of dsRNA enrichment protocols [3]. Moreover, these techniques strengthen the idea that mycovirus genomes are primarily composed of RNA and completely disregard viruses that may be composed of DNA [2]. Purified dsRNA is also normally visualized using agarose gel electrophoresis, which may result in false negatives in cases where there are only low-titer infections.

Culture-based methods are also used to evaluate the effect of mycoviral infection on the fungal host. Infected cultures often exhibit reduced growth and sporulation, along with alterations in pigmentation and morphology [6]. For instance, *C. parasitica* infected with hypoviruses from the species *Alphahypovirus cryphonectriae* display a loss of the characteristic orange pigmentation seen in non-infected fungal strains, while infected *S. sclerotiorum* strains exhibit abnormal colony morphology and produce smaller and fewer sclerotia [6]. However, this is not always the case. Some mycoviruses that induce hypovirulence in vitro, may induce hypervirulence in planta [85].

Consequently, dsRNA profiling is usually used alongside in silico methods to identify and characterize mycoviruses [2, 438].

In-silico Based Detection

Thanks to the development of new technologies, it has become easier to detect mycoviruses and gain insight into their structure and biology [2, 20, 439]. One such approach is the in-silico method, which leverages metatranscriptomic datasets of fungal hosts to identify sequences with homology to mycoviral proteins, enabling the detection of putative mycoviruses. One example of such a protein is the RdRp, which is essential for viral transcription and replication, and is thus ubiquitous in RNA viruses [2]. Other proteins which are also found in the genomes of some, but not all mycoviruses, are viral helicases, glycosyl transferases, methyl transferases, and capsid proteins [212, 440, 441]. Because the in-silico approach to detection does not make use of agarose gel electrophoresis, it is more sensitive to low titer mycoviral infections than in vitro-based approaches [2]. The greater accessibility of fungal transcriptomic datasets on openaccess platforms like the NCBI sequencing reads archive (SRA), as a result of decreased sequencing costs, is another benefit of these approaches [62]. Researchers can now examine publicly available fungal datasets for the presence of these viruses, further elucidating the diversity and prevalence of mycoviruses in under-researched fungal groups.

In contemporary studies, researchers are increasingly employing advanced approaches, such as genome-wide linkage analysis, to unravel the underlying mechanisms governing the effects of mycoviruses on their fungal hosts [434]. For instance, RNA-seq-based genome-wide expression analyses showed distinct expression patterns in response to infection by four phylogenetically different mycoviruses in *F. graminearum* (FgV1-4) [434]. While all these mycoviruses induced changes in the transcriptome expression of the host, only FgV1 and FgV2 produced observable alterations in the host phenotype [434]. Mycoviruses rely on various host factors, as well as the intricate pathways and processes related to metabolism, transport, RNA processing, and signalling [3]. However, not all of these interactions lead to noticeable phenotypic changes in the host. Mycoviruses and their hosts often interact in complex ways, so more detailed research is needed to better understand these interactions.

Mycoviruses of Ceratocystidaceae

Plant pathogenic fungi occupy positions of great importance in both agriculture and natural plant communities [442]. Some fungal plant pathogens may occur naturally and play a role in regulating plant community structure [443]. The majority, however, are responsible for significant economic losses in agricultural crops and forestry systems worldwide [443, 444].

Ceratocystidaceae is a fungal family which includes insect symbionts, wood-degrading agents, and plant pathogens [445-447]. *Endoconidiophora* and *Ambrosiella*, which contain symbionts of insects, may negatively affect plant health, either by causing disease after transmission, or indirectly by facilitating the survival of insect pests [448, 449]. Although the genus *Huntiella* contains mainly saprobes, some are also known to cause disease in plants [450]. The majority of serious plant pathogenic fungi, however, are found in the genera *Ceratocystis*, *Thielaviopsis*, *Chalaropsis* and *Davidsoniella* [447, 451-453].

For many years, chemical fungicides have been used to control diseases caused by plant pathogenic fungi [454]. While these chemicals are effective in controlling various fungal diseases, including those caused by members of this family, they are also harmful to human health and the environment [455, 456]. In response, agricultural product safety standards have been strengthened, and public awareness has increased, creating urgent demand for efficient and environmentally friendly alternatives [457]. The development of suitable biocontrol strategies has thus become increasingly popular in recent years. Studies have shown that certain bacteria and fungi can produce volatile organic compounds and bioactive metabolites with antifungal activity, thus making them suitable biocontrol agents of plant pathogenic fungi [458, 459]. Another promising alternative to chemical fungicides is fungal viruses, or mycoviruses, due to the ability of some to reduce the virulence, growth and sporulation of their plant pathogenic hosts [410].

To date, only three species in the Ceratocystidaceae have been found to associate with any mycoviruses. Two of these are *Endoconidiophora resinifera* and *Endoconidiophora polonica*, both associating with two different strains of the same partitivirus [460]. A third fungus, *Thielaviopsis basicola*, is associated with mitochondrial viruses from the species *Unuamitovirus thba1* (Thielaviopsis basicola mitovirus) [190]. As Ceratocystidaceae is relatively under-researched with regards to mycoviruses, further studies are needed to elucidate the mycoviral landscape within this family, and to help aid in the discovery of future biocontrol strategies against the plant pathogenic members.

Conclusion

Mycoviruses are ubiquitous within the fungal kingdom, and recent studies have revealed their associations with most of the major fungal taxa. Yet, the number of mycoviruses that have been fully characterized is low in comparison to economically important plant and animal viruses. This is most likely due to their cryptic nature, which means that it is often difficult to distinguish

whether a fungal host is infected by empirical observation. Mycoviruses have been commonly detected with in vitro-based approaches, however, these are known to suffer from several disadvantages. The rapid advances in high throughput sequencing however, particularly RNA sequencing, which has become mainstream, has led to an exponential increase in the number of mycoviruses that have been discovered. Fungal transcriptomes can now be mined for mycoviral sequences, and this allows for the study of the complex interactions between fungal hosts and mycoviruses. Unlike most other viruses, mycoviruses do not always elicit measurable changes to host phenotypes. It is now clear that some mycoviruses cause hypovirulence and may be potential biocontrol agents of plant pathogenic fungi, which makes their discovery and characterization in such hosts even more important. There are, however, still numerous challenges that need to be addressed before their widespread use. Research is ongoing in pursuing the use of mycoviruses as biocontrol agents, and it is conceivable that these challenges will be overcome in the future.

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Chapter 2: Identification and Characterization of Mycoviruses in Publicly available Transcriptomes from the fungal family Ceratocystidaceae

Abstract

Mycoviruses are ubiquitous within the fungal kingdom, yet the diversity of mycoviruses in several fungal families and genera remains unexplored. In this study, 11 publicly available fungal transcriptomes from Ceratocystidaceae were analysed for the presence of mycoviruses. To date, only four genera in this family are known to contain mycoviruses. This study shed light on the mycoviral diversity found within Ceratocystidaceae, uncovering the identification of 10 novel mycoviruses across 5 of these isolates. The majority of mycoviruses were composed of positive sense single stranded RNA and were putatively assigned to the viral families *Mitoviridae* (with tentative classification into the genera *Unuamitovirus* and *Duamitovirus*) and *Endornaviridae*. The double stranded RNA viruses however, only associated with the family *Totiviridae* (with tentative classification into the genus *Victorivirus*). Furthermore, this study unveiled the remarkable detection of an identical unuamitovirus within *Thielaviopsis ethacetica* and *Thielaviopsis paradoxa*, respectively. This finding was particularly unusual, given that these two fungal isolates were obtained from distinct geographical locations. This study not only broadens our knowledge of the host range for mycoviruses but also marks the first identification of mycoviruses within *Ceratocystis platani*, *Thielaviopsis paradoxa*, *Thielaviopsis ethacetic*a and two isolates of *Huntiella omanensis*.

Introduction

Viruses are among the most abundant and diverse biological entities found on Earth, capable of infecting a wide range of life forms, including animals, plants, bacteria, protists, and fungi [1-5]. The study of viruses is of paramount importance in the context of managing and preventing viral disease, understanding viral evolution and ecological impact, and harnessing their potential in therapeutics and biotechnology [6-9]. However, the majority of virus-related research has primarily focused on economically significant plant and animal viruses, often neglecting other important groups, such as fungal viruses (mycoviruses).

Mycoviruses are prevalent throughout the fungal kingdom. They have been found in most of the major fungal taxonomic groups, including some of the early diverging lineages such as Mucoromycota and Zoopagomycota [10, 11]. They also exhibit great diversity and have been

classified into 23 recognized viral families and one unclassified genus by the International Committee on the Taxonomy of Viruses (ICTV) [12]. Classification is based on multiple factors, such as genome characteristics (e.g., nucleic acid type, genome orientation, length, and organization), host specificity, and percent identity to closely related viruses [12]. While the majority of mycoviruses consist of double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA) genomes, a small number with single-stranded DNA genomes have also been documented [11]. For example, members of the species *Gemycircularvirus sclero1* and *Gemytripvirus fugra1* belong to the family *Genomoviridae*, which consists of DNA viruses [13, 14].

Mycoviruses may cause several different effects on their fungal hosts. While the majority of known mycoviruses induce asymptomatic infections, some may also evoke hypervirulence (enhanced virulence) or hypovirulence (attenuated virulence) in their respective hosts [15]. The first documentation of hypovirulence-inducing mycoviruses occurred in the 1960s, when they were recognized as the primary cause of La France disease [16]. This condition had significant detrimental effects on the cultivation of commercially produced mushrooms (*Agaricus bisporus*) [16, 17]. Mushrooms were infected with Agaricus bisporus virus 1 (AbV1), a chrysovirus which was later identified as the primary causal agent of this disease, and displayed reduced mycelial growth, malformed fruiting bodies, and rapid deterioration of these fruiting bodies after harvesting [18, 19]. Within this particular framework, mycoviruses can be perceived as harmful because they cause economic losses in the production of edible mushrooms [19].

Mycoviruses resulting in fungal disease can also present potential benefits from a biocontrol standpoint, as they have the capacity to reduce the impact of plant pathogenic fungi and thus plant disease. A notable example is the use of viruses from the species *Alphahypovirus cryphonectriae* (Cryphonectria parasitica hypovirus 1; CHV-1) in the control the chestnut blight pathogen, *Cryphonectria parasitica*, in certain regions of Europe [20-23]. Consequently, a significant portion of mycovirus related research has revolved around the discovery and characterization of mycoviruses which are capable of inducing hypovirulence in plant pathogenic fungi [24-27]. This research is also driven by the need for alternatives to chemical fungicides, which are becoming increasingly ineffective due to fungal resistance, and this poses risks to agriculture and human health [28-31].

The rapid advancement in Next-Generation Sequencing (NGS) technologies, coupled with various bioinformatics tools, have significantly increased the discovery of mycoviruses [3234]. Moreover, this has resulted in an increased availability of fungal transcriptomic datasets on open-

access platforms like the National Centre for Biotechnology Information (NCBI) sequencing reads archive (SRA), which enables the identification of mycoviruses within these transcriptomes [35, 36]. However, our current understanding of mycoviral diversity in various fungal genera and families remains inadequate, highlighting the need for further research to obtain a more comprehensive understanding of mycoviral diversity within these groups.

Ceratocystidaceae encompasses a diverse group of fungi, including those with substantial economic impact, such as plant pathogens, insect symbionts, and agents responsible for timber degradation [37-40]. While some members of Ceratocystidaceae have been found to harbour mycoviruses, the knowledge in this regard is limited. Two members of the genus *Endoconidiophora* for example, are known to associate with a partitivirus [41]. Recently, mycoviruses have also been found in publicly available transcriptomes from *Ceratocystis fimbriata* and *Ceratocystis cacaofunesta* [42]. However, a comprehensive understanding of mycoviral diversity within Ceratocystidaceae is still lacking, necessitating further research to explore and characterize the full extent of mycoviral diversity in this fungal family.

The aim of this study was to gain a deeper understanding of mycoviral diversity within Ceratocystidaceae. To achieve this, publicly available transcriptomes from members of this family were analysed for the presence of mycoviruses using bioinformatics approaches. The genomes of any potential mycoviruses were then characterized, and their phylogenetic relationships with closely related viruses were investigated in order to elucidate their taxonomy and evolutionary connections. By achieving these objectives, the study provides a comprehensive and detailed overview of mycoviral diversity within the Ceratocystidaceae family. Furthermore, this study facilitated the identification of mycoviruses that could be further evaluated as potential biocontrol agents in future research.

Materials and methods

Identifying fungal transcriptomes from publicly available databases

Unassembled transcriptomic datasets from members of Ceratocystidaceae were retrieved from the NCBI SRA database [43] and can be found in Table 1. To ensure data integrity, the quality of these datasets was assessed using FastQC [44] and processed with Trimmomatic (version 0.36) [45], with a minimum Phred score of 33 and 20 for sequences generated with IonTorrent and Illumina platforms, respectively.

Table 1. Overview of publicly available fungal transcriptomes used for the discovery of mycoviruses in Ceratocystidaceae. Fungal isolates representing five genera within the Ceratocystidaceae family were procured from the Sequencing Reads Archive. Each dataset is identified by a distinct SRA ID, which serves as a unique accession number. In cases where a single isolate was associated with multiple datasets, multiple accession numbers are shown. The table also includes information about the fungal plant/insect host, origin, the type of instrument used and, where applicable, the selection step used in library preparation.

Genus	Isolate	SRA ID	Fungal plant/insect host and origin	Instrument and selection step used
Ambrosiella	*Ambrosiella xylebori* CBS 110.61	SRR5865576	*Xyleborus compactus* gallery in *Coffea canephora* - Ivory Coast	Illumina HiSeq 2000 - unknown
Bretziella	*Bretziella fagacearum* C519	SRR13083620	*Quercus rubra* - Unknown origin	Illumina NovaSeq 6000 - unknown
Ceratocystis	*Ceratocystis platani* MS580	SRR13858934	*Platanus* x *hispanica* - Tuscany, Italy	Illumina MiSeq – unknown
Huntiella	*Huntiella abstrusa* CMW21092	SRR22044985 SRR22044986 SRR22044987	*Eucalyptus* - Teso East, Riau province, Indonesia	Ion Torrent Proton – mRNA enrichment
	Huntiella omanensis CMW44442	SRR5640136 SRR5640137 SRR5640138	Lab strain - South Africa	Ion Torrent Proton – mRNA enriched
	Huntiella omanensis CMW44450	SRR5639921 SRR5639922 SRR5639923	Lab strain - South Africa	Ion Torrent Proton - mRNA enriched
	Huntiella omanensis CMW 11056	SRR5640139 SRR5640140 SRR5640141	*Mangifera indica* - Oman	Ion Torrent Proton - mRNA enriched
	Huntiella moniliformis CMW 36919	SRR5640143 SRR5640144 SRR5640145 SRR5640146 SRR5640147 SRR5640148	*Theobroma cacao* - Cameroon	Ion Torrent Proton - mRNA enriched
Thielaviopsis	*Thielaviopsis paradoxa* R-189	SRR15533162	*Elaeis guineensis* - Meta, Colombia	Illumina HiSeq 2000 – poly (A) selection
	Thielaviopsis ethacetica JCM 6961	SRR12744487 SRR12744488 SRR12744489 SRR12744490	Saccharum officinarum - Sertãozinho, São Paulo, Brazil	Illumina HiSeq 2500 – poly (A) selection

Bioinformatics pipeline for the discovery of mycoviruses

The trimmed datasets were *de novo* assembled with rnaviralSPAdes (version 3.15.0) [46]. The assembled contigs were then imported into CLC genomics workbench 22 (Qiagen Bioinformatics, Aarhus, Denmark), where they were translated from nucleotide sequences to protein sequences in all conceivable reading frames utilizing the 'Translate to Protein' tool. These sequences were aligned to viral protein sequences within a custom viral protein database, using the BLAST toolkit within CLC genomics workbench. This custom database was created in

March 2022, and comprised viral protein sequences from all known viral families containing mycoviruses, as well as unclassified mycoviruses. These protein sequences were obtained from the NCBI protein database and were regularly updated as new viral families and genera were released (Last updated in April 2023). The output from this was manually inspected, and putative mycoviral contigs were selected for downstream analysis based on, alignment length and E-value (E-value $<10^{-15}$). To differentiate real viral sequences from host sequences, the selected contigs were then subjected to BLAST evaluation against a non-redundant protein sequence database using the NCBI BLASTp tool [47].

Genomic characterization and phylogenetic analysis of mycoviruses

The open reading frames (ORFs) of all putative mycoviruses were determined with NCBI ORF finder [48]. In instances where putative mycoviruses showed homology to mitoviruses or narnaviruses, the mitochondrial genetic code was used to identify ORFs, while the conventional genetic code was used for all other viruses. Where necessary, the web-based version of Mfold [49] was used to predict the secondary 5' and 3' UTRs of certain mycoviruses. The web-based version of the tool ProbKnot [50] was also used to predict pseudoknots in the ORF-junction region of putative totiviruses. Subsequently, the conserved protein domains within the mycoviral ORFs were identified using the NCBI BLASTp tool. To perform phylogenetic analysis, the ORFs containing RNA-dependent RNA polymerase (RdRp) from these mycoviruses were aligned with reference sequences of closely related viral protein sequences obtained from the NCBI GenBank. The ClustalW program in MEGA 11 was used to perform these alignments [51]. Additionally, MEGA 11 was used to determine the best-fit protein model for each alignment. Using the obtained alignments as input, Maximum Likelihood (ML) phylogenetic trees were constructed with IQ-TREE (version 2.1.3), using the best-fit model for the analysis [52]. Multiple sequence alignments of the RdRp amino acid sequences from characterized mycoviruses and a subset of the most closely related viruses were then aligned utilizing the web-based version of the Clustal Omega tool [53], to determine conserved motifs within this protein. The same tool was then used to construct percent identity matrices based on these alignments, to compare sequence homology between viruses. Where pertinent, alignments of the amino acid sequences of coat proteins (CP) from the identified viruses and other closely related viruses were also used to construct percent identity matrices.

Results

Summary of identified viral sequences

From the 10 fungal isolates evaluated in this study, five were found to be associated with putative mycoviruses. A total of six were +ssRNA viruses, and three were dsRNA viruses. An overview of these mycoviruses and their characteristics can be found in Table 2.

Table 2. Taxonomic and genomic overview of putative mycoviruses identified in the transcriptomes of fungi from Ceratocystidaceae.

Host	Genome type	Virus Family	Putative virus name	Virus abbrev.	Contig Length (nucleotide)	Contig coverage	Protein ID	Protein length (amino acid)
Ceratocystis platani MS580	+ssRNA	*Mitoviridae*	Ceratocystis platani RNA virus 1	CpRV-1	3150	19.78	Mitovir RNA pol	373
Ceratocystis platani MS580	+ssRNA	*Endornaviridae*	Ceratocystis platani RNA virus 2	CpRV-2	857	1.39		
Huntiella omanensis CMW44450	dsRNA	*Totiviridae*	Huntiella omanensis RNA Virus 1	HoRV-1	2322	9.22	RdRP 4	457
*▼*Huntiella omanensis* CMW44450	dsRNA	*Totiviridae*	Huntiella omanensis RNA Segment 1	Ho_RNA_Seg1	1852	27.18	Totivirus coat	499
Huntiella omanensis CMW44442	dsRNA	*Totiviridae*	Huntiella omanensis RNA Virus 3	HoRV-3	547	1.62		
Thielaviopsis paradoxa R-189	+ssRNA	*Mitoviridae*	Thielaviopsis paradoxa RNA virus 1	TpRV-1	2599	339.41	Mitovir RNA pol	465
Thielaviopsis paradoxa R-189	dsRNA	*Totiviridae*	Thielaviopsis paradoxa RNA virus 2	TpRV-2	5460	42.42	Totivirus coat RdRP 4	716 465
Thielaviopsis ethacetica JCM -6961	+ssRNA	*Mitoviridae*	Thielaviopsis ethacetica RNA virus 1	TeRV-1	2620	2094.42	Mitovir RNA pol	465
Thielaviopsis ethacetica JCM -6961	+ssRNA	*Mitoviridae*	Thielaviopsis ethacetica RNA virus 2	TeRV-2	2616	147.73	Mitovir RNA pol	477
Thielaviopsis ethacetica JCM -6961	+ssRNA	*Mitoviridae*	Thielaviopsis ethacetica RNA virus 3	TeRV-3	2991	2145.99	Mitovir RNA pol	371

Truncated contigs are indicated with an asterisk (*), and in cases where no conserved protein domains could be found, Protein ID and length were left blank. Viral segments without an RdRp domain, and which may belong to other truncated viruses in the assemblies are denoted with a triangle (▼).

Characterization of positive sense single stranded RNA mycoviruses

1. Identification of mycoviruses from *Mitoviridae* and *Endornaviridae*

The majority of mycoviruses analysed in this study possessed +ssRNA genomes. The fungal transcriptomes derived from *C. platani*, *T. paradoxa* and *T. ethacetica* were all associated with mycoviruses which displayed similarity to the viral family *Mitoviridae*. The fungi *C. platani* and *T. paradoxa* were both associated with single mitoviruses, putatively named Ceratocystis platani RNA virus 1 (CpRV-1) and Thielaviopsis paradoxa RNA virus 1 (TpRV-1), respectively. *T. ethacetica* on the other hand, was found to harbour three mitoviruses, with the putative names Thielaviopsis ethacetica RNA virus 1 (TeRV-1), Thielaviopsis ethacetica RNA virus 2 (TeRV2), and Thielaviopsis ethacetica RNA virus 3 (TeRV-3). In addition to CpRV-1, a mycovirus demonstrating similarity to the family *Endornaviridae* was also discovered in *C. platani* and was putatively named Ceratocystis platani RNA virus 2 (CpRV-2). However, CpRV-2 genome was severely truncated on both ends and was thus omitted from subsequent genomic characterization and phylogenetic analyses.

1.1. Phylogenetic classification of mycoviruses from *Mitoviridae*

To characterize the relationships of the mitoviruses, a phylogenetic tree was constructed based on the RdRp amino acid sequences of these five viruses and cognate sequences derived from references obtained from GenBank (Fig.1). The analysis revealed that TeRV-1 and TpRV-1 clustered most closely with members of the genus *Unuamitovirus*, while CpRV-1 and TeRV-3 clustered with viruses from *Duamitovirus*. Additionally, TeRV-2 appeared to cluster with unclassified mitoviruses that displayed significant sequence similarity to the genus *Unuamitovirus*. In the present study, a comparative analysis of the RdRp sequences of various mitoviruses and closely related viruses was conducted, and a percent identity matrix was constructed to delineate distinct viral species (Fig. 2). The percent identities of the RdRps for CpRV-1, TeRV-3, and TeRV-2 in comparison to other mitoviruses were found to be below the designated threshold of 90%, which serves as the criterion for species demarcation within the *Mitoviridae* family [54]. This suggests the novel nature of these mycoviruses, classifying them within the genera *Duamitovirus* (CpRV-1 and TeRV-3) and the unclassified unuamitovirus-like mycoviruses (TeRV-2). Notably, although TeRV-1 and TpRV-1 are both considered novel mitoviruses within the genus *Unuamitovirus*, they exhibit a remarkable 98.53% identity to one another at the RdRp level, indicating that they are the same virus. This virus was thus given the collective name Thielaviopsis RNA Virus 1 (TRV-1).

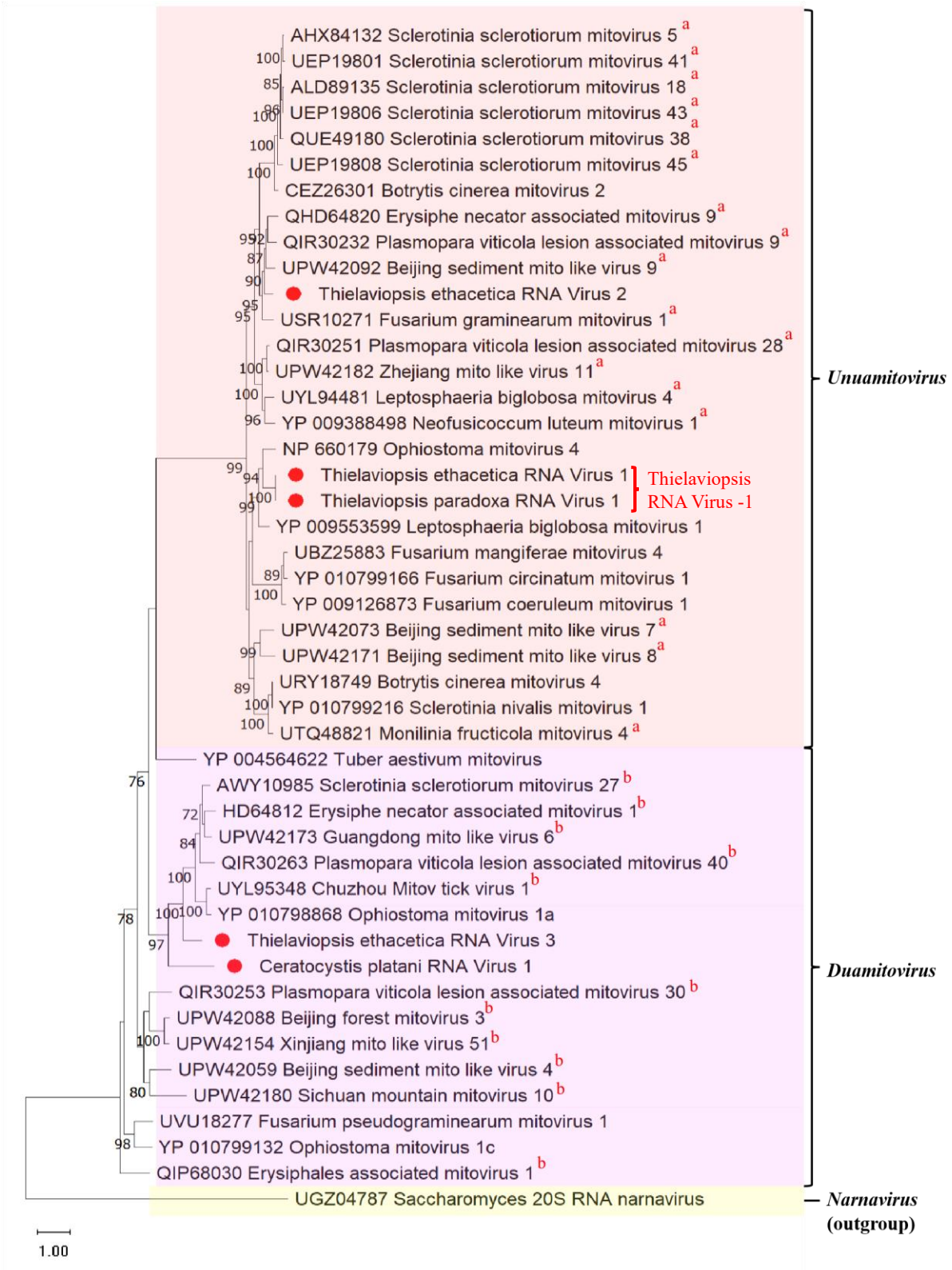

Fig.1 Phylogenetic tree showing maximum likelihood analysis of the RdRp protein sequences from the mitoviruses discovered in this study, with those of related viruses. The phylogeny was generated using IQ-TREE, using the Whelan and Goldman model with frequencies, invariant sites, and gamma distribution. Bootstrapping with 1000 replicates was performed, and corresponding percentages are indicated next to the branches. Only bootstrap values equal to or exceeding 70 are displayed. The Saccharomyces 20S RNA Narnavirus was used as an outgroup. The red circles (●) indicate mycoviruses discovered in this study. a: Denotes unuamitoviruses and b: duamitoviruses which have not yet been taxonomically assigned by the ICTV (https://ictv.global/taxonomy). These mitoviruses exhibit genetic characteristics and phylogenetic relationships that strongly suggest their classification within these genera.

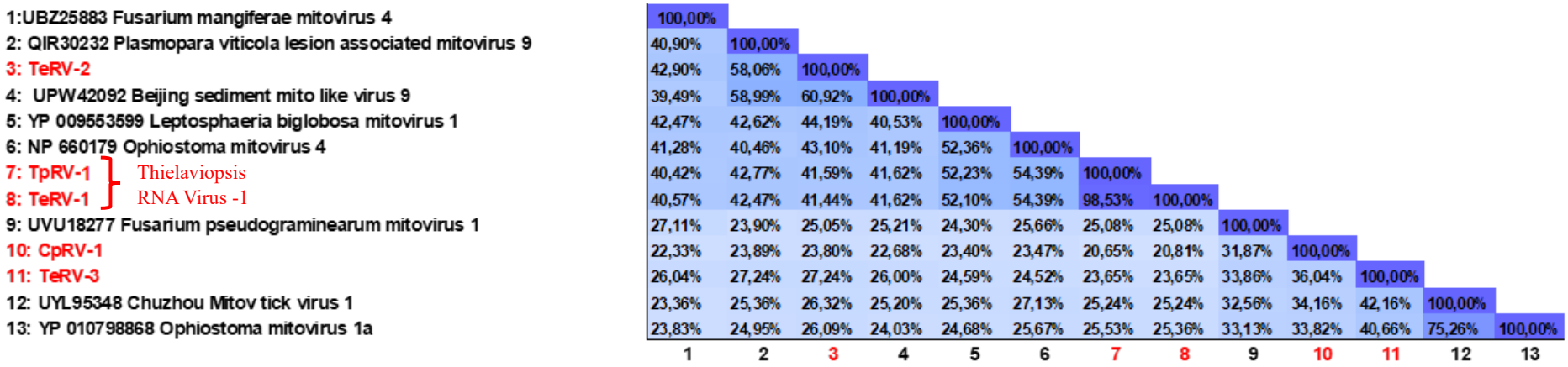

Fig. 2 Percent Identity Matrix of the mitoviruses from this study, and other viral members from *Mitoviridae*, generated using Clustal Omega. The colour gradients represent percent identities, with darker blue shades indicating higher identities and lighter shades indicating lower identities. The mitoviruses are numbered from 1 to 13, and their corresponding labels are displayed at the bottom of each column. Mitoviruses from this study are highlighted in red for better readability.

1.2. Genomic characterization of mycoviruses from *Mitoviridae*

The genomes of all putative mitoviruses were characterized and a more detailed diagrammatic representation of their genome organizations can be found in Fig. 3. All five putative mitoviral genomes were composed of a single ORF, which contained an RdRp protein domain.

The genome organizations, including the length of the ORF and all associated protein domains, of the unuamitoviruses TpRV-1, TeRV-1, and TeRV-2 is indicated in Fig. 3a, 3b, and 3c, respectively. The predicted mass of the protein product produced by ORFs of these viruses were 87.52 kDa for TpRV-1, 87.53 kDa for TeRV-1, and 78.23 kDa for TeRV-2. A Blastp analysis of the RdRp protein domain from TeRV-2 exhibited the highest percent identity to Beijing sediment mito-like virus 9 (61.47% identity, 98% coverage, 0.0 E-value), while those of TpRV-1 and TeRV-1 displayed the highest similarity to Ophiostoma mitovirus 4 (52.49% identity, 99% coverage, and 0.0 E-value). All unuamitoviruses displayed 5' and 3' UTRs of varying lengths, and the secondary structures, as well as the associated free energy of these were assessed and is indicated in Fig. S3, Fig. S4, and Fig. S5 for TpRV-1, TeRV-1, and TeRV-2, respectively. The analysis revealed the formation of multiple stem loop structures in all three mitoviruses, with free energy values promoting the formation of stable stem-loop structures. Additionally, the terminal ends of all three unuamitoviruses showed reverse complementarity, and may thus potentially form panhandle structures, albeit with varying free energy values. These are indicated in Fig. S3C (TpRV-1), Fig. S4C (TeRV-1), and Fig. S5C (TeRV-2).

The genome organizations of TeRV-3 and CpRV-1, putative members of the genus *Duamitovirus*, can be found in Fig. 3d, and Fig. 3e, respectively. The predicted protein mass for the protein product produced by the ORF of TeRV-3 was 86.75 kDa, and the RdRp protein when analysed with Blastp displayed the highest percent identity to Guangdong mito-like virus 6 (43.19% identity, 95% coverage, 1e-180 value). The remaining duamitovirus, CpRV-1, displayed a predicted protein mass of 85.78 kDa, and similarly to TeRV-3, also displayed an RdRp percent identity that was closer to that of the Guangdong mito-like virus 6 (39.00% identity, 74% coverage, 3e-106 E-value). As with the unuamitoviruses from this study, both duamitoviruses contained 5' and 3' UTRs of varying lengths, which formed stable stem-loop structures with varying free energy values. These structures, as well as all associated energy values, are indicated in Fig. S6 for TeRV-3, and Fig. S7 for CpRV-1. Based on the complementarity of the 3' and 5' UTRs, and the predicted free energy values, both

duamitoviruses may potentially form a panhandle structure, as indicated in Fig. S6C for TeRV-3 and Fig. S7C for CpRV-1.

Amino acid alignments of the RdRp sequences from all mitoviruses analysed in this study, and other closely related viruses also revealed six conserved protein motifs (I to VI), typical of mitoviral RdRps [55, 56] (Fig. S1).

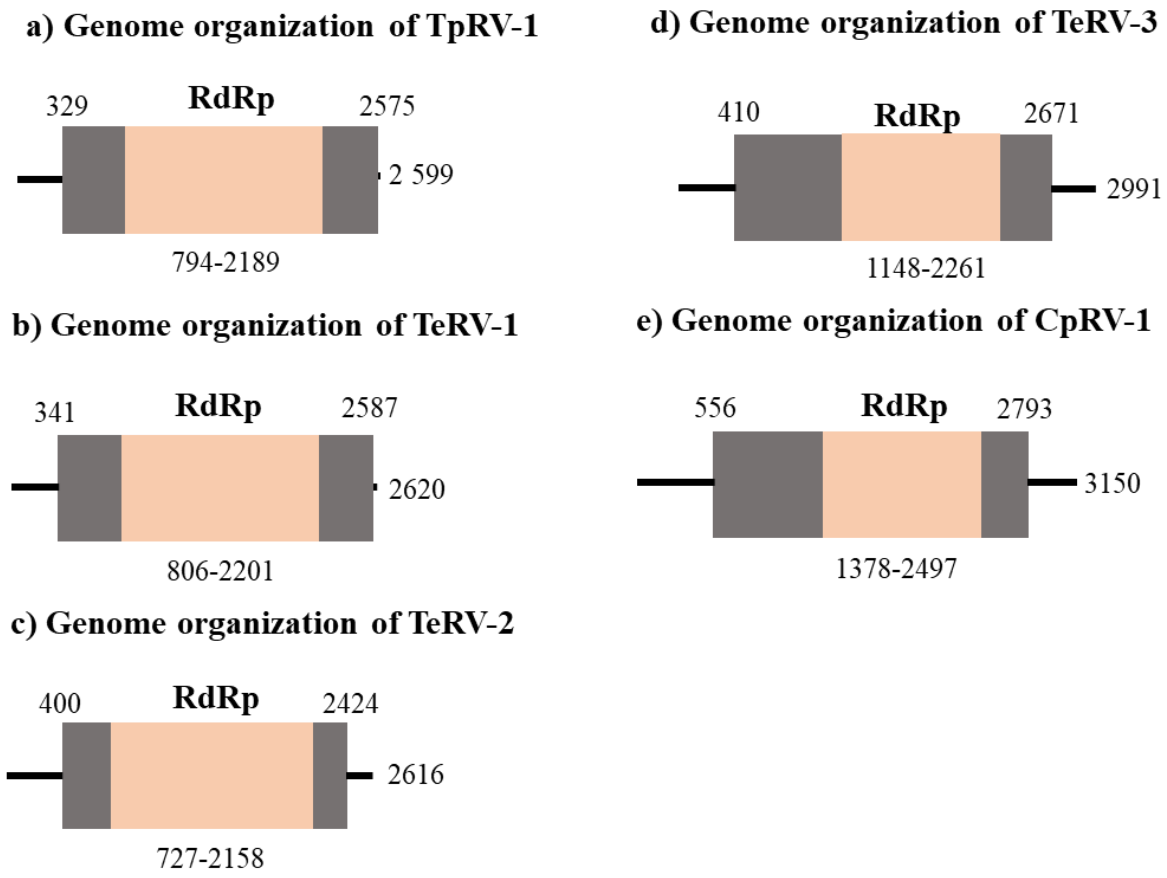

Fig. 3 Schematic representation of the genomic organization of a) TpRV-1, b) TeRV-1, c) TeRV-2, d) TeRV-3, and e) CpRV-1. The open bars represent single open reading frames (ORFs), while the single lines represent untranslated regions (UTRs). The length of the ORF is indicated for all mitoviruses, and the position of the RdRp domain encoded on the ORF is also shown.

Characterization of double stranded RNA mycoviruses

1. Identification of mycoviruses from *Totiviridae*

Three putative mycoviruses with dsRNA genomes were found in the *H. omanensis* CMW 44450, *H. omanensis* CMW 44442, and *T. paradoxa* transcriptomes. Notably, these mycoviruses exhibited significant similarity to viruses belonging to the family *Totiviridae*. The totivirus from *H. omanensis* CMW 44450 were provisionally named Huntiella omanensis RNA Virus 1 (HoRV-1). An additional segment was found in the transcriptome of this fungus and was named Huntiella omanensis RNA Segment 1 (Ho_RNA_Seg_1). The totiviruses

associating with *H. omanensis* CMW 44442 and *T. paradoxa* was tentatively named Huntiella omanensis RNA Virus 3 (HoRV-3) and Thielaviopsis paradoxa RNA Virus 2 (TpRV-2), respectively.

1.1. Phylogenetic classification of mycoviruses from *Totiviridae*

To elucidate the relationships among the totiviruses, a phylogenetic tree was constructed using the RdRp amino acid sequences from TpRV-2 and HoRV-1, and cognate sequences derived from references obtained from GenBank. The totivirus HoRV-3, and viral segment Ho_RNA_Seg_1, were omitted from further analysis due to the RdRp being incomplete or missing from the assembled contigs. Their exclusion ensured the integrity and accuracy of the phylogenetic tree, enabling a more reliable representation of the relationships among the characterized viruses and their related counterparts. The resulting phylogenetic tree (Fig. 4) showed clustering of both TpRV-2 and HoRV-1 with viral members belonging to the genus *Victorivirus*. A comparative analysis of the RdRp and CP sequences of various totiviruses and closely related viruses was conducted, and percent identity matrices were constructed to delineate distinct viral species (Fig. 5). The findings revealed that the RdRp percent identities of HoRV-1 and TpRV-2 (Fig. 5A), in comparison to other totiviruses, fell below the designated threshold of 60%, which is a critical criterion for species demarcation within the *Totiviridae* family. Moreover, for TpRV-2, all percent identities for the CP sequences were also below 60% (Fig. 5B) [57], further supporting its classification as a distinct species Thus, TpRV-2 can be considered a potential member of the *Victorivirus* genus. It is worth noting that HoRV-1 did not have a CP due to truncation, hence a CP percent identity matrix was not generated for this virus.

1.2. Genomic characterization of mycoviruses from *Totiviridae*

Due to the lack of complete RdRp and CP ORFs, the putative totiviruses HoRV-1, 2 and 3 were excluded from further genomic characterisation. The totivirus TpRV-2 however, had a genome consisting of a complete totivirus CP domain and another ORF encoding for a complete RdRp protein domain. The genome organization of TpRV-2, as well as the length of all ORFs and protein domains, can be found in Fig. 6. Amino acid alignments of the RdRp sequences from HoRV-1, TpRV-2 and other closely related viruses also revealed eight conserved protein motifs (I to VIII), typical of victorivirus RdRps (Fig. S2).

The genome of TpRV-2 consists of a 5' and 3' UTR, as well as two overlapping ORFs. The first ORF, which is in the +3-reading frame, encodes for a CP, with a predicted molecular mass of 86.59 kDa. This protein, when analysed with Blastp, was found to exhibit significant sequence

similarity to Totiviridae sp. (35.28% identity, 80% coverage, 9e-93 E-value). The C-terminal ends of the TpRV-2 protein also lacks an Ala/Gly/Pro rich region which is sometimes present in the ORF encoding the CP in victoriviruses. ORF 1 and 2 overlaps with a stop-initiation codon (UAAUG), and a pseudoknot is predicted to span in this region, as indicated by Fig.S8. ORF 2, which is in the +2-reading frame, encodes for an RdRp protein with a predicted mass of 91.72 kDa. This RdRp protein also has a higher percent identity to Fusarium sambucinum victorivirus 1 (49.64% identity, 98% coverage, 0.0 E-value).

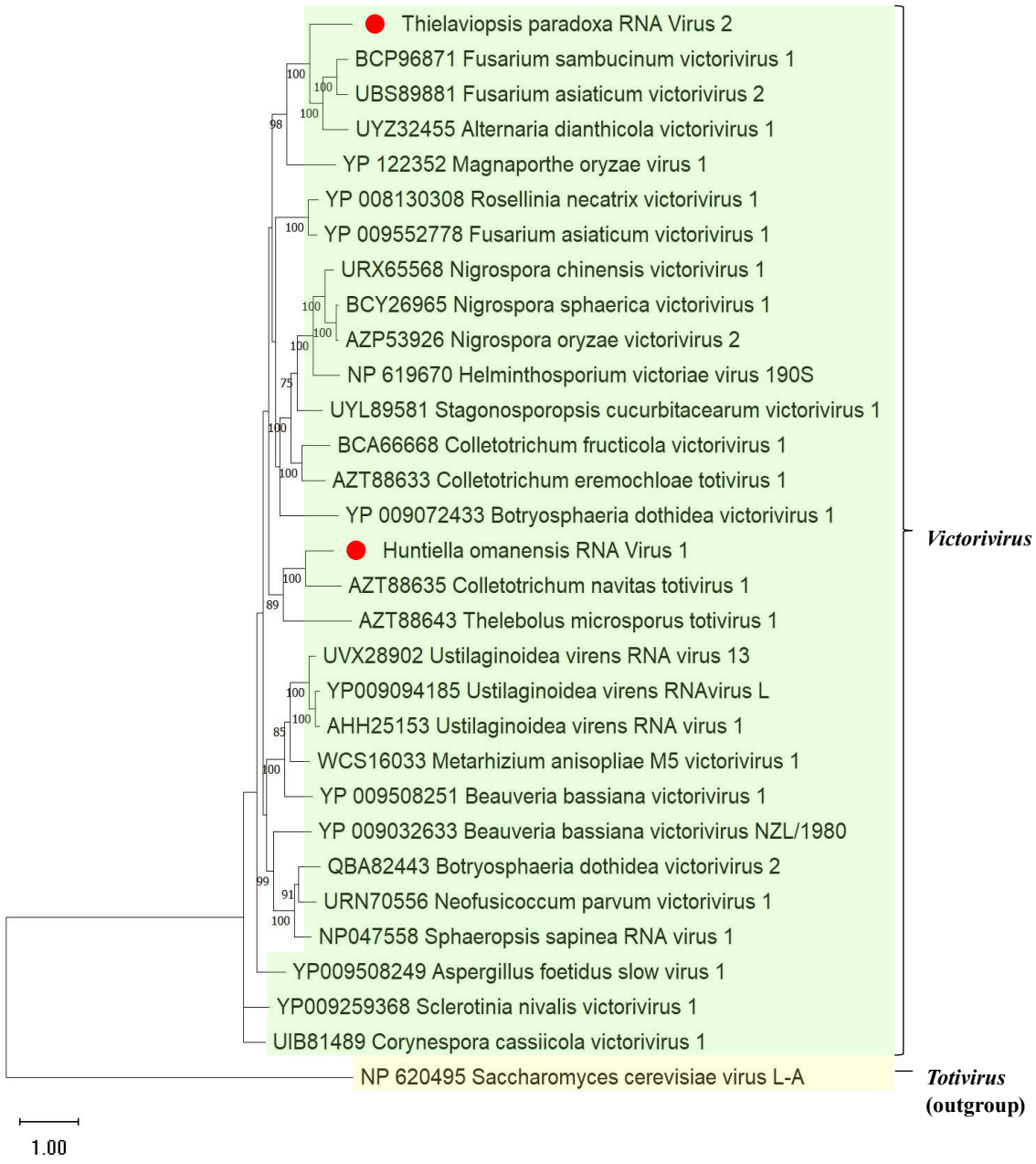

Fig. 4 Maximum likelihood phylogenetic tree constructed from the alignment of RdRp protein sequences from totiviruses discovered in this study, with those of related viruses. The phylogeny was generated using IQ-TREE, using the Le Gauss model with frequencies, invariant sites, and gamma distribution. Bootstrapping with 1000 replicates was performed, and corresponding percentages are indicated next to the branches. Only bootstrap values equal to or exceeding 70 are displayed. The Saccharomyces cerevisiae virus L-A was used as an outgroup. The red circles (●) indicate the totiviruses discovered in this study.

A)

1: TpRV-2	100,00%							
2: UYZ32455 Alternaria dianthicola victorivirus 1	46,07%	100,00%						
3: BCP96871 Fusarium sambucinum victorivirus 1	49,28%	56,73%	100,00%					
4: YP 009072433 Botryosphaeria dothidea victorivirus	39,90%	37,08%	37,83%	100,00%				
5: AZT88643 Thelebolus microsporus totivirus 1	35,51%	36,23%	35,46%	36,02%	100,00%			
6: YP 009552778 Fusarium asiaticum victorivirus 1	38,59%	41,50%	42,08%	42,55%	41,08%	100,00%		
7: HoRV-1	39,55%	40,68%	41,33%	42,82%	42,44%	46,08%	100,00%	
8: AZT88635 Colletotrichum navitas totivirus 1	37,79%	37,56%	38,14%	38,26%	39,83%	41,48%	53,73%	100,00%
	1	2	3	4	5	6	7	8

B)

1: TpRV-2	100,00%						
2: QDH91164 Totiviridae sp.	34,47%	100,00%					
3: BCA66667 Colletotrichum fructicola victorivirus 1	33,11%	50,27%	100,00%				
4: QJW70224 Bipolaris maydis victorivirus 1d	33,81%	57,75%	65,16%	100,00%			
5: NP 619669 Helminthosporium victoriae virus 190S	32,71%	54,00%	61,41%	97,03%	100,00%		
6: AXB26763 Bipolaris maydis victorivirus 1	32,71%	54,13%	61,80%	96,88%	97,93%	100,00%	
7: QJW70222 Bipolaris maydis victorivirus 1c	32,57%	53,73%	61,41%	96,72%	97,80%	98,58%	100,00%
	1	2	3	4	5	6	7

Fig. 5 Percent Identity Matrix of the totiviruses from this study, and other viral members from the genus *Victorivirus*. The Matrix was generated using Clustal-Omega 1.2.2, with the colour gradients serving to represent the respective percent identities of the totiviruses from this study to other related viruses. The percent identity matrixes are presented as A, denoting the alignments of the (A) RdRp and (B) those of the CP amino acid sequences. The percent identities are visualized with varying shades of blue and pink in the RdRp and CP alignments, respectively. Higher percentages are indicated in darker shades, while lower percentages are indicated in lighter hues. For clarity, the totiviruses from this study are numbered as 1 to 7 in the CP alignments and as 1 to 8 in the RdRp alignments. Corresponding labels for each of these totiviruses are displayed at the bottom of their respective columns. To enhance readability, totiviruses from this study have been highlighted in red. It is important to note that HoRV-1, due to the absence of a full-length genome and thus a CP, has been excluded from the CP alignments.

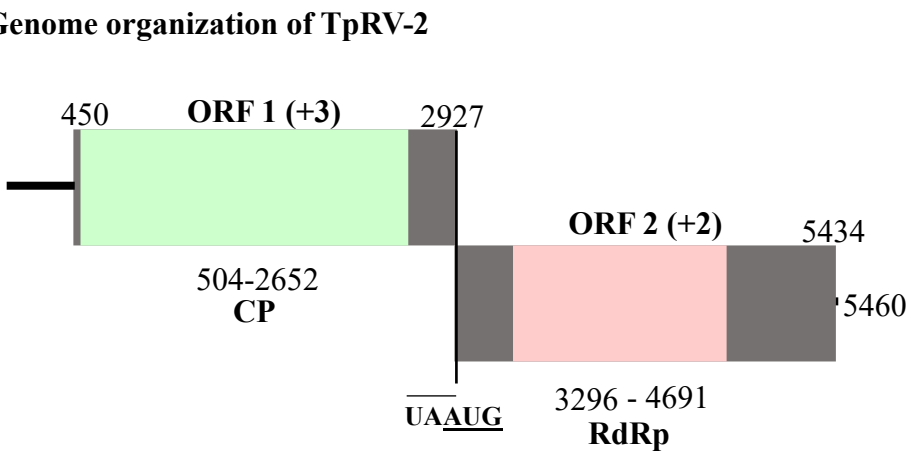

Fig. 6 Schematic representation of the genome organization of TpRV-2. The open bars signify individual ORFs, and the single lines represent the 5' and 3' UTRs. The putative victorivirus consists of two ORFs, and their respective lengths are indicated. Moreover, the positions of the RdRp and CP domains encoded within their corresponding ORFs are shown. The reading frames for each ORF are clearly indicated, and the stop-initiation codon required for the translation of ORF 2 is also depicted in the figure.

Discussion

The current study demonstrates the efficacy of utilizing metatranscriptomic approaches to investigate fungal transcriptomes, including those retrieved from publicly available repositories, for the discovery of mycoviruses. This method has been instrumental in the successful identification of numerous mycoviruses in other studies [32, 35, 36], and has resulted in the discovery of 10 novel mycoviruses in 5 fungal transcriptomes from Ceratocystidaceae in this study.

Mycoviruses with +ssRNA genomes were more prevalent in this study when compared to those with dsRNA genomes. In the past, mycoviruses with +ssRNA genomes were less frequently detected than mycoviruses with dsRNA genomes. This disparity can be attributed to the conventional culture-based methods used at the time, which relied on the purification of dsRNA or a dsRNA intermediate [58]. These techniques were less sensitive to the detection of ssRNA viruses, as the recovery of intermediate dsRNA was less efficient than that of genomic dsRNA, leading to an under representation of many ssRNA mycoviruses [59, 60]. The findings from this study suggests that +ssRNA viruses may be more prevalent in the fungal isolates evaluated in this study and underscore the suitability of employing transcriptome analysis for the discovery of ssRNA viruses.

The +ssRNA mycoviruses showed similarity to members of the viral families *Mitoviridae* and *Endornaviridae*. *Mitoviridae* has recently earned recognition as a distinct family by the ICTV, which has led to the formation of 4 new genera (https://ictv.global/taxonomy). Mitoviruses

generally possess genomes ranging in size from 2.0 to 4.5 Kb and are characterized by a single ORF that encodes an RdRp protein [61, 64], which was consistent with the genome organizations of the five mitoviruses analysed in this study. Furthermore, alignments of the RdRp protein sequence of the mitoviruses from this study against those of other mitoviruses also revealed the presence of 6 protein motifs, which included a well conserved Glycine-Aspartic Acid-Aspartic Acid (GDD) motif. These were consistent with the 6 protein motifs which are commonly conserved among viruses belonging to *Mitoviridae* [55, 61, 64, 65].

Another unique feature of mitoviruses is their ability to utilize the mitochondrial genetic code during replication, where the codon UGA, typically a stop codon in the standard genetic code, also encodes for the amino acid tryptophan [66]. This has allowed mitoviruses to adapt to environmental conditions in the mitochondria of their fungal hosts, where these viruses reside [67]. The findings from this study supports this notion since the genomes of the identified mitoviruses only revealed complete ORFs when the mitochondrial genetic code was applied for ORF prediction.

Another characteristic commonly exhibited by the 3' and 5' UTRs of mitoviruses, is the formation of stable stem-loop structures, which can potentially form a panhandle structure through inverted complementarity [55, 68-70]. All the mitoviruses which were analysed in this study were found to have stable stem-loop structures in their UTRs, suggesting the conservation of this genomic feature across the identified mycoviruses. These secondary structures have been hypothesized to play important roles in replication and translation of the mitoviruses and may also act as protective elements against degradation [56, 71]. The presence of such structures may contribute to the stability and viability of mitoviruses within their host organisms. Among the mitoviruses from this study, TpRV-1 had limited inverted complementarity, and the formation of a panhandle is thus less likely. Nevertheless, the absence of panhandle structures is not uncommon in mitoviruses and has been reported in other studies [71-73]. This suggests that these structures may not be essential for replication of the virus. However, it is important to note that a limitation of this study is the lack of cultures for the fungal isolates under investigation. As a result, it remains uncertain whether the UTRs of all viruses under investigation were complete, as 3' and 5' Rapid Amplification of cDNA Ends (RACE) PCRs, which are normally used to elucidate the terminal ends of the viral genome, could not be performed.

Phylogenetic analysis of all the mitoviruses from this study revealed that these viruses belonged to two established genera in the family *Mitoviridae*, namely *Unuamitovirus* and *Duamitovirus*. The criteria for assigning viruses to these genera are primarily based on their phylogenetic relationships, as official guidelines for taxonomic classification are currently lacking. Species demarcation within this family, however, dictates that the amino acid sequence identities of RdRp proteins among any putative mitoviruses should be below 90% when compared to other closely related viruses [54]. The mitoviruses TeRV-3 and CpRV-1 meet this criterion and are thus novel mycoviruses which may potentially belong to the genus *Duamitovirus*. Similarly, the mitovirus TeRV-2 also meets this criterion and thus a novel mycovirus which is a putative member of the genus *Unuamitovirus*.

Despite having RdRp percent identities lower than 90% when compared to closely affiliated mitoviruses within the *Unuamitovirus* genus, the percent identity matrix of TpRV-1 and TeRV-1 revealed that these viruses exhibited a 98% identity to each other. This striking similarity raises the possibility that TpRV-1 and TeRV-1 represents the same virus. Thus, the name TRV-1, was putatively chosen to denote this virus. A more detailed look into the origins of the isolates utilized to create these datasets revealed that *T. ethacetica* and *T. paradoxa* were sourced from Brazil (Sertãozinho) and Colombia (Bogotá), respectively. These fungal species are also known to infect some of the same host plants, including sugarcane [74, 75] and oil palm [76, 77]. This suggests the possibility that these fungal hosts might have come into contact at some point, potentially leading to the exchange of the mitovirus between the two species, possibly through hyphal anastomosis. Interestingly, cases have been documented where different fungal species are naturally infected by the same mycovirus. For example, members of the species *Botrytis porri botybirnavirus* 1(Botrytis porri RNA virus 1; BpRV1), a mycovirus initially identified in *Botrytis porri*, was also detected in *Botrytis squamosa* and *S. sclerotiorum* [78]. These occurrences suggest that certain mycoviruses can propagate between fungi within the same genus or even the same family.

Another possible explanation is cross-kingdom transfer, where a fungal virus could be transmitted to a plant host and subsequently to another fungal host. For instance, *Cucumber mosaic virus* (CMV), a plant virus, was recently found to naturally infect *Rhizoctonia solani* [78]. This study demonstrated that CMV could also be transmitted from the infected fungus to an uninfected host plant. Similarly, Bian, et al. [80] has demonstrated the cross-kingdom transfer of CHV-1 to *Nicotiana tabacum*, facilitated by the plant virus *Tobacco mosaic virus*

(TMV). These researchers also showed that CHV-1 could be transferred from the infected plant to a vegetatively incompatible fungus, *Fusarium graminearum*. These findings raise the possibility that the mitovirus present in *T. paradoxa* and *T. ethacetica* might have spread through a yet unidentified mechanism. It's important to note that the other mycoviruses from *T. ethacetica* (TeRV-2 and TeRV-3) were not found in the datasets of *T. paradoxa*, and the reasons for these discrepancies remain unclear. Collectively, these findings suggest the likelihood that TRV-1 is a novel member of the *Unuamitovirus* genus which may infect both *T. paradoxa* and *T. ethacetica*. However, further exploration into the mechanisms underpinning the transfer of the virus between these two isolates is warranted to fully elucidate this phenomenon.

The remaining +ssRNA virus CpRV-2, showed similarity to members of the viral family *Endornaviridae*. Notably, viruses within this family are recognized for their large genomes, ranging from 9.7 to 17.6 Kb [81]. The genomes encode for a single polyprotein, which always contains an RdRp domain, and may also encode for other proteins such as viral helicases, methyl transferases, glycosyl transferases, and capsular polysaccharide synthases [81]. However, the putative endornavirus CpRV-2 was associated with a contig only 857 nucleotides in length. Additionally, this genome fragment did not yield significant alignments with any known proteins when subjected to a Blast analysis. It was thus not possible to include CpRV-2 in either the phylogenetic analysis or genomic characterization. The reason for the truncation of the CpRV-2 genome might be linked to the selection strategy employed during library preparation. While precise details regarding the library preparation method for the *C. platani* isolate is limited, it is conceivable that poly-A selection might have been utilized to generate sequencing libraries (mRNA-sequencing). This method is inclined toward capturing RNA molecules with poly-A tails [82]. Given that endornaviruses typically lack a poly-A tail [83-85], their representation in these datasets may have been reduced. One effective approach to address this challenge involves incorporating a ribo-depletion step during library selection (RNA-sequencing). This method specifically targets and eliminates ribosomal RNA, the predominant component in total RNA [82]. This approach thus allows for the enrichment of non-ribosomal RNA molecules and will not exclude mycoviruses without a poly-A tail [82]. Another potential explanation is that CpRV-2 might have been present in low abundance within the fungal host, resulting in insufficient genome coverage during sequencing.

The study also unveiled the presence of four mycoviruses consisting of dsRNA genomes, all of which exhibited homology to existing members of the viral family *Totiviridae*. The putative totivirus genomes originating from the *Huntiella* datasets, namely HoRV-1 and HoRV-3, were likely truncated. These putative totiviruses featured genome sizes falling below the established range of 4.6 to 7.0 kb range typically observed among members of the *Totiviridae* family [11]. As previously discussed, the truncation of contigs could potentially be attributed to the selection step utilized in library preparation, and low viral abundance in the host cells. However, in the case of HoRV-1 and 3, it's important to acknowledge the potential influence of the chosen sequencing platform, specifically Ion Torrent Proton. This platform introduces limitations and challenges which may negatively impact the assembly of full length contigs. Of notable concern is the presence of sequence errors, particularly those associated with homopolymers, which represents a recognized limitation inherent to Ion Torrent sequencing technology [86]. These sequence errors, often manifested as insertions or deletions in regions with consecutive identical nucleotides, can cause frameshifts or create gaps within the assembled sequences [87]. Consequently, these errors result can in truncated contigs which do not represent the full viral genome.

Two partial totiviral genomes, HoRV-1 and Ho_RNA_Seg_1, each featured a single ORF, encoding an RdRp and a CP, respectively. The genomes of totiviruses are usually nonsegmented and consist of two ORFs which encode for a CP and RdRp protein, respectively [11]. It may be possible that HoRV-1 and Ho_RNA_Seg_1 potentially represent the same virus, which was not assembled into a complete genome. The separation of the two ORFs might be attributed to the influence of complex secondary structures impeding the reverse transcription step during library preparation. It's worth noting that a pseudoknot, frequently situated upstream of the RdRp-containing ORF, aids in stop/restart translation of most victoriviruses [88]. Remarkably, the CP-containing ORF of Ho_RNA_Seg_1 lacks a complete domain for the CP and an "AUGA" overlap region [88]. This tetranucleotide sequence normally lies upstream of the pseudoknot and acts as the initiation-termination codon which plays a role in the translation of the downstream RdRp containing ORF [88]. This raises the possibility of a pseudoknot-like structure within the genome of this virus, potentially contributing to the premature truncation of the CP protein and its separation from the RdRp containing ORF. It is essential to acknowledge however, that the coverage for these genomes was relatively low (9.22 for HoRV-1 and 27.88 for Ho_RNA_Seg_1). Therefore, it's also possible that the assembler could not bridge the gap between these two contigs due to insufficient data.

The remaining totivirus, TpRV-2 was the only dsRNA virus with a genome conforming to the expected genome size and organization of members within the *Totiviridae* family. Phylogenetic analysis revealed that TpRV-2 clustered with members of the genus *Victorivirus* and could thus be a member of this genus. Similar to other members of this genus, the genome of TpRV-2 contains an H-type pseudoknot structure upstream of the RdRp encoding ORF (which is essential for the translation of this protein) and contains a UAAUG overlap region [89-91]. The requirement for species demarcation within this genus is an amino acid sequence identity percent of less than 60% with other closely related viruses at the RdRp and Cp protein level [57]. Since TpRV-2 meets these requirements, it is likely a novel member of the genus *Victorivirus*.

Despite the absence of an ORF encoding a CP, HoRV-1 was included in phylogenetic analysis due the presence of a complete ORF encoding for an RdRp domain. This putative totivirus was also found to cluster with members of the genus *Victorivirus* and had an amino acid sequence identity less than 60% to that of related victoriviruses at the RdRp level. However, a complete genome is required to confirm this.

Overall, this study enhances our understanding of mycoviral diversity within the fungal family Ceratocystidaceae. However, it should be noted that this study's scope was limited to publicly available transcriptomes, which resulted in the analysis of only a subset of species within the Ceratocystidaceae family. To gain a more comprehensive understanding of mycoviral diversity, acquiring additional sequencing data for a broader range of family members would be advantageous. Nevertheless, the present study has identified novel mycoviruses in 4 additional species from this family, namely *C. platani*, *H. omanensis*, *T. paradoxa* and *T. ethacetica*.

Notably, *C. platani*, *T. paradoxa*, and *T. ethacetica* are significant fungal pathogens of plants, known for their detrimental impact on economically important crops and trees [38, 74, 77, 9295]. Several studies have shown that mycoviruses show potential as biocontrol agents against plant pathogenic fungi, due to the ability of some to induce hypovirulence in the host [96-99]. Therefore, the identification of mycoviruses within these fungal species may contribute to the future development of biocontrol strategies against them. Interestingly, the majority of mycoviruses that were present in these genera were mitoviruses. Several studies have shown that some mitoviruses possess the capability to induce hypovirulence in their plant pathogenic hosts [71, 73, 100]. Research by Shackelton and Holmes [101] suggests that hypovirulent mitoviruses might have originated from plant hosts, which deployed them as a strategic defence

mechanism against invasive fungal threats. Studies have also characterized hypovirulence inducing mycoviruses in the families *Totiviridae* and *Endornaviridae* [102-105]. It is thus possible that the mycoviruses from this study may also confer hypovirulence to their plant pathogenic fungal hosts, although this will have to be evaluated in future research.

Conclusion

In summary, this investigation has enhanced our knowledge of the mycoviral landscape within the relatively unexplored fungal family Ceratocystidaceae. The study marked the discovery of the first mycoviruses in several fungal species, including *C. platani, T. ethacetica*, *T. paradoxa*, and two *H. omanensis* isolates. The utilization of fungal transcriptomes from publicly available databases proved to be a valuable approach, resulting in the analysis of 10 fungal transcriptomes and the identification of nine novel mycoviruses. These were primarily +ssRNA viruses, mainly belonging to the *Mitoviridae* and *Endornaviridae* viral families. Additionally, three mycoviruses with dsRNA genomes from the *Totiviridae* family were identified, with phylogenetic analysis conducted for two of these. However, limitations included restricted transcriptomic datasets, the utilization of mRNA-seq for the majority of fungal isolates evaluated, and the need for further molecular techniques to confirm and explore these mycoviruses in their original hosts. Future research should assess the impact of these mycoviruses on their fungal hosts and consider their potential as biocontrol agents for the fungal isolates examined in this study.

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Supplementary Information

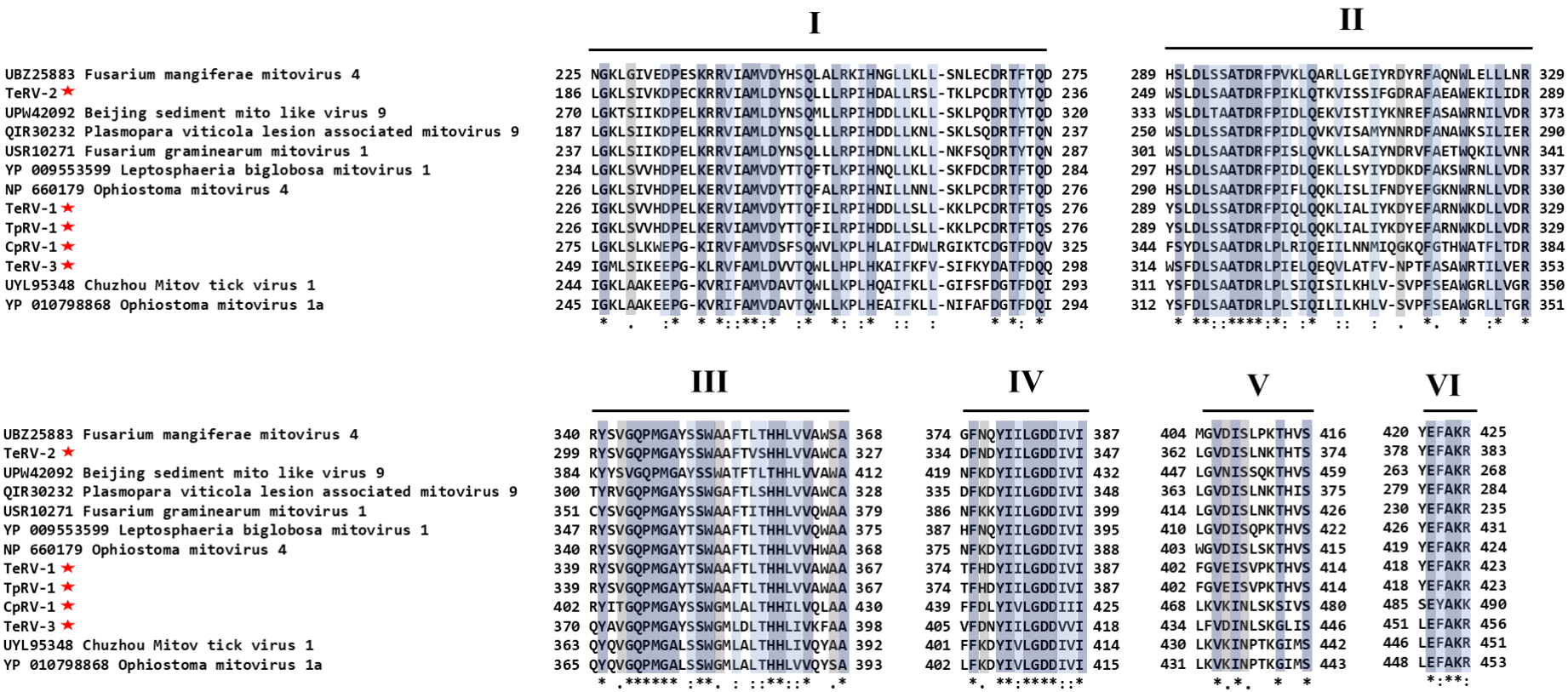


Fig. S1 Multiple sequence alignment of the RdRp amino acid sequences from the mitoviruses investigated in Chapter 2 (Indicated by the red stars), and other selected viruses from *Mitoviridae*. The alignment was generated using Clustal Omega 1.2.1 with default parameters. Conserved residues are denoted by an asterisk (*) and are highlighted in dark blue. Colons (:) and dots (.) signify conserved and semiconserved residues, respectively, and are coloured light blue and grey. There were 6 conserved motifs in total, and these are numbered from I to VI.

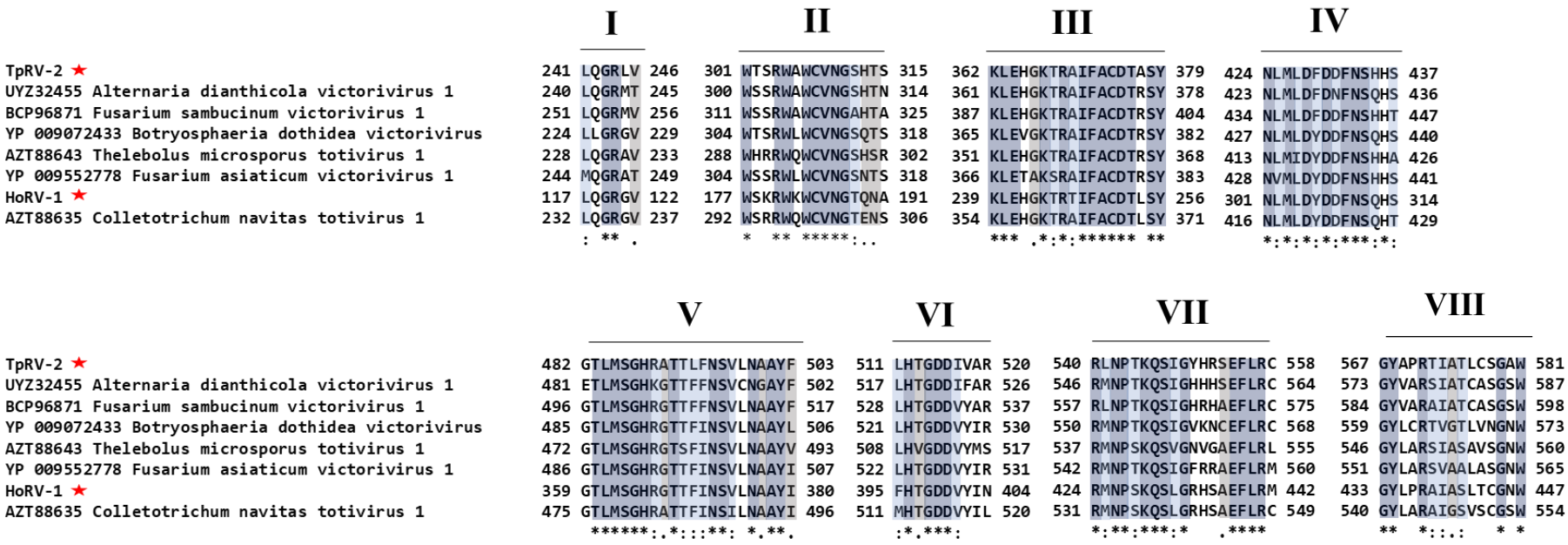


Fig. S2 Multiple sequence alignment of the RdRp amino acid Sequences from the totiviruses investigated in this study (Indicated by the red stars), and other selected viruses from the genus *Victorivirus*. The alignment was generated using Clustal Omega 1.2.1 with default parameters. Conserved residues are denoted by an asterisk (*) and are highlighted in dark blue. Colons (:) and dots (.) signify conserved and semi-conserved residues, respectively, and are coloured light blue and grey. There were 8 conserved motifs in total, and these are numbered from I to VIII.

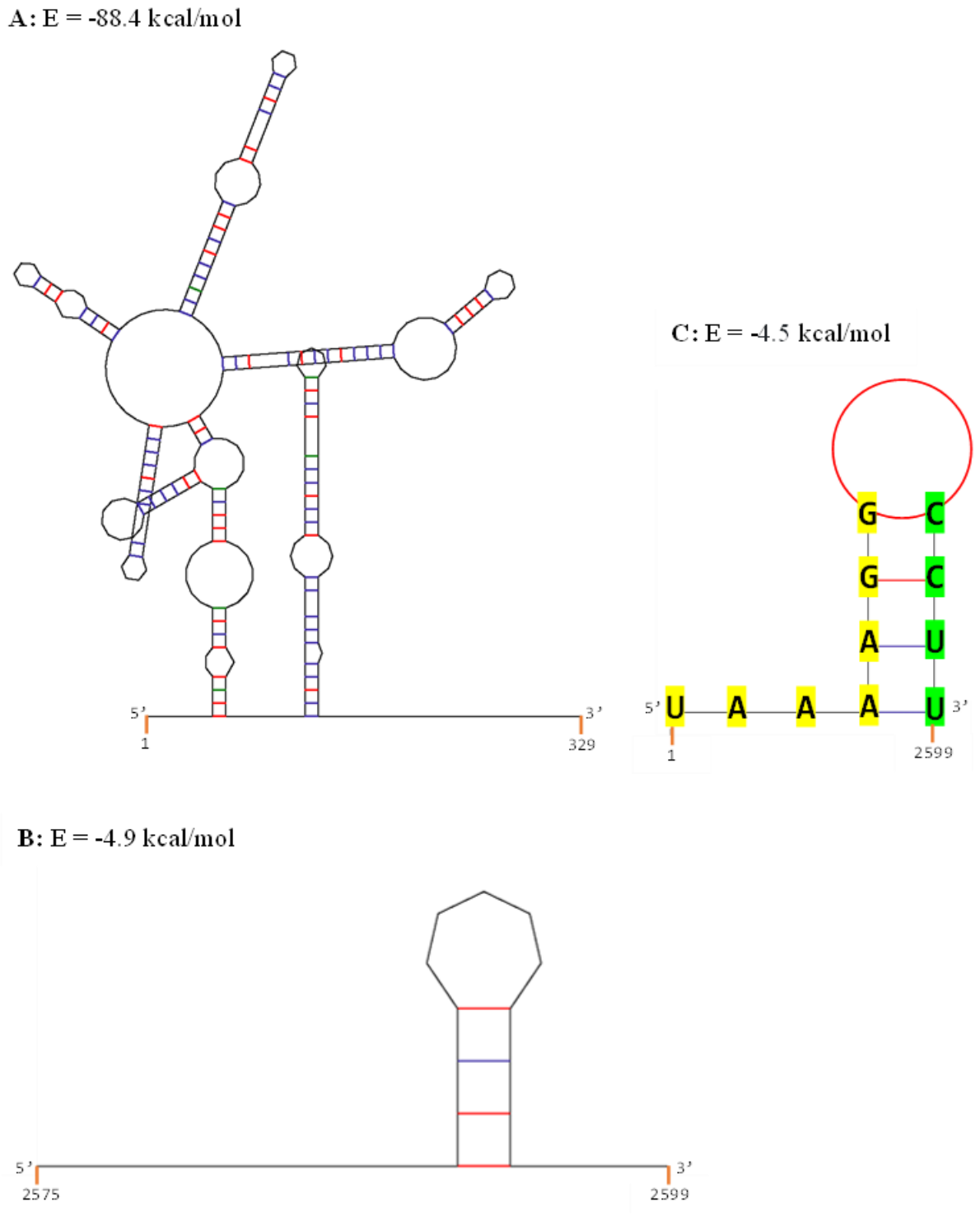


Fig. S3 Predicted secondary structures of the 5' (A) and 3' (B) terminal UTRs of TpRV-1, as well as the predicted panhandle structure (C) formed through complementary pairing between the two UTRs. Nucleotide positions 1 and 2599 represent the first and last nucleotide in the 5' and 3' UTRs, respectively. In the panhandle structure, complementary bases between sections of the 5' and 3' UTRs are highlighted: yellow for the 5' UTR and green for the 3' UTR. For the 3' UTR, complementary base pairing starts at position 2596, and ends at position 2599. Complementary base pairing for the 5' UTR starts at position 4 and ends at position 7. The red circle (not drawn to scale) represents the inner RNA Molecule. The secondary structures were predicted using Mfold.

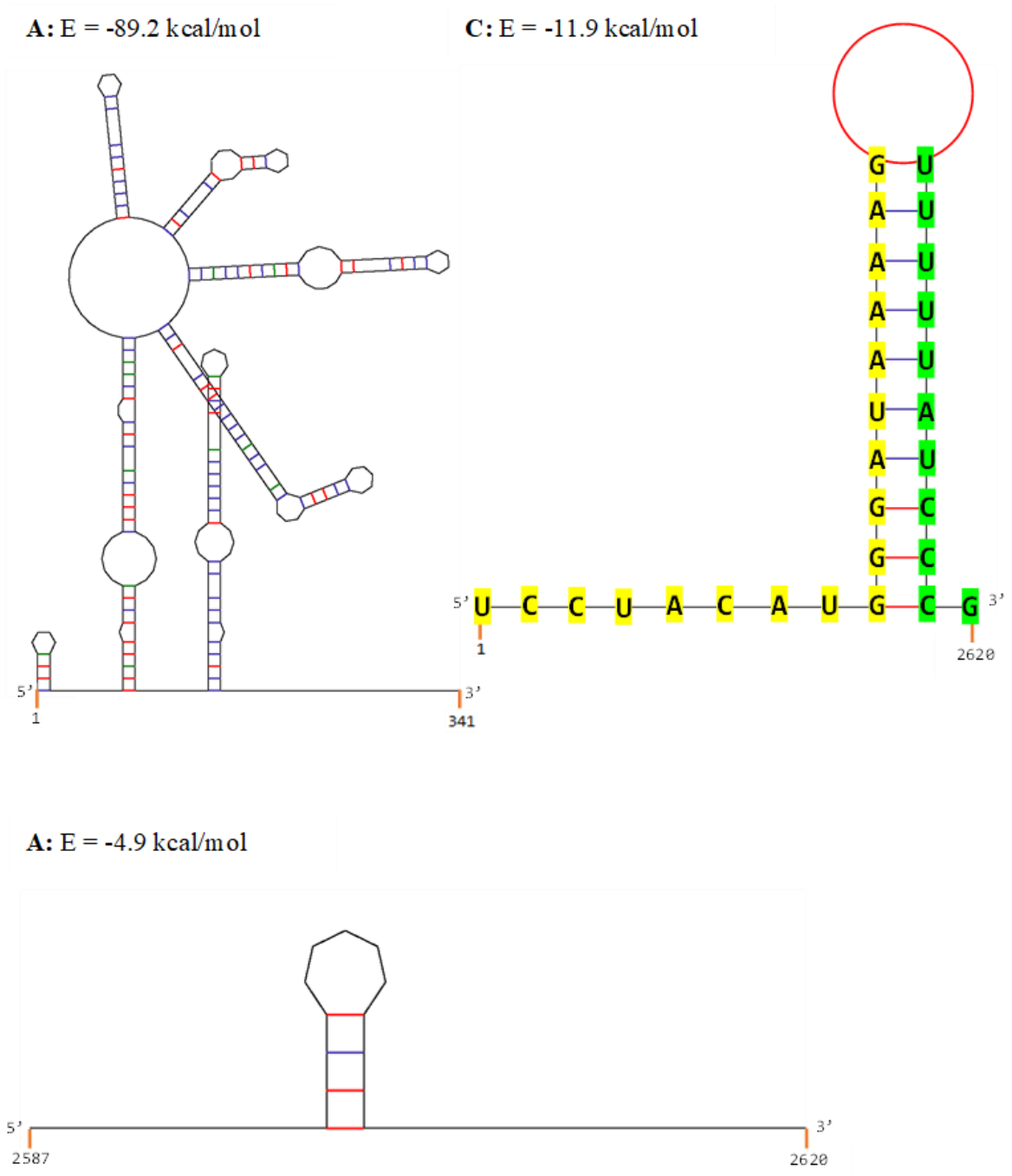


Fig. S4 Predicted secondary structures of the 5' (A) and 3' (B) terminal UTRs of TeRV-1, as well as the predicted panhandle structure (C) formed through complementary pairing between the two UTRs. Nucleotide positions 1 and 2620 represent the first and last nucleotide in the 5' and 3' UTRs, respectively. In the panhandle structure, complementary bases between sections of the 5' and 3' UTRs are highlighted: yellow for the 5' UTR and green for the 3' UTR. For the 3' UTR, complementary base pairing starts at position 2610, and ends at position 2619. Complementary base pairing for the 5' UTR starts at position 9 and ends at position 18. The red circle (not drawn to scale) represents the inner RNA Molecule. The secondary structures were predicted using Mfold.

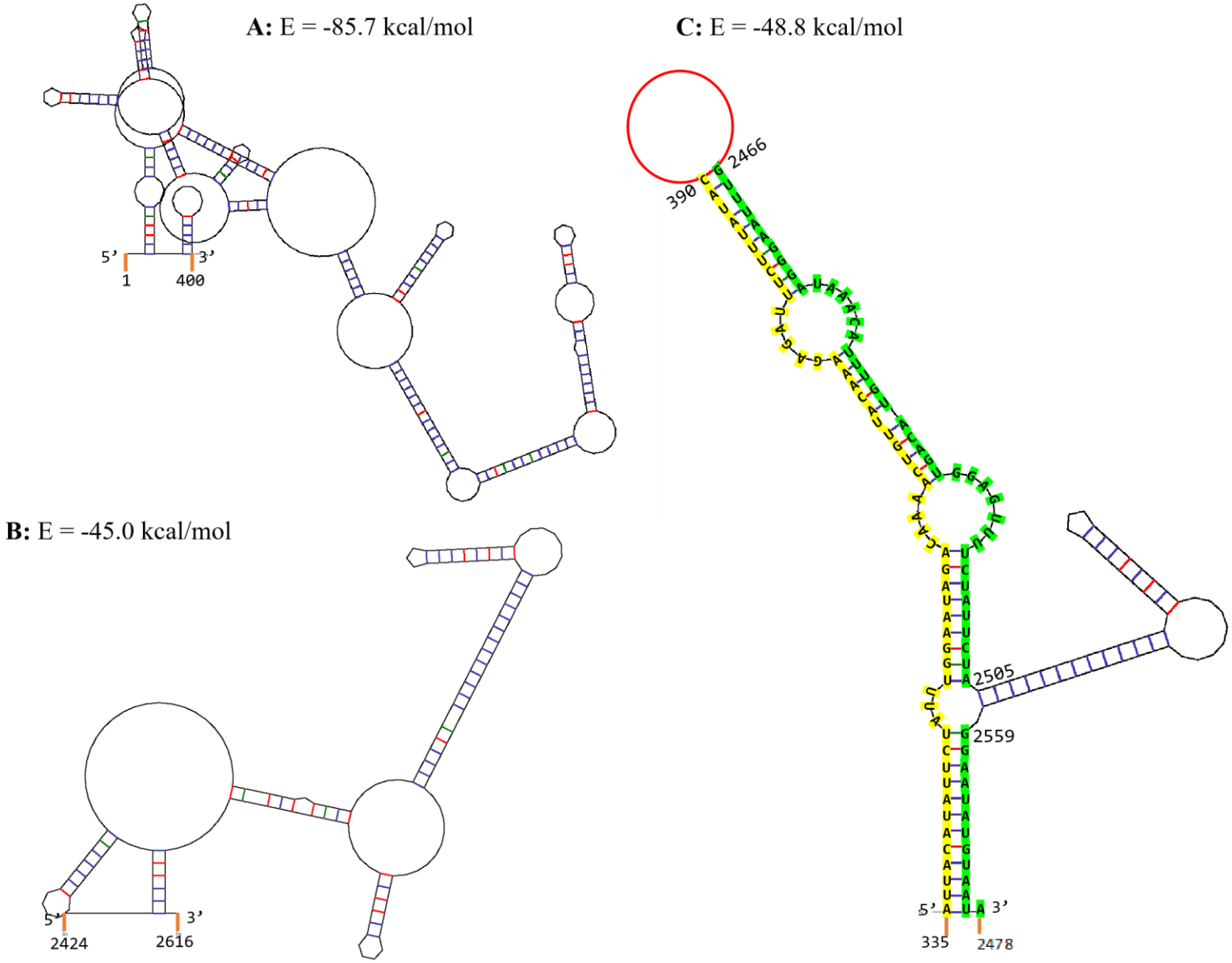


Fig. S5 Predicted secondary structures of the TeRV-2 5' (A) and 3' (B) terminal UTRs, as well as the predicted panhandle structure (C) formed through complementary pairing between the two UTRs. In the panhandle structure, complementary bases between sections of the 5' and 3' UTRs are highlighted: yellow for the 5' UTR and green for the 3' UTR. Nucleotide position 1 and 2616 represent the first and last nucleotide in the 5' and 3' UTRs, respectively. For the 3' UTR, complementary base pairing starts at position 2466, and ends at position 2505. This is followed by self-complementarity and the formation of secondary structures of the 3' terminal end, which ends at position 2559. Complementary base pairing between the 3' UTR and the 5' UTR then continues and ends at position 2477. Complementary base pairing for the 5' UTR starts at position 335 and ends at position 390. The red circle (not drawn to scale) represents the rest of the inner RA molecule. The secondary structures were predicted using Mfold.

A: E = -109.6 kcal/mol

B: E = -80.1 kcal/mol

C: E = -28.3 kcal/mol

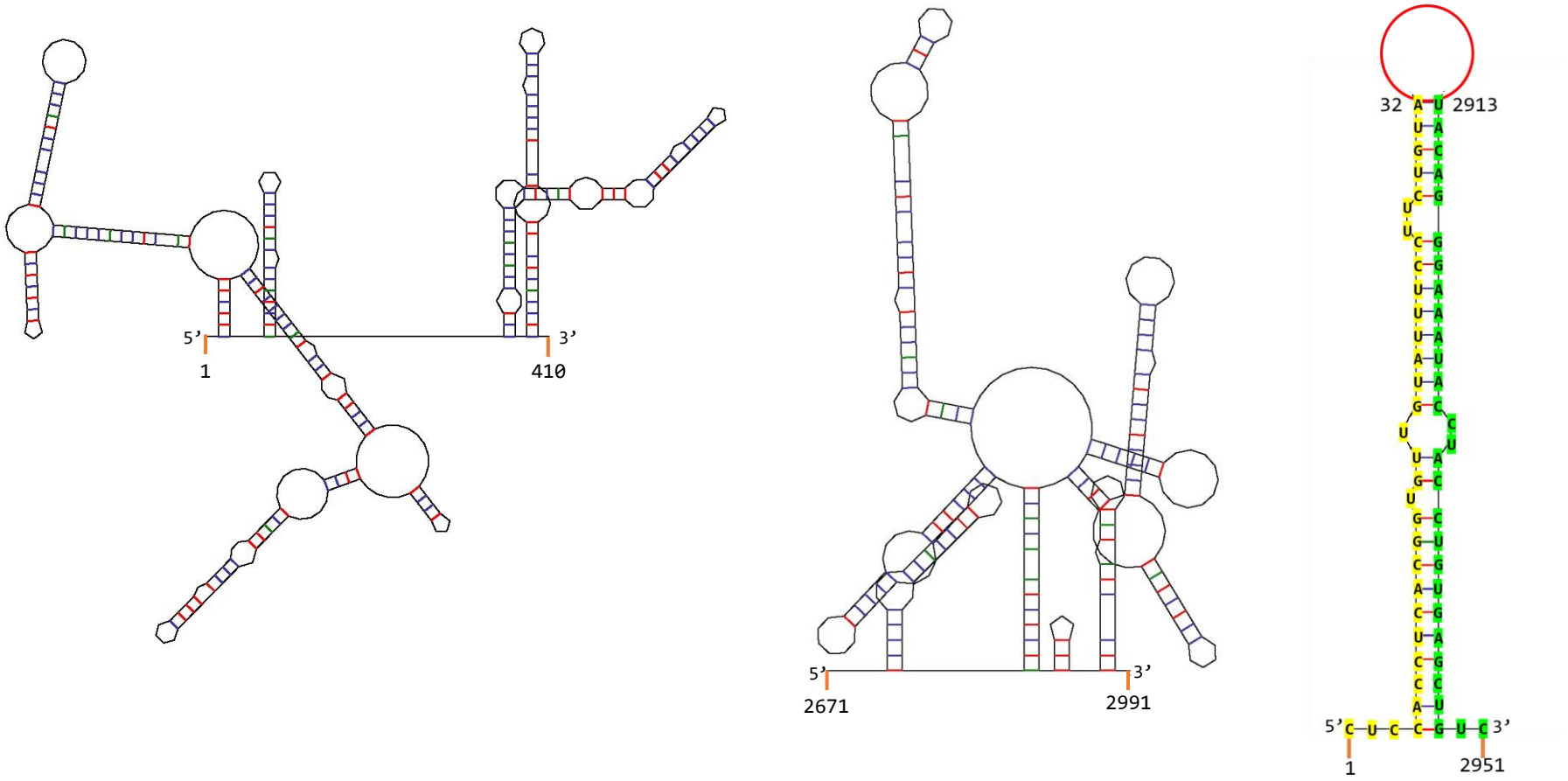


Fig. S6 Predicted secondary structures of the TeRV-3 5' (A) and 3' (B) terminal UTRs, as well as the predicted panhandle structure (C) formed through complementary pairing between the two UTRs. In the panhandle structure, complementary bases between sections of the 5' and 3' UTRs are highlighted: yellow for the 5' UTR and green for the 3' UTR. Nucleotide positions 1 and 2991 represent the first and last nucleotide in the 5’ and 3’ UTRs, respectively. For the 3’ UTR, complementary base pairing starts at position 2913, and ends at position 2949. Complementary base pairing for the 5’ UTR starts at position 4 and ends at position 32. The red circle (not drawn to scale) represents the rest of the inner RNA molecule. The secondary structures were predicted using Mfold.

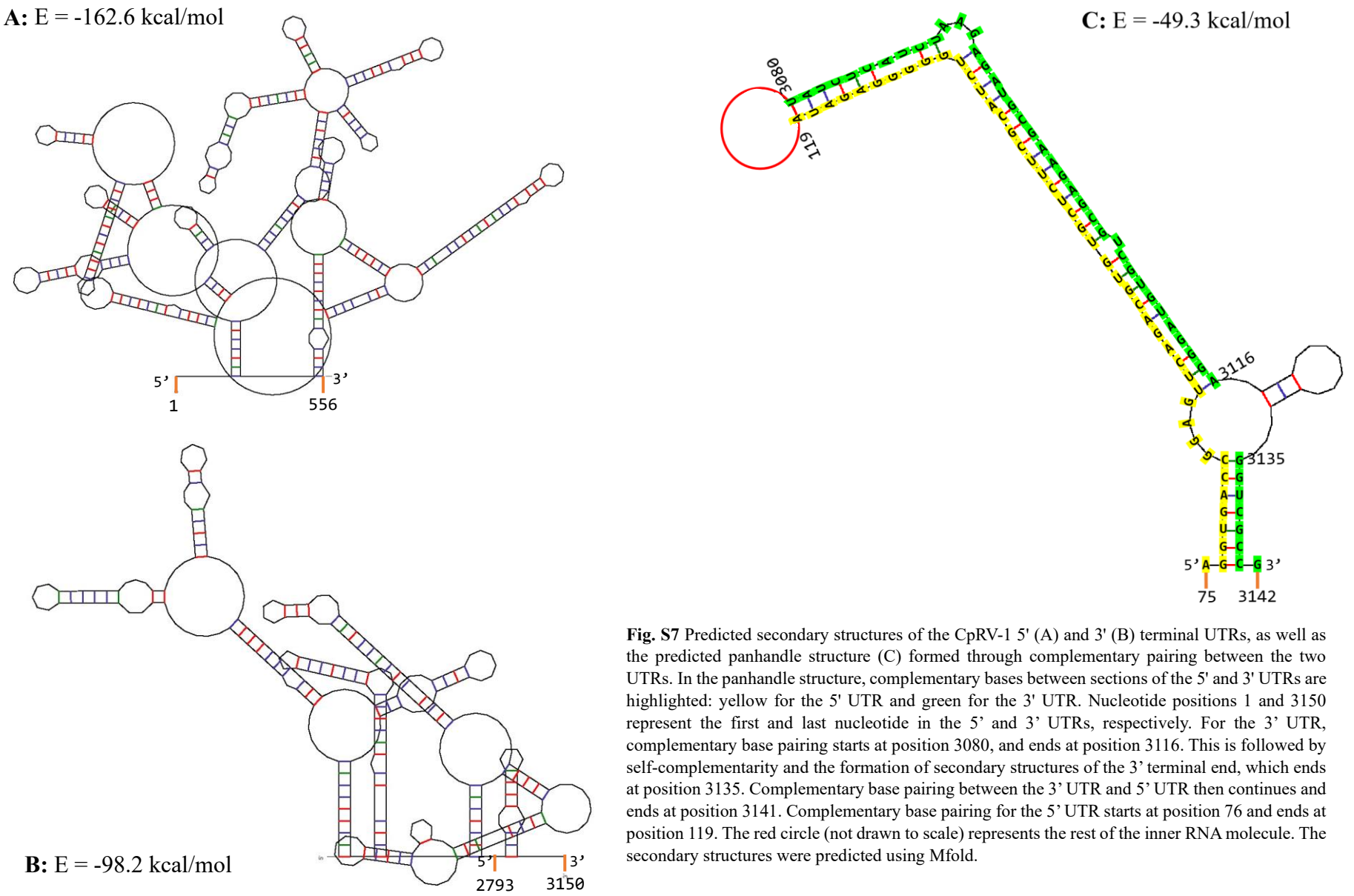


Fig. S7 Predicted secondary structures of the CpRV-1 5' (A) and 3' (B) terminal UTRs, as well as the predicted panhandle structure (C) formed through complementary pairing between the two UTRs. In the panhandle structure, complementary bases between sections of the 5' and 3' UTRs are highlighted: yellow for the 5' UTR and green for the 3' UTR. Nucleotide positions 1 and 3150 represent the first and last nucleotide in the 5' and 3' UTRs, respectively. For the 3' UTR, complementary base pairing starts at position 3080, and ends at position 3116. This is followed by self-complementarity and the formation of secondary structures of the 3' terminal end, which ends at position 3135. Complementary base pairing between the 3' UTR and 5' UTR then continues and ends at position 3141. Complementary base pairing for the 5' UTR starts at position 76 and ends at position 119. The red circle (not drawn to scale) represents the rest of the inner RNA molecule. The secondary structures were predicted using Mfold.

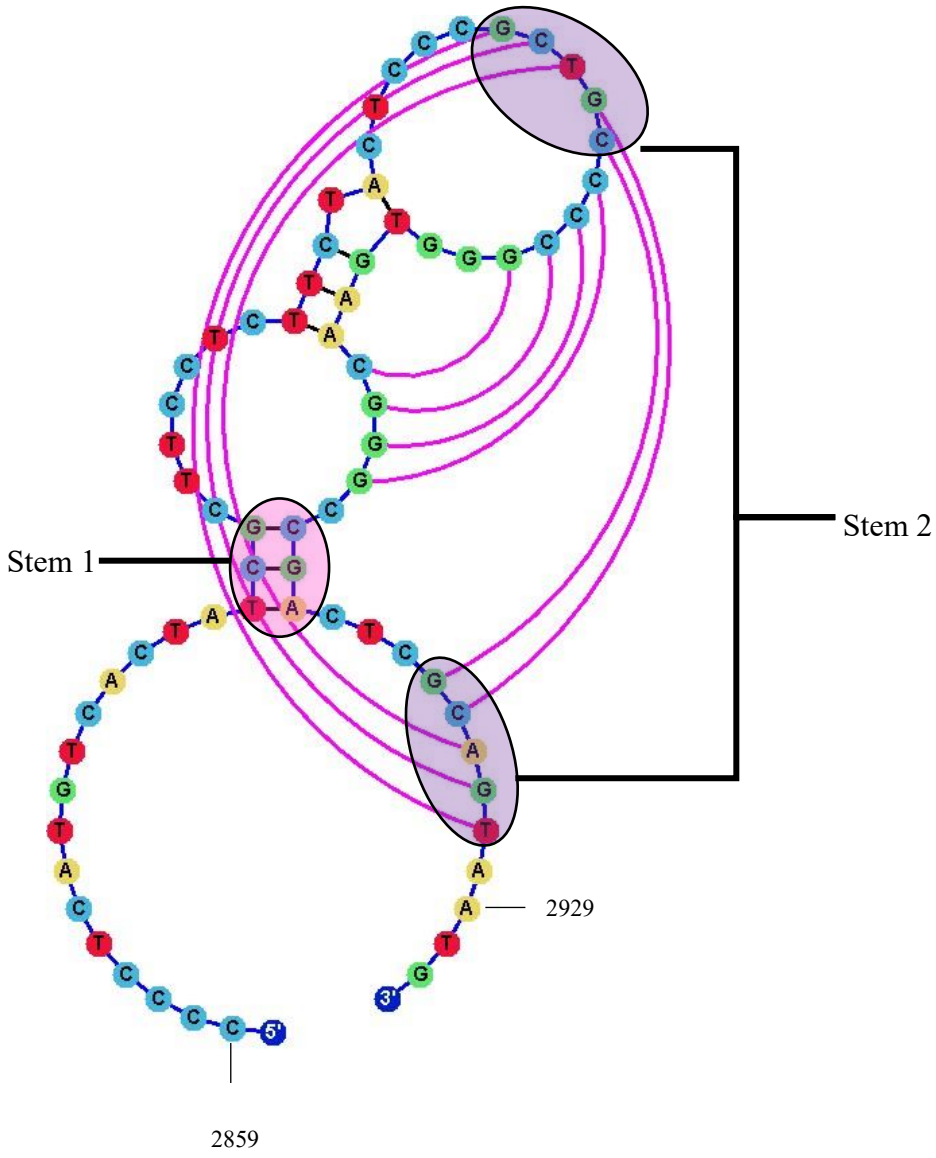


Fig S8. Predicted pseudoknot structure spanning the stop-start region of TpRV-2. The nucleotide positions are indicated, and Stem 1 and Stem 2 are indicated by purple and pink ovals, respectively. Structures were predicted with ProbKnot and visualized with jViz.RNA 4.0.

Chapter 3: Mycovirus Profiling in Ribo-Depleted Transcriptomes from the Fungal Genus *Ceratocystis*

Abstract

Mycoviruses hold significant importance in the realm of plant pathology, due to the ability of some to attenuate the pathogenicity of their plant-pathogenic fungal hosts. Extensive research has been devoted to investigating and characterizing mycoviruses in plant-pathogenic fungi. *Ceratocystis*, a fungal genus within the Ceratocystidaceae family, encompasses several economically important plant pathogens but has been relatively understudied in the context of mycoviruses. By analysing four fungal datasets from *Ceratocystis*, which included a ribosomal depletion step during library preparation, we sought to elucidate mycoviral diversity within this genus. The use of ribo-depletion in RNA-sequencing, in contrast to the standard poly-A selection method used in mRNA-sequencing, has proven to be more effective in uncovering mycoviruses that lack a poly-A tail. Within the framework of this investigation, ribo-depletion proved to be an invaluable tool for resolving the complete genome of three endornaviruses in *C. fimbriata*, whose genomes had remained unresolved in prior research efforts. Apart from the three endornaviruses, an additional 15 mycoviruses were discovered in this investigation. Among these, 14 were tentatively assigned to viral families, namely *Endornaviridae* (*Alphaendornavirus* genus), *Mitoviridae* (*Unuamitovirus* and *Duamitovirus* genera), *Mymonaviridae*, and *Totiviridae* (*Victorivirus* genus). Additionally, one of the identified mycoviruses remains unclassified but exhibits virgalike characteristics. This research represents the first identification of mycoviruses in *C. eucalypticola*, *C. manginecans*, and *C. albifundus*. Moreover, this research revealed the existence of identical mitoviruses in *C. fimbriata, C. manginecans,* and *C. eucalypticola*. This discovery is particularly surprising because these fungi infect different plant hosts. This study makes a substantial contribution to our knowledge of mycoviruses in *Ceratocystis* and, by extension, the family Ceratocystidaceae. The insights gained from this research could potentially lay the foundation for future investigations focused on developing biocontrol methods to address the challenges posed by these fungi.

Introduction

The fungal family Ceratocystidaceae encompasses numerous genera that house fungi of significant economic importance [1-5]. Among these is the genus *Ceratocystis*, which contains several members that cause economic losses in natural forests and agricultural crops

worldwide. Notable examples include *Ceratocystis fimbriata*, responsible for black rot in sweet potatoes [6]; *Ceratocystis manginecans*, which induces sudden decline disease in mango trees [7]; *Ceratocystis eucalypticola*, causal agent of ceratocystis wilt in *Eucalyptus* [8]; and *Ceratocystis albifundus*, responsible for wattle wilt of Acacia trees [9]. Conventionally, plant pathogenic fungi have been managed through the use of synthetic fungicides [10-13]. As a result of increased fungal resistance however, these approaches are becoming less effective [14-18]. Additionally, mounting concerns over the harmful effects of synthetic fungicides on the environment and human health [19-21] have prompted the search for alternatives [22-24]. One approach that has been suggested involves the use of mycoviruses (viruses that infect fungi) as biocontrol agents against their plant pathogenic fungal hosts [25-27].

The most widely recognized example of successful mycovirus-mediated biocontrol has been the use of members of the species *Alphahypovirus cryphonectriae* (Cryphonectria hypovirus 1; CHV-1) to combat the chestnut blight pathogen, *Cryphonectria parasitica*, in Europe [28]. However, employing mycoviruses as biocontrol agents comes with its own set of challenges. While some single stranded DNA mycoviruses, such as those from the species *Gemycircularvirus sclero1* (Sclerotinia sclerotiorum hypovirulence associated DNA virus 1; SsHADV-1) [29], are the exceptions, the majority of mycoviruses are not infectious in the traditional sense and lack an extracellular infection route [30]. Instead, they disseminate intracellularly through hyphal anastomosis, which involves the fusion of hyphae from vegetatively compatible fungi [30]. Consequently, it is challenging for mycoviruses to achieve successful transmission between fungi that are vegetatively incompatible. Nonetheless, researchers are actively seeking solutions to this issue, and have made a lot of progress in this regard (For in-depth reviews see [31-33]). Mycovirus-mediated biocontrol of a broad spectrum of plant pathogenic fungi could thus be realized in the future. Consequently, the discovery and identification of mycoviruses in plant pathogenic fungi has become the central focus of mycovirus related research.

To detect mycoviruses within their fungal hosts, numerous methods have been employed. Culture-based approaches like cellulose chromatography have traditionally been used to purify mycoviral double-stranded RNA (dsRNA). Subsequently, these dsRNA molecules could be visualized through agarose gel electrophoresis [34-36]. However, this approach leans heavily on the premise that most mycoviruses contain either a double-stranded dsRNA genome or possess a dsRNA intermediate. Consequently, mycoviruses with single-stranded RNA (ssRNA) genomes have been inadequately represented in the past due to the less efficient

purification of their dsRNA intermediates as compared to the dsRNA genomes of dsRNA viruses [37]. Additionally, this approach proves less effective in detecting mycoviruses present in low abundance [38].

More recently, the landscape of mycovirus discovery has been dramatically reshaped by the rapid advancements in Next Generation Sequencing (NGS). Now, with the aid of a suitable bioinformatics pipeline, mycoviruses can be discerned within the transcriptomes of their fungal hosts, providing a more comprehensive and efficient approach to their detection and characterization [39-41]. Furthermore, these advances have made it possible to characterize mycoviruses using publicly available fungal transcriptomes, eliminating the need for traditional culturing [39, 40, 42-44]. The use of such datasets comes with inherent limitations however, as they were originally created for different purposes and may not be optimized for mycovirus discovery. For example, the specific selection step employed during the creation of sequencing libraries could potentially influence mycovirus discovery within a given dataset. The majority of these datasets typically include a poly-A selection step during library preparation, which could exclude viruses that lack poly-A tails [39, 45]. Therefore, a more advantageous approach might involve utilizing datasets where ribo-depletion was employed during library construction. This technique effectively removes ribosomal RNA, which makes up a significant portion of total RNA, while retaining mRNA and viral RNA [46].

In chapter 2, publicly available transcriptomes from members of *Ceratocystidaceae* were evaluated for the presence of mycoviruses. The primary aim of that study was to provide insights into the extent of mycoviral diversity within this particular family. The primary aim of this study was to expand on this, by identifying and characterizing mycoviruses in the transcriptomes from four isolates from species in the genus *Ceratocystis*. Unlike the datasets obtained from public repositories, these transcriptomes have been generated with ribodepletion during the library selection step, in an attempt to reduce any potential biases.

Methods

Procurement of cultures

To assess mycoviral diversity within *Ceratocystis*, three existing datasets were obtained from a fellow researcher. These datasets incorporated ribodepletion during the library selection step, and included *Ceratocystis fimbriata* CMW 14799, *Ceratocystis manginecans* CMW 46461, and *Ceratocystis eucalypticola* CMW 53160. Additionally, cultures of both *C. fimbriata* CMW 14799 and *Ceratocystis albifundus* CMW 4068 were sourced from the fungal culture collection

(CMW) at the Forestry and Agriculture Biotechnology Institute (FABI). To augment the available datasets, the cultures from *C. albifundus* were processed and sent for high throughput sequencing. The *C. fimbriata* culture, however, was specifically procured for the purpose of viral confirmation and terminal nucleotide determination of a select number of mycoviruses in the original culture.

Fungal culturing and RNA extraction

The fungal isolates obtained from the FABI CMW culture collection, *C. fimbriata* CMW 14799 and *C. albifundus* CMW 4068, were cultivated on 2% malt extract agar (MEA) plates, which was supplemented with 150 mg/L streptomycin and covered with a cellophane overlay. When the desired biomass was reached, fungal mycelia were carefully harvested and subsequently ground into a fine powder by using a sterile mortar and liquid nitrogen. Total RNA was then extracted from this material using the RNeasy Plant Minikit (Qiagen, Valencia, CA), according to manufacturer instructions. For the datasets obtained from a fellow researcher (*C. manginecans* CMW 46461, *C. fimbriata* CMW 14799 and *C. eucalypticola* CMW 53160), total RNA was extracted from the original fungal cultures by using a modified version of the CTAB protocol [47].

Generation of RNA sequencing library and NGS sequencing

Paired-end sequencing libraries were generated by employing ribosomal RNA (rRNA) depletion techniques for all cultures. The Zymo-seq RiboFree Total RNA library kit (Zymo Research, Irvine, CA) was used for library construction of *C. albifundus* CMW 4068, using 500 ng of total RNA extract as input. Subsequently, these libraries were subjected to sequencing using an Illumina NextSeq 2000 instrument (University of Leeds, UK). Total RNA from *C. fimbriata* CMW 14799, *C. manginecans* CMW 46461, and *C. eucalypticola* CMW 53160 were sent to Macrogen Europe for sequencing. The TruSeq Stranded Total RNA with Ribo-Zero H/M/R_Gold kit (Illumina, San Diego, CA) was used to construct libraries for these isolates, and subsequent sequencing was carried out on an Illumina NovaSeq 2000 platform. To ensure data integrity, the quality of these datasets was assessed using FastQC [48] and trimmed with Trimmomatic (version 0.36) [49], with a minimum Phred score of 20.

Bioinformatics pipeline for the discovery of mycoviruses

The trimmed datasets were de novo assembled with the rnaviralSPAdes software (version 3.15.0) [50]. The resulting contigs were then imported into CLC genomics workbench 22 (Qiagen Bioinformatics, Aarhus, Denmark), where they were translated from nucleotide

sequences to amino acid sequences, in all possible reading frames, with the 'Translate to Protein' tool. A custom viral database was subsequently created using viral protein sequences from the NCBI protein database, covering all known viral families with mycoviruses as well as unclassified mycoviruses. This database was established in March 2022 and was regularly updated to include newly established viral families and genera, with the latest update in April 2023. The BLAST toolkit within CLC genomics workbench was used to align the assembled contigs to viral proteins within this custom database, and putative mycoviral contigs were then selected for downstream analysis based on alignment length and E-value (E-value <10-15). This E-value cutoff was selected because any hits with higher E-values did not correspond to actual viruses but rather corresponded to fungal proteins. To differentiate real viral sequences from fungal host sequences, the chosen contigs underwent BLAST analysis against a nonredundant protein sequence database using NCBI's BLASTp tool [51].

Genomic characterization and phylogenetic analysis of mycoviruses

The open reading frames (ORFs) of putative mycoviruses were determined using the National Center for Biotechnology Information (NCBI) ORF finder [52]. The mitochondrial genetic code was used to identify ORFs in putative mycoviruses that exhibited similarities to mitoviruses or narnaviruses, while the conventional genetic code was used for other viruses. For mitoviruses, the secondary 5' and 3' untranslated regions (UTRs) were predicted using Mfold [53]. The conserved protein domains within the ORFs of mycoviruses were subsequently identified using the NCBI BLASTp tool. To conduct phylogenetic analysis, the ORFs containing RNA-dependent RNA polymerase (RdRp) from these mycoviruses were aligned with closely related viral protein sequences obtained from NCBI GenBank. The alignments were carried out using the ClustalW program within MEGA 11 [54]. Additionally, MEGA 11 was employed to select the optimal protein model for each alignment. Using these alignments as input data, Maximum Likelihood (ML) phylogenetic trees were constructed with IQ-TREE (version 2.1.3), utilizing the appropriate model for the analysis [55]. To identify conserved motifs within the RdRp amino acid sequences of characterized mycoviruses and closely related viruses, multiple sequence alignments were created using the web based ClustalOmega tool [56]. This tool was also used to generate percent identity matrices based on these alignments, facilitating the comparison of sequence similarity between viruses. Where applicable, the NCBI Blastn tool was also used to compare overall nucleotide percent identities of any potential endornaviruses from this study.

Determination of the 3' and 5' UTRs of CfRV-1, CfRV-2 and CfRV-3

To confirm the 5' terminal ends of 3 endornaviruses in *C. fimbriata* CMW 14799, the 5' RACE System for Rapid Amplification of cDNA Ends (RACE), version 2.0 (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturers specifications. The 3' terminal ends were also confirmed, by use of the 3' RACE System for Rapid Amplification of cDNA Ends, version 2.0 (Invitrogen, Carlsbad, CA, USA). Primer sequences for these RACE PCRs were manually determined by analysing the viral sequences on CLC genomics workbench. Subsequently, the these were submitted to Inqaba Biotech (Inqaba Biotechnical Industries, Muckleneuk, Pretoria, South Africa) for primer synthesis.

For the 5' RACE PCR, the following gene-specific primers were used: For Ceratocystis fimbriata RNA Virus 1, CfRV1-GSP1 (5'-AGT CAG CTG CTC GGT GAT TGA AAT GC-3') and CfRV1-GSP2 (5'-GAC TGT GTG GAC GCA CTC TTT GC-3'). For Ceratocystis fimbriata RNA Virus 2, CfRV2-GSP1 (5'-GGA CAC CTC CCT TGT GTT TGA ATG C-3') and CfRV2-GSP2 (5'-CCC TGA TCT GGA CCG TTT GCT TTG C-3'). For Ceratocystis fimbriata RNA Virus 3, CfRV3-GSP1 (5'-AGC CAT TTG CTG AGA CTT GGA TTG C-3') and CfRV3-GSP2 (5'-TCT GGC GTT TTG TAA TCC AGT GTG CTT -3').

For the 3' RACE PCR. poly-A tailing was initially performed on the input RNA using *E. coli* Poly (A) Polymerase (New England Biolabs, Ipswich, MA, USA), following the manufacturer's specifications. This step was necessary as endornaviruses typically lack a 3' poly-A tail, which is required for attachment of the oligo(dT)-containing adapter primer in the 3' RACE PCR reaction. The gene specific primers used in the 3' RACE PCR were CfRV1-GSPA for CfRV1 (5'-AAG CCA CCG TCG AAA ACC TGC AA-3'), CfRV2-GSPB for CfRV2 (5'-CGA CAA CCT GAT GCT GGT TGA AAA GCA -3'), and CfRV3-GSPC for CfRV3 (5'-CGT CAA TAG CAC AGG ACT CCG CAA A-3'). The resulting PCR products were then analysed on a 2% agarose gel. Once the success of the amplification was confirmed, a PCR cleanup was performed by adding 2 µl of ExoSap-IT reagent (Applied Biosystems, Foster City, CA, USA) to 5 µl of PCR reagent. Sanger sequencing was then performed on the purified products. For the purified 5' RACE PCR products, sequencing employed the specific GSP-2 primer unique to each virus. Similarly, sequencing of the purified 3' RACE PCR products was carried out using the corresponding specific GSP primer for each virus. Each sequencing reaction comprised 1 µl of BigDye™ Terminator v3.1 mix (Applied Biosystems, Foster City, CA, USA), 2.25 µl of BigDye™ v3.1 sequencing buffer (Applied Biosystems, Foster City, CA, USA), 0.75 µl of each 2 µM primer, and PCR-grade water, making up a final volume of 10 µl.

The sequencing reaction conditions followed the manufacturer's protocol. Subsequently, sequencing products underwent precipitation using a standard Sodium acetate/EDTA clean-up protocol. These samples were then sequenced at the DNA Sanger Sequencing Facility at the University of Pretoria. The results were then compared with the viral sequences which were previously assembled from the associated RNA-seq dataset, by using CLC genomics workbench 22.

Confirmation RT-PCRs of CfRV1, CfRV2 and CfRV3

Total RNA which was previously extracted from *C. fimbriata* CMW 14799 was used as input for a two-step Reverse Transcription (RT) PCR to confirm the presence of CfRV1, CfRV2 and CfRV3. These reactions were carried out using M-MuLV Reverse Transcriptase and the OneTaq® 2X Master Mix (NEB, Ipswich, Massachusetts, United States). Primer sequences for these PCRs were manually determined by analysing the viral sequences on CLC genomics workbench. Subsequently, these sequences were submitted to Inqaba Biotech (Inqaba Biotechnical Industries, Muckleneuk, Pretoria, South Africa) for primer synthesis. These had the following sequences: For CfRV1, CfRV1-F (5'-AAA CCT CGG TCC AGG AGA ATG CT-3') and CfRV1-R (5'-GAC TGT GTG GAC GCA CTC TTT GC-3'). For CfRV2, CfRV2-F (5'-TGG ATG TCA CAA CAC GCA AAG CA-3') and CfRV2-R (5'-CAC TCT

GGA GAC TTC TCC TGC TT-3'). For CfRV3, CfRV3-F (5'-GAA GGC GAG GTG TGC TTG AGC TT-3') and CfRV3-R (5'-CCT CGG CAG GTC CGC TAG TGC TT-3). The resulting PCR products were confirmed on a 2% agarose gel. Upon confirmation of successful amplification, a PCR cleanup was conducted by combining 2 µl of ExoSap-IT reagent (Applied Biosystems, Foster City, CA, USA) with 5 µl of PCR reagent. Subsequently, Sanger sequencing was carried out on the purified products. Each sequencing reaction included 1 µl of BigDye™ Terminator v3.1 mix (Applied Biosystems, Foster City, CA, USA), 2.25 µl of BigDye™ v3.1 sequencing buffer (Applied Biosystems, Foster City, CA, USA), PCR-grade water, and 0.75 µl of the reverse primer (2 µl) corresponding to each virus, making a total volume of 10 µl. The sequencing reaction conditions adhered to the manufacturer's protocol. Following this, the sequencing products underwent precipitation using a standard Sodium acetate/EDTA clean-up protocol. Subsequent sequencing was conducted at the DNA Sanger Sequencing Facility at the University of Pretoria. The obtained results were then compared with viral sequences previously assembled from the associated RNA-seq dataset using CLC Genomics Workbench 22.

Results

Summary of identified viral sequences

During this study, four fungal isolates from the genus *Ceratocystis* were examined for the presence of mycoviruses. Overall, a total of 18 viruses were detected, and all four fungal isolates were found to be associated with viruses. Most mycoviruses were composed of ssRNA (77.78%) and dsRNA (16.67%), with only one virus having a negative sense single stranded RNA (ssRNA) genome. An overview of these mycoviruses and their genomic characteristics are available in Table 1.

Table 1. Taxonomic and genomic overview of putative mycoviruses identified in the transcriptomes of fungi from *Ceratocystis*.

Host	Genome type	Virus Family	Virus name	Virus abbrev.	Contig length (nucleotide)	Contig coverage	Protein ID	Protein length (amino acid)
**C. albifundus* CMW 4068	+ssRNA	*Endornaviridae*	Ceratocystis albifundus RNA Virus 1	CaRV-1	4383	187663.83	RdRP 2	273
C. albifundus CMW 4068	+ssRNA	*Endornaviridae*	Ceratocystis albifundus RNA Virus 2	CaRV-2	11028	105727.05	Viral helicase 1 RdRP 2	255 361
▼**C. albifundus* CMW 4068	+ssRNA	*Endornaviridae*	Ceratocystis albifundus RNA Segment 3	Ca RNA Seg3	8809	141076.9 5	GT1 Gtf -like	333
**C. albifundus* CMW 4068	-ssRNA	*Mymonaviridae*	Ceratocystis albifundus RNA Virus 4	CaRV-4	4938	67.07		
C.mangine cans CMW 46461	+ssRNA	*Mitoviridae*	Ceratocystis manginecans RNA Virus 1	CmRV-1	2856	270787.48	Mitovir RNA pol	493
C.mangine cans CMW 46461	+ssRNA	*Mitoviridae*	Ceratocystis manginecans RNA Virus 2	CmRV-2	2777	8.84	Mitovir RNA pol	485
C.mangine cans CMW 46461	+ssRNA	*Mitoviridae*	Ceratocystis manginecans RNA Virus 3	CmRV-3	3080	457441.89	Mitovir RNA pol	336
C.mangine cans CMW 46461	+ssRNA	*Endornaviridae*	Ceratocystis manginecans RNA Virus 4	CmRV-4	10948	1415.29	Viral helicase 1 RdRP 2	256 238

Truncated contigs are indicated with an asterisk (*), and in cases where no conserved protein domains could be found, Protein ID and length were left blank. Viral segments without an RdRp domain, and which may belong to other truncated viruses in the assemblies are denoted with a triangle (▼).

Table 1 continued. Taxonomic and genomic overview of putative mycoviruses identified in the transcriptomes of fungi from *Ceratocystis*.

Host	Genome type	Virus Family	Virus name	Virus abbrev.	Contig Length(nucleotides)	Protein ID	Protein length (amino acids)
C.eucalypticola CMW 53160	+ssRNA	*Mitoviridae*	Ceratocystis eucalypticola RNA Virus 1	CeRV-1	2851	Mitovir RNA pol	495
C.eucalypticola CMW 53160	+ssRNA	*Mitoviridae*	Ceratocystis eucalypticola RNA Virus 2	CeRV-2	2742	Mitovir RNA pol	485
C.eucalypticola CMW 53160	+ssRNA	*Virgaviridae*	Ceratocystis eucalypticola RNA Virus 3	CeRV-3	9539	Viral helicase 1 RdRP 2	244 414
▼*C.eucalypticola* CMW 53160	dsRNA	*Totiviridae*	Ceratocystis eucalypticola RNA segment 4	Ce RNA Seg4	2397	Totivirus coat	
▼*C.eucalypticola* CMW 53160	dsRNA	*Totiviridae*	Ceratocystis eucalypticola RNA Segment 5	Ce RNA Seg5	2216	Totivirus coat	
C.eucalypticola CMW 53160	dsRNA	*Totiviridae*	Ceratocystis eucalypticola RNA Virus 6	CeRV-6	2921	RdRp 4	466
C.eucalypticola CMW 53160	dsRNA	*Totiviridae*	Ceratocystis eucalypticola RNA Virus 7	CeRV-7	2049	RdRp 4	458
C.fimbriata CMW 14799	+ssRNA	*Endornaviridae*	Ceratocystis fimbriata RNA Virus 1	CfRV-1	11033	AAA 30 domain RdRp 2	101 215
C.fimbriata CMW 14799	+ssRNA	*Endornaviridae*	Ceratocystis fimbriata RNA Virus 2	CfRV-2	13619	Viral helicase 1 GT1 Gtf - like RdRP 2	229 375 178
C.fimbriata CMW 14799	+ssRNA	*Endornaviridae*	Ceratocystis fimbriata RNA Virus 3	CfRV-3	13539	Viral helicase 1 GT1 Gtf - like RdRP 2	259 376 236
C.fimbriata CMW 14799	+ssRNA	*Mitoviridae*	Ceratocystis fimbriata RNA Virus 4	CfRV-4	2864	Mitovir RNA pol	
C.fimbriata CMW 14799	+ssRNA	*Mitoviridae*	Ceratocystis fimbriata RNA Virus 5	CfRV-5	2783	Mitovir RNA pol	485
C.fimbriata CMW 14799	dsRNA	*Totiviridae*	Ceratocystis fimbriata RNA Virus 6	CfRV-6	2357	RdRp 4	408
▼*C.fimbriata* CMW 14799	dsRNA	*Totiviridae*	Ceratocystis fimbriata RNA Segment 7	Cf RNA Seg7	1545	Totivirus coat	
▼*C.fimbriata CMW 14799	dsRNA	*Totiviridae*	Ceratocystis fimbriata RNA segment 8	Cf RNA Seg8	1044	LA-virus coat	

Characterization of positive sense and negative sense single stranded RNA mycoviruses

Mycoviruses with +ssRNA genomes were identified in the transcriptomes of all four fungal isolates. These showed similarity with other viruses from the families *Mitoviridae*, *Endornaviridae*, and *Virgaviridae*. Only 1 mycovirus with a -ssRNA genome was identified in the transcriptome of *C. albifundus*. This particular virus exhibited similarity to viruses from the *Mymonaviridae* family. However, this virus, termed Ceratocystis albifundus RNA Virus 4 (CaRV-4), was significantly truncated and did not display any conserved domains. Consequently, CaRV-4 was not included in phylogenetic analysis or genome characterization.

1. Identification of mycoviruses from *Mitoviridae*

The majority of mycoviruses identified in this study belonged to the *Mitoviridae* family. Three mitoviruses were present in the transcriptome of *C. manginecans*, namely Ceratocystis manginecans RNA Virus 1 (CmRV-1), Ceratocystis manginecans RNA Virus 2 (CmRV-2), and Ceratocystis manginecans RNA Virus 3 (CmRV-3). The transcriptome of *C. eucalypticola* contained two mitoviruses, specifically Ceratocystis eucalypticola RNA Virus 1 (CeRV-1) and Ceratocystis eucalypticola RNA Virus 2 (CeRV-2). The transcriptome of *C. fimbriata* also contained two mitoviruses, putatively named Ceratocystis fimbriata RNA Virus 4 (CfRV-4) and Ceratocystis fimbriata RNA Virus 5 (CfRV-5). No mitoviruses were found in the transcriptome of *C. albifundus*.

1.1. Phylogenetic classification of mycoviruses from *Mitoviridae*

Phylogenetic analysis was based on the putative amino acid sequences of RdRP proteins of CeRV-1, CeRV-2, CfRV-4, CfRV-5, CmRV-1, CmRV-2, and CmRV-3 as well as other cognate sequences derived from GenBank (Fig.1). The mitoviruses CeRV-1, CfRV-4, and CmRV-1 clustered most closely with members of the genus *Unuamitovirus*, and thus likely belong to this group. The remaining mitoviruses, CeRV-2, CmRV-2, CfRV-4 and CmRV-3, appeared to cluster more with mitoviruses from the genus *Duamitovirus*. Therefore, it is a strong possibility that CeRV-2, CmRV-2, CmRV-3, and CfRV-4 may belong to *Duamitovirus*.

A percent identity matrix was also constructed to compare the conserved RdRp sequences of the seven mitoviruses under investigation to that of other related viruses, thus facilitating the differentiation of distinct viral species (Fig. 2). The RdRp sequence identities of CmRV-1 and CmRV-3 fell below the established threshold of 90%, a benchmark for species demarcation within the *Mitoviridae* family [57]. Interestingly, CfRV-4 and CeRV-1 displayed 100% RdRp sequence identity to one another, and 99.86% to *Unuamitovirus cefi 1* (UMVcefi1). This

observation strongly suggests that these three mitoviruses represent variants of the species *Unuamitovirus cefi 1*. Similarly, CmRV-2, CeRV-2, and CfRV-5 exhibited an identical 100% RdRp sequence identity to one another, and 89.09% identity to *Mitovirus cefi 1* (MVcefi1). This indicated that CmRV-2, CeRV-2, CfRV-5 and MVcefi1 are the variants of the same species.

1.2 Genomic characterization of mycoviruses from *Mitoviridae*

All mitoviruses from this study were characterized, and a diagrammatic representation of their genomic structure is shown in Fig.3. Notably, the genomes of all seven mitoviruses were found to be comprised of a single ORF encoding for an RdRp protein and flanked by 3' and 5' untranslated terminal regions of varying lengths (UTRs). Amino acid alignments of the RdRp protein from these viruses, and the RdRp sequences of other cognate mitoviruses, also revealed 6 conserved protein motifs (I to VI) (Fig S1.).

The genomic arrangements, encompassing the ORF length and associated protein domains, for unuamitoviruses are illustrated in Fig.3a, Fig. 3d, and Fig. 3f, corresponding to CmRV-1, CeRV-1, and CfRV-4, respectively. Each of the protein products produced by CmRV-1, CeRV-1 and CeRV-4 is predicted to have a mass of approximately 83.00 kDa. A Blastp analysis uncovered that these proteins exhibit the closest similarity to UMVcefi1, with 100% coverage, a 0.0 E-value, and identities of 87.99% for CmRV-1 and 99.86% for CfRV-4 and CeRV-1. Interestingly, all three unuamitoviruses also showed high percent identities (around 60%) with Thielaviopsis basicola mitovirus. The secondary structures of the 3' and 5' UTRs in all three unuamitoviruses were subsequently analysed and revealed the formation of stable stem-loop structures with distinct free energy values. These structures, along with their corresponding energy values, are graphically represented in Fig. S5 for CmRV-1, Fig. S6 for CeRV-1, and Fig. S7 for CeRV-4. Moreover, the terminal ends of these unuamitoviruses exhibited reverse complementarity, suggesting the potential formation of panhandle structures, each characterized by varying free energy values. These findings are visually depicted in Fig. S5C, Fig. S6C, and Fig. S7C for CmRV-1, CeRV-1, and CeRV-4, respectively.

A more detailed depiction of the genomic organizations of CmRV-2, CmRV-3, CeRV-2, and CfRV-5 can be found in Fig. 3b, Fig. 3c, Fig.3e, and Fig. 3g, respectively. The genomes of these duamitoviruses produce protein products with an estimated weight of 84.11 kDa for CmRV-2 and CfRV-5, 88.88 kDa for CmRV-3, and 86.75 kDa for CeRV-2. The protein encoded by CmRV-3 exhibited the highest similarity to Guangdong mito-like virus 6 (73% coverage, a 2e112 E-value, and an 40.41% identity) when analysed with Blastp, while those of CmRV-2, CeRV-2,

and CfRV-5 share the highest percent identity to that of MVcefi1 (63% coverage, 0.0 E-value, and 98.70% identity). The secondary structures of all duamitoviruses in this study were subsequently evaluated and revealed the presence of stable stem-loop structures with varying free energy values. The depictions of these structures, as well as their associated free energy values, can be found in Fig. S8, Fig. S9, Fig. S10, and Fig. S11 for CmRV-2, CmRV-3, CeRV-2, and CfRV-5, respectively. Moreover, panhandle structures, formed by inverted complimentary sequences in the 3' and 5' UTRs of these viruses were also predicted. These are presented in Fig. S8C for CmRV-2, Fig. S9C for CmRV-3, Fig. S10C for CeRV-2, and Fig. S11C for CfRV-5. It should be noted that CmRV-3 displayed only limited 3' and 5' complementarity, having a free energy value of only -1.10 kcal/mol, thus making the formation of a panhandle structure less likely.

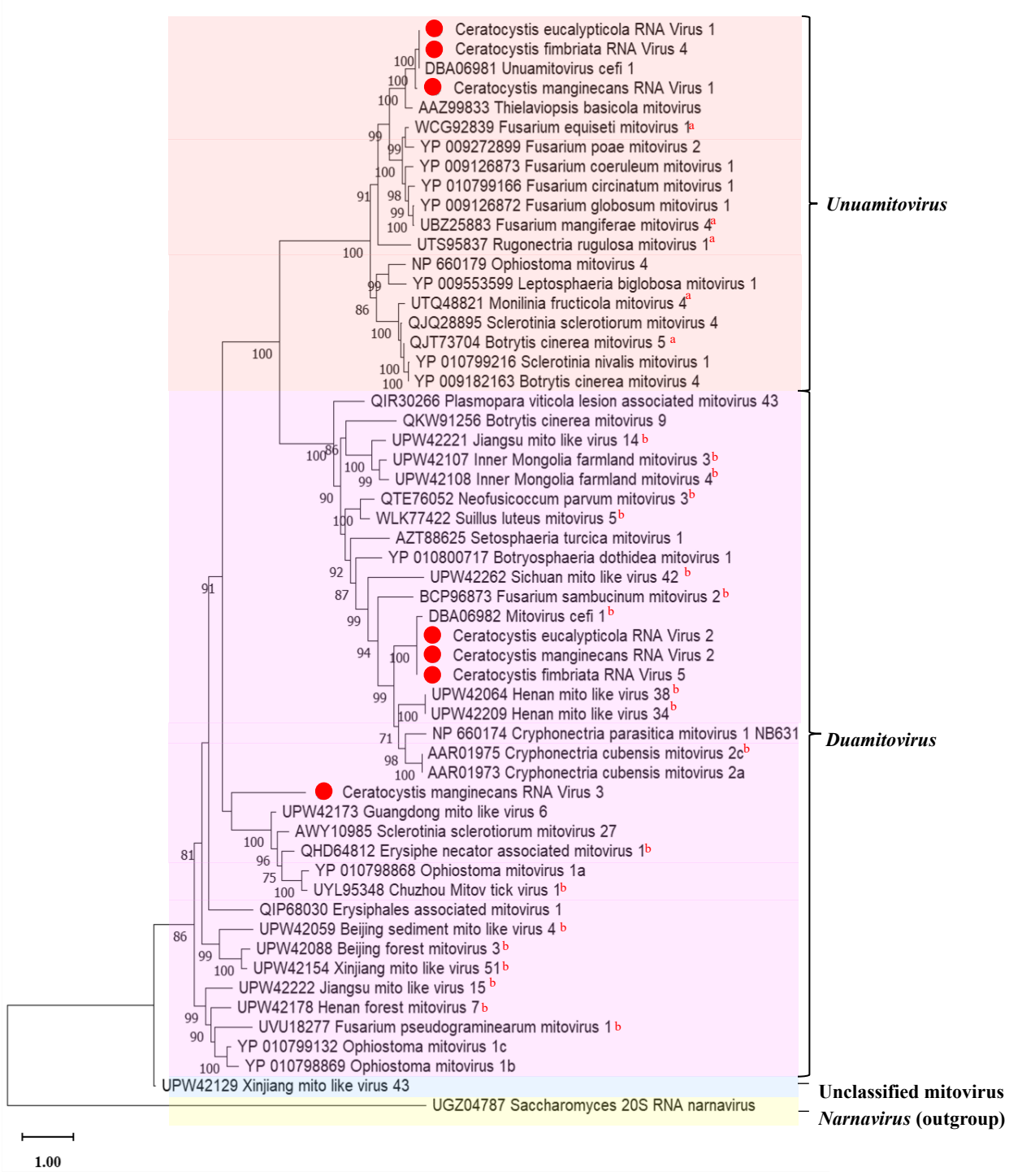
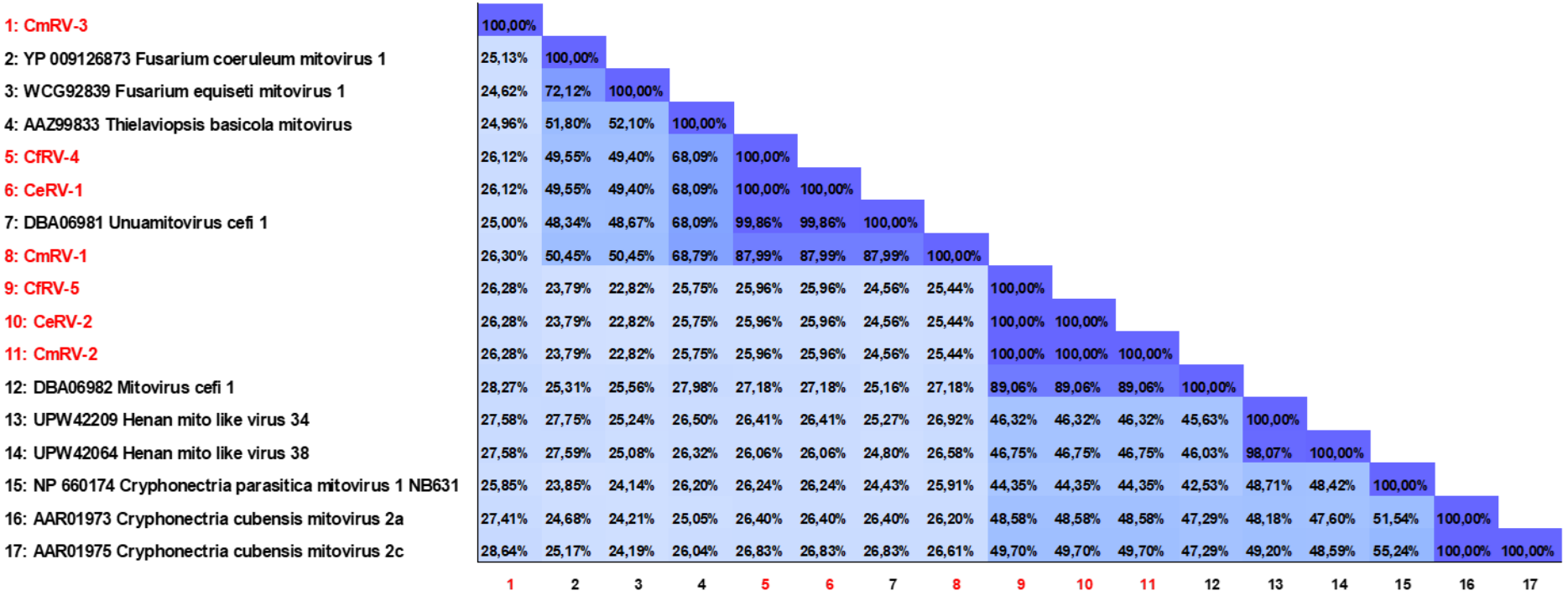


Fig. 1 Phylogenetic tree showing maximum likelihood analysis of the RdRp protein sequences from the mitoviruses discovered in this study, with those of related viruses. The phylogeny was generated using IQ-TREE, using the Whelan and Goldman model with frequencies, invariant sites, and gamma distribution. Bootstrapping with 1000 replicates was performed, and corresponding percentages are indicated next to the branches. Only bootstrap values equal to or exceeding 70 are displayed. The Saccharomyces 20S RNA Narnavirus was used as an outgroup. The red circles (●) indicate mycoviruses discovered in this study. a: Denotes unuamitoviruses and b: duamitoviruses which have not yet been taxonomically assigned by the ICTV (https://ictv.global/taxonomy). These mitoviruses exhibit genetic characteristics and phylogenetic relationships that strongly suggest their classification within these genera.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1: CmRV-3	100,00%																
2: YP 009126873 Fusarium coeruleum mitovirus 1	25,13%	100,00%															
3: WCG92839 Fusarium equiseti mitovirus 1	24,62%	72,12%	100,00%														
4: AAZ99833 Thielaviopsis basicola mitovirus	24,96%	51,80%	52,10%	100,00%													
5: CfRV-4	26,12%	49,55%	49,40%	68,09%	100,00%												
6: CeRV-1	26,12%	49,55%	49,40%	68,09%	100,00%	100,00%											
7: DBA06981 Unuamitovirus cefi 1	25,00%	48,34%	48,67%	68,09%	99,86%	99,86%	100,00%										
8: CmRV-1	26,30%	50,45%	50,45%	68,79%	87,99%	87,99%	87,99%	100,00%									
9: CfRV-5	26,28%	23,79%	22,82%	25,75%	25,96%	25,96%	24,56%	25,44%	100,00%								
10: CeRV-2	26,28%	23,79%	22,82%	25,75%	25,96%	25,96%	24,56%	25,44%	100,00%	100,00%							
11: CmRV-2	26,28%	23,79%	22,82%	25,75%	25,96%	25,96%	24,56%	25,44%	100,00%	100,00%	100,00%						
12: DBA06982 Mitovirus cefi 1	28,27%	25,31%	25,56%	27,98%	27,18%	27,18%	25,16%	27,18%	89,06%	89,06%	89,06%	100,00%					
13: UPW42209 Henan mito like virus 34	27,58%	27,75%	25,24%	26,50%	26,41%	26,41%	25,27%	26,92%	46,32%	46,32%	46,32%	45,63%	100,00%				
14: UPW42064 Henan mito like virus 38	27,58%	27,59%	25,08%	26,32%	26,06%	26,06%	24,80%	26,58%	46,75%	46,75%	46,75%	46,03%	98,07%	100,00%			
15: NP 660174 Cryphonectria parasitica mitovirus 1 NB631	25,85%	23,85%	24,14%	26,20%	26,24%	26,24%	24,43%	25,91%	44,35%	44,35%	44,35%	42,53%	48,71%	48,42%	100,00%		
16: AAR01973 Cryphonectria cubensis mitovirus 2a	27,41%	24,68%	24,21%	25,05%	26,40%	26,40%	26,40%	26,20%	48,58%	48,58%	48,58%	47,29%	48,18%	47,60%	51,54%	100,00%	
17: AAR01975 Cryphonectria cubensis mitovirus 2c	28,64%	25,17%	24,19%	26,04%	26,83%	26,83%	26,83%	26,61%	49,70%	49,70%	49,70%	47,29%	49,20%	48,59%	55,24%	100,00%	100,00%

Fig. 2 Percent Identity Matrix of the mitoviruses from this study, and other viral members from *Mitoviridae*, generated using Clustal Omega. The colour gradients represent percent identities, with darker blue shades indicating higher identities and lighter shades indicating lower identities. The mitoviruses are numbered from 1 to 17, and their corresponding labels are displayed at the bottom of each column. Mitoviruses from this study are highlighted in red for better readability.

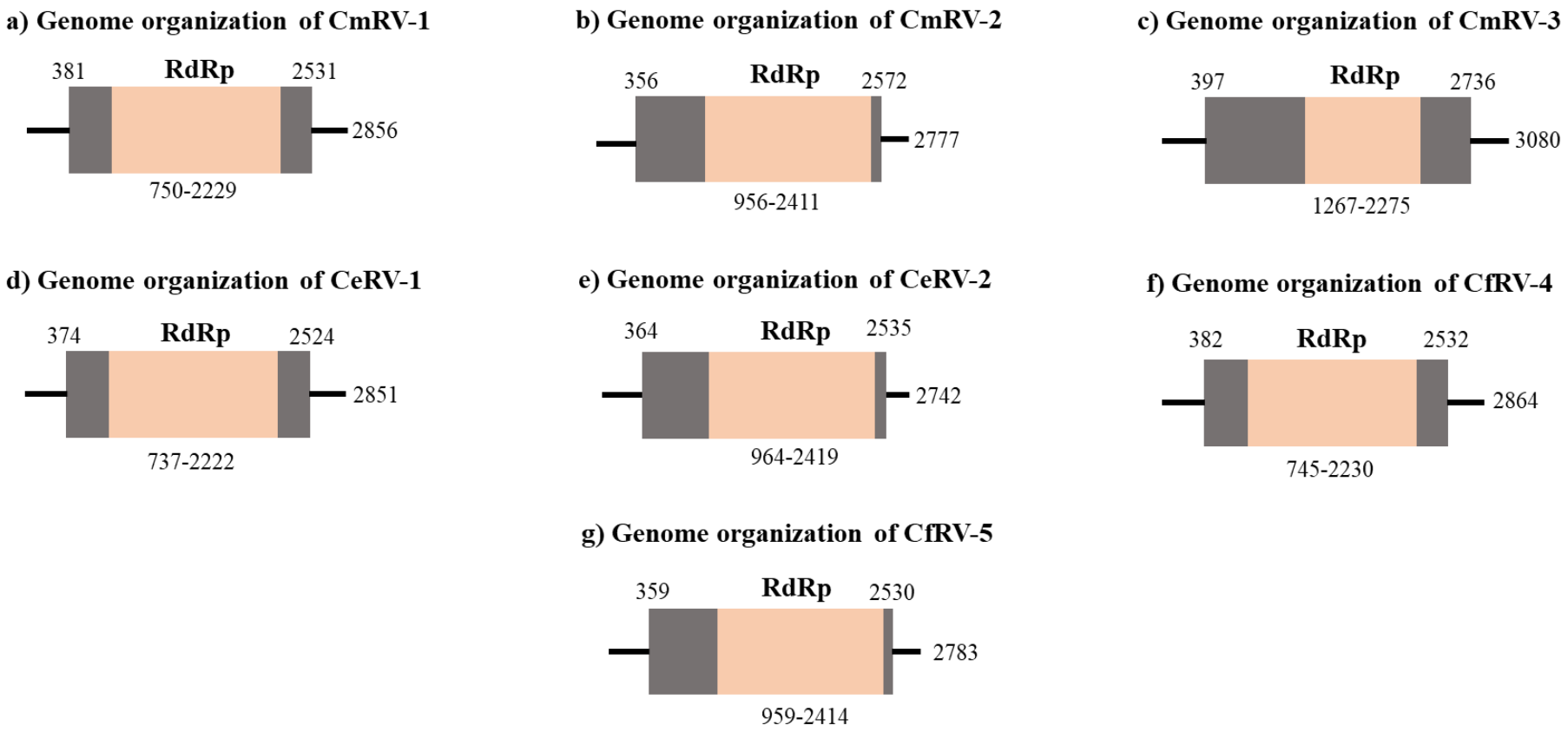


Fig. 3 Schematic representation of the genomic organization of all 7 mitoviruses (a-g). The open bars represent single open reading frames (ORFs), while the single lines represent untranslated regions (UTRs). The length of the ORF is indicated for all mitoviruses, and the position of the RdRp domain encoded on the ORF is also shown.

2. Identification of mycoviruses from *Endornaviridae*

Within the dataset from *C. albifundus*, two endornaviruses were identified, specifically Ceratocystis albifundus RNA virus 1 (CaRV-1) and Ceratocystis albifundus RNA virus 2 (CaRV-2). It is important to note that the genome of CaRV-1 was considered incomplete as it lacked a stop codon, resulting in a truncated ORF. This endornavirus was thus excluded from further genomic characterization. Nevertheless, CaRV-1 contained a fully intact RdRp domain, and was thus included in phylogenetic analyses. An additional viral segment with a glucosyltransferase (Gtf) domain was identified within the *C. albifundus* datasets. This particular segment was designated as Ceratocystis albifundus RNA segment 3 (Ca RNA Seg 3). It is worth mentioning that Ca RNA Seg 3 did not exhibit any notable similarity with other endornaviruses present in the dataset. Therefore, due to its truncation and the absence of an RdRp domain, Ca RNA Seg 3 could not be included in either phylogenetic analyses or subjected to comprehensive genomic characterization. However, it is a possibility that this segment may belong to the truncated genome of CaRV-1; hence, it could not be classified as a novel endornavirus. One endornavirus was identified in the *C. manginecans* dataset, which has been provisionally named Ceratocystis manginecans RNA virus 4 (CmRV-4). The datasets pertaining to *C. fimbriata* harboured three distinct endornaviruses, specifically Ceratocystis fimbriata RNA virus 1 (CfRV-1), Ceratocystis fimbriata RNA virus 2 (CfRV-2), and Ceratocystis RNA virus 3 (CfRV-3).

2.1 Phylogenetic classification of mycoviruses from *Endornaviridae*

The characterize the relationships of the endornaviruses, a phylogenetic tree was constructed based on the RdRp amino acid sequences of CaRV-1, CaRV-2, CmRV-4, CfRV-1, CfRV-2, CfRV-3, and cognate sequences derived from references that were obtained from GenBank (Fig. 4). Notably, all six endornaviruses formed a distinct cluster alongside other members belonging to the genus *Alphaendornavirus*. This clustering pattern strongly supports their provisional classification within this genus. A comparative analysis of the polyprotein amino acid sequences of various endornaviruses and closely related viruses was also conducted (Fig. 5). The analysis revealed that none of the six endornaviruses examined in this study exhibited significant amino acid sequence identity with other endornaviruses.

Species demarcation for members of *Alphaendornavirus* also requires an overall genome nucleotide sequence percent identity of less than 75% [58]. Due to having a truncated genome, CaRV-1 was not included in this analysis. CaRV-2 on the other hand, displayed an overall nucleotide sequence identity of 69% with Alphaendornavirus fimbriatae-3 (18% coverage, and 2e-123 E-value). The genome coverage of this endornavirus with other reference viruses were

very low (1% or less), thus CaRV-2 is likely a novel alphaendornavirus of the family *Endornaviridae*. The endornavirus CmRV-4 displayed 73.41% nucleotide identity to Alphaendornavirus fimbriatae-3 (47% coverage, and 0.0 E-value), and may also represent a novel alphaendornavirus. On the other hand, CfRV-1 exhibited an overall nucleotide percent identity of 99.77% to Alphaendornavirus fimbriatae-3 (AEVf-3; 51% coverage, and 0.0 Evalue), indicating that these viruses are identical. Similarly, CfRV-2 showed 99.80% nucleotide identity to Alphaendornavirus fimbriatae-2 (AEVf-2; 55% coverage, and 0.0 E-value) suggesting that they are the same virus. Finally, CfRV-3 displayed 99.90% nucleotide identity to Alphaendornavirus fimbriatae-1 (AEVf-1; 76% coverage, and 0.0 E-value). This also indicates that these two viruses are identical.

2.2 Genomic characterization of mycoviruses from *Endornaviridae*

The genomes of all putative endornaviruses were characterized and a diagrammatic representation of their genome organizations can be found in Fig. 6. All six putative endornaviral genomes contained a single ORF, which encodes for a polyprotein with various protein domains. Notably, a sequence comprising cytosine residues was also situated at the C-terminal end across most of the endornaviral genomes, with the exception of CmRV-4. Additionally, the ORFs of these endornaviruses were flanked by 5' and 3' untranslated regions (UTRs) of variable lengths. Amino acid alignments of the protein sequences from the endornavirus of interest and other closely related viruses also revealed five conserved protein motifs (I to V), typical of endornaviruses (Fig. S2).

For a more comprehensive understanding of the genomic organization, protein lengths, and protein domains of all endornaviruses evaluated in this study, refer to the diagrams presented in Fig. 7a (CaRV-2), Fig. 7b (CmRV-4), Fig. 7c (CfRV-1), Fig. 7d (CfRV-2), and Fig. 7e (CfRV-3). The molecular weights of the proteins produced by each virus were as follows: 402.97 kDa for CaRV-2, 399.52 kDa for CmRV-4, 404.05 kDa for CfRV-1, 506.18 kDa for CfRV-2, and 503.79 kDa for CfRV-3. An RdRp domain was also omnipresent in the ORFs of all five endornaviruses. However, a few unique features distinguished these viruses; specifically, glucosyltransferases (Gtfs) were exclusively found in the ORFs of CfRV-2 and CfRV-3. In addition, with the exception of CfRV-1, all alphaendornaviruses examined in this study featured a viral helicase (Hel) domain in the ORF region. Remarkably, CfRV-1, lacking the Hel domain, stood out with the inclusion of an AAA domain, a distinctive characteristic absent in the other endornaviruses.

To ascertain the terminal ends of CfRV-1, CfRV-2, and CfRV-3, a 3' and 5' RACE PCR was conducted, thus successfully determining the terminal ends and confirming the completeness of the genomes of these viruses. Additionally, an RT-PCR assay was performed to validate the presence of these three viruses within their original host. These experiments were carried out in in order to facilitate the taxonomic classification of these viruses by the ICTV, and the publication of the full-length genomes of these viral sequences on NCBI.

Fig. 4 Phylogenetic tree showing maximum likelihood analysis of the RdRp protein sequences from the endornaviruses discovered in this study, with those of related viruses. The phylogeny was generated using IQTREE, using the Whelan and Goldman model with frequencies, invariant sites, and gamma distribution. Bootstrapping with 1000 replicates was performed, and corresponding percentages are indicated next to the branches. Only bootstrap values equal to or exceeding 70 are displayed. The Botrytis cinerea betaendornavirus 1 was used as an outgroup. The red circles (●) indicate the endornaviruses discovered in this study a. Denotes unclassified endornaviruses which may belong to the genus *Alphaendornavirus* but has not yet been recognized by the ICTV (https://ictv.global/taxonomy). These endornaviruses exhibit genetic characteristics and phylogenetic relationships that suggest their classification within this genus.

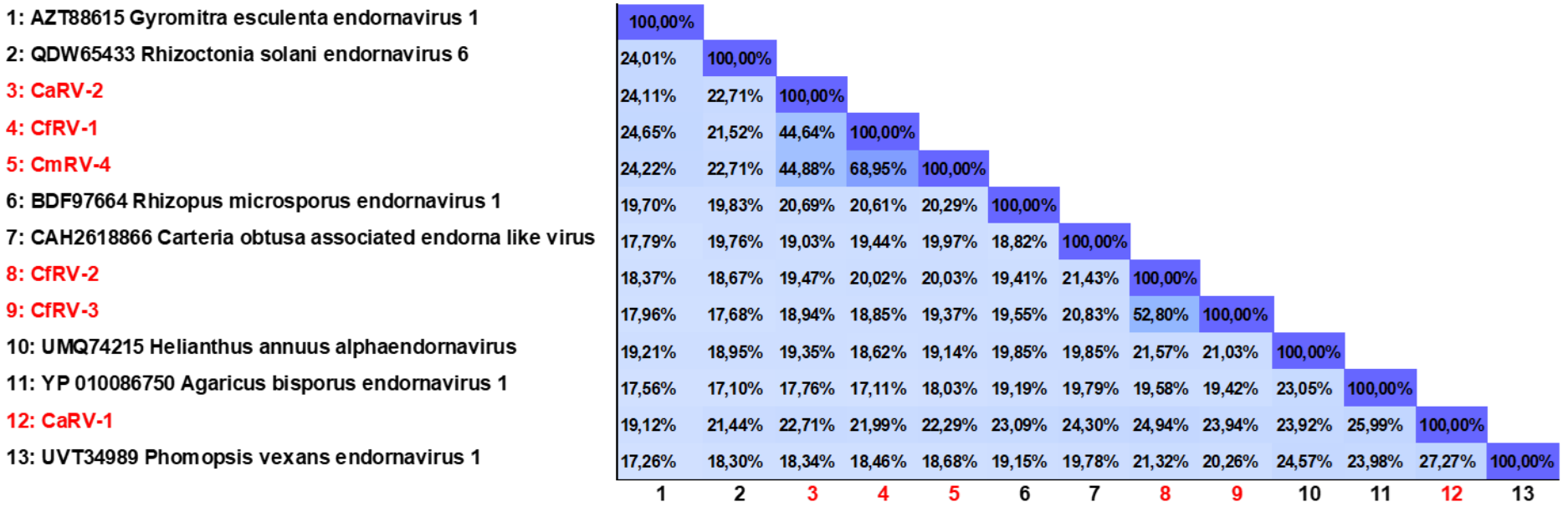


Fig. 5 Percent Identity Matrix of the endornaviruses from this study, and other viral members from *Endornaviridae* generated using Clustal Omega. The colour gradients represent percent identities, with darker blue shades indicating higher identities and lighter shades indicating lower identities. The endornaviruses are numbered from 1 to 13, and their corresponding labels are displayed at the bottom of each column. Endornaviruses from this study are highlighted in red for better readability.

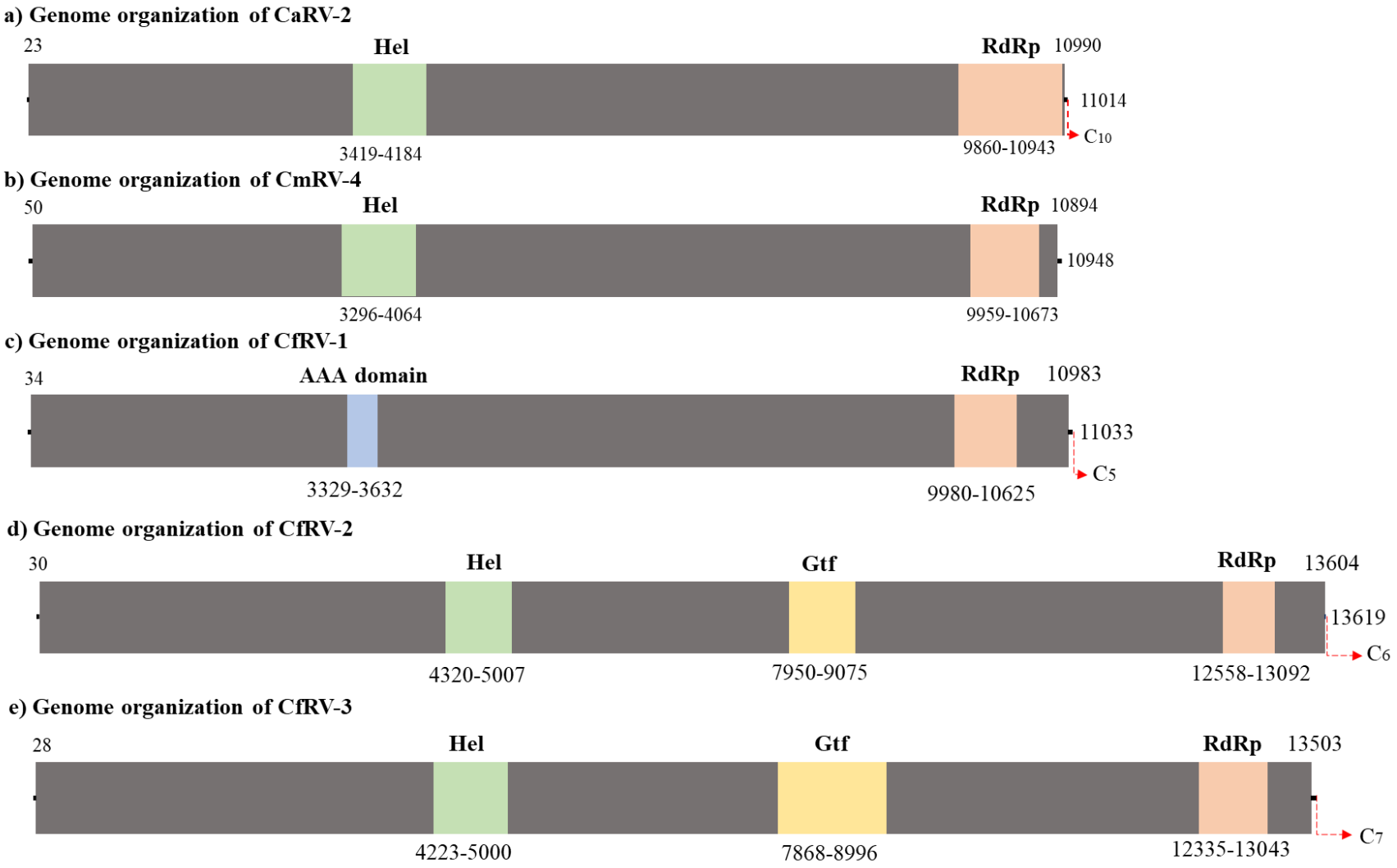


Fig. 6 Schematic representation of the genomic organization of all 5 endornaviruses (a-e). The open bars represent single open reading frames (ORFs), while the single lines represent untranslated regions (UTRs). The length of the ORF is indicated for all endornaviruses, and the position of the protein domains encoded on the ORF is also shown.

3. Phylogenetic and genomic characterization of a mycovirus from *Virgaviridae*

A virga-like virus was discovered in the transcriptome of *C. eucalypticola* and was provisionally designated as Ceratocystis eucalypticola RNA virus 3 (CeRV-3). To elucidate the taxonomic position of CeRV-3, a phylogenetic tree was constructed using amino acid sequences of the RdRp from this virga-like virus, along with analogous sequences retrieved from the GenBank database (Fig. 7). The phylogenetic analysis revealed that CeRV-3 clustered more closely with unclassified members of the family *Virgaviridae*, as well as other Virga-like viruses. Additionally, a comparative analysis of the polyprotein sequences of CeRV-3 and closely related viruses was conducted, and a percent identity matrix was constructed (Fig. 8). This analysis demonstrated the absence of significant sequence similarity between CeRV-3 and any closely related viral sequences.

Subsequently, the genome of CeRV-3 was comprehensively characterized, and a graphical representation is provided in Fig. 9. Within this genome, a single ORF encoded a polyprotein spanning, with a molecular weight of 350.43 kDa. Notably, this polyprotein contained specific domains, including a Hel and an RdRp domain. A Blastp search also showed that this polyprotein shares the highest degree of similarity to that of Plasmopara viticola lesion-associated virga-like virus 1, with a coverage of 30%, an E-value of 2e-137, and an identity of 33.51%. This indicates that CeRV-3 is a novel member of the family *Virgaviridae*. Multiple sequence alignment was conducted on the amino acid sequence of the conserved RdRp domain of CeRV-3, along with the corresponding sequences of representative viruses belonging to the family *Virgaviridae* (Fig. S3). The alignment analysis showed that the protein encoded by CaRV-3 exhibits the 8 conserved domains shared with closely related viruses. This includes the identification of a putative glycine aspartic acid-aspartic acid (GDD) motif, which is recognized as a hallmark feature among RdRp proteins.

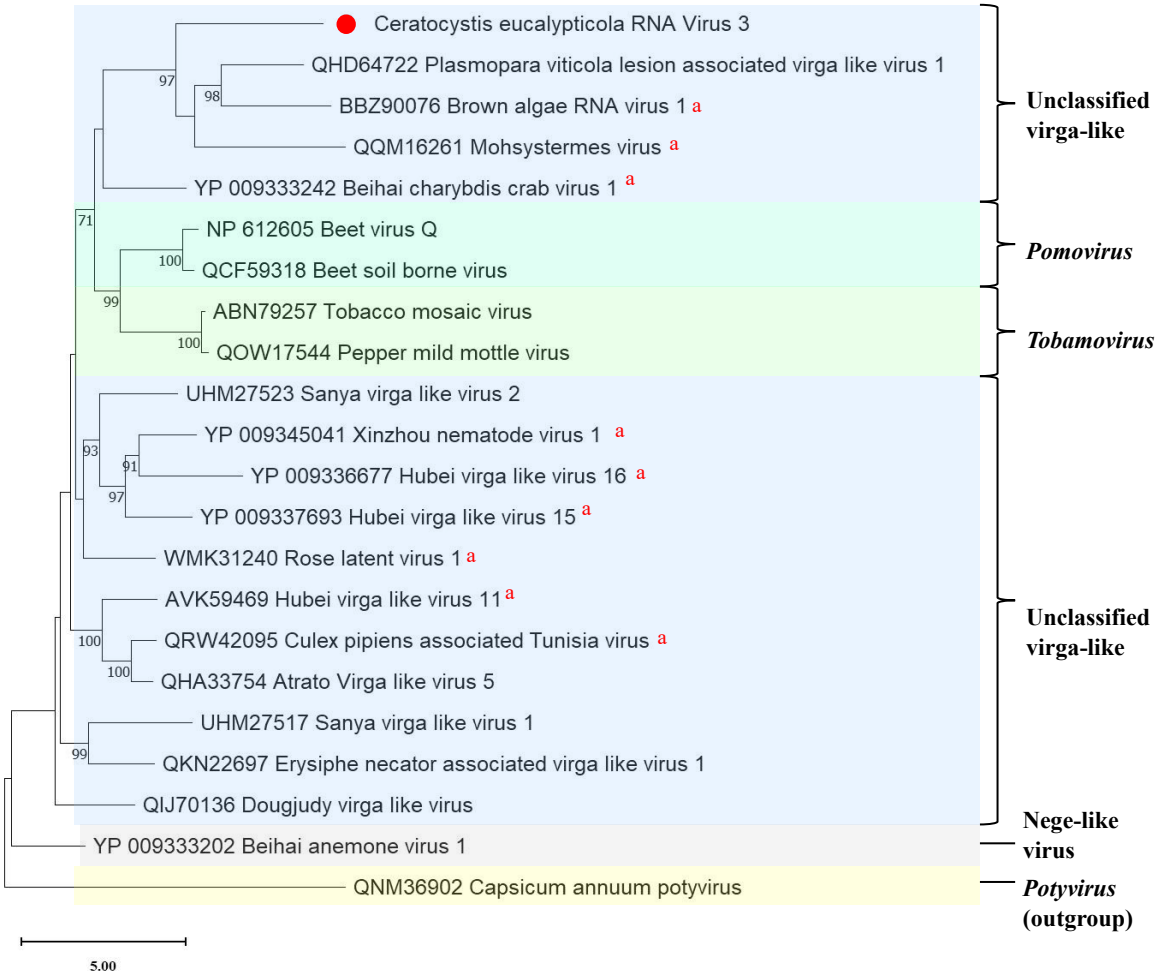


Fig. 7 Phylogenetic tree showing maximum likelihood analysis of the RdRp protein sequences from the virgavirus discovered in this study, with those of related viruses. The phylogeny was generated using IQTREE, using the Le Gauss model with frequencies, invariant sites, and gamma distribution. Bootstrapping with 1000 replicates was performed, and corresponding percentages are indicated next to the branches. Only bootstrap values equal to or exceeding 70 are displayed. The Capsicum annuum potyvirus was used as an outgroup. The red circle (●) indicates the virg-like virus discovered in this study. a. Denotes unclassified viruses which may belong to the family *Virgaviridae* but have not formally been classified into this family. These viruses exhibit genetic characteristics and phylogenetic relationships that suggest their classification within this family.

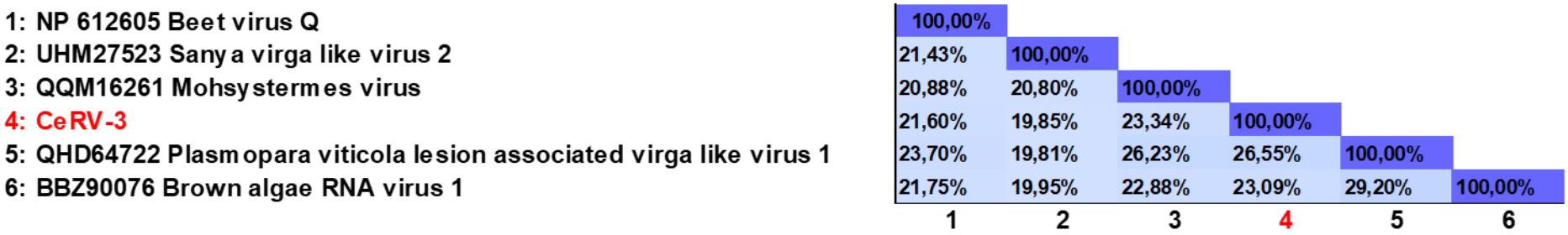


Fig. 8 Percent Identity Matrix of the virga-like from this study, and other viral members of *Virgaviridae* generated using Clustal Omega. The colour gradients represent percent identities, with darker blue shades indicating higher identities and lighter shades indicating lower identities. The viruses are numbered from 1 to 6, and their corresponding labels are displayed at the bottom of each column. CeRV-3 is highlighted in red for better readability.

Fig. 9. Schematic representation of the genomic organization of CeRV-3. The open bar represents a single open reading frame (ORF), while the single lines represent untranslated regions (UTRs). The length of the ORF is indicated for CeRV-3, and the position of the protein domains encoded on the ORF is also shown.

Characterization of double stranded RNA viruses

1. Identification of mycoviruses from *Totiviridae*

Two mycoviruses with double-stranded RNA genomes were identified within the transcriptome of *C. eucalypticola*, and one within the transcriptome of *C. fimbriata*. All three of these viruses exhibited sequence similarity with other viruses belonging to the family *Totiviridae*. The totiviruses originating from *C. eucalypticola* were provisionally designated as Ceratocystis eucalypticola RNA virus 6 (CeRV-6) and Ceratocystis eucalypticola RNA virus 7 (CeRV-7). Notably, while both CeRV-6 and CeRV-7 contained an intact RdRP encoding ORF, they lacked the ORF typically associated with the totivirus coat protein (CP). Consequently, they were excluded from genomic characterization. Additionally, two other viral segments were present within the *C. eucalypticola* transcriptome, designated as C. eucalypticola RNA segment 4 (Ce RNA Seg 4) and C. eucalypticola RNA segment 5 (Ce RNA Seg 5). These segments encoded totivirus CPs, albeit in a truncated form. Due to their truncation and the absence of an RdRp domain, Ce RNA Seg 4 and Ce RNA Seg 5 could not be included in either phylogenetic analyses or subjected to comprehensive genomic characterization. There is a possibility that these segments may belong to the truncated genomes of either CeRV-6 or CeRV-7; thus, they were not considered to be distinct totiviruses.

Similarly, within the *C. fimbriata* transcriptome, a totivirus was identified and designated as C. fimbriata RNA virus 6 (CfRV-6). While CfRV-6 possessed an intact ORF with an RdRp domain, it was found to lack the ORF typically associated with the totivirus CP, leading to its exclusion from further genomic characterization. Furthermore, the transcriptome associated with *C. fimbriata* contained two viral segments designated as C. fimbriata RNA Segment 7 (Cf RNA Seg 7) and C. fimbriata RNA Segment 8 (Cf RNA Seg 8). These segments also encoded for a truncated totivirus CP, with no alignment observed to CfRV-6 or to each other. It is plausible that these segments may still be part of the genome of CfRV-6; hence, they could not be considered as distinct totiviruses.

1.1 Phylogenetic classification of mycoviruses from *Totiviridae*

To elucidate the relationships among the totiviruses, a phylogenetic tree was constructed utilizing the amino acid sequences of the RdRps from CeRV-6, CeRV-7, CfRV-6, and corresponding sequences sourced from references available in GenBank (Fig. 10). The resulting phylogenetic tree displayed a clustering pattern wherein all three totiviruses grouped together with viral members from the genus *Victorivirus*.

Subsequently, a comparative analysis of the RdRp amino acid sequences of these viruses, along with those of closely related viruses, was conducted. Percent identity matrices were then generated to distinguish distinct viral species (Fig. 11). The findings indicated that the RdRp percent identities of CeRV-7 and CeRV-8, when compared to other totiviruses, fell below the established threshold of 60%, which serves as a critical criterion for species demarcation within *Victorivirus* (Totiviridae | ICTV). In contrast, the totivirus CfRV-6 exhibited 100% amino acid sequence identity to a virus known as *Victorivirus fimbriatae* (VVf), suggesting that they are the variants of the same species. Moreover, amino acid alignments of the RdRp sequences from the totiviruses investigated in this study, as well as other closely related viruses, revealed eight conserved protein motifs denoted as I to VIII, which are characteristic features of victorivirus RdRps (Fig. S4).

It is worth noting that due to the absence of a major CP, conducting a phylogenetic analysis on the CPs of these three totiviruses was not feasible. Nevertheless, the results of this study strongly indicate that CeRV-6 and CeRV-7 are novel victoriviruses, while CfRV-6 is a victorivirus which is identical to VVf.

Fig. 10 Phylogenetic tree showing maximum likelihood analysis of the RdRp protein sequences from the totiviruses discovered in this study, with those of related viruses. The phylogeny was generated using IQTREE, using the Le Gauss model with frequencies, invariant sites, and gamma distribution. Bootstrapping with 1000 replicates was performed, and corresponding percentages are indicated next to the branches. Only bootstrap values equal to or exceeding 70 are displayed. The Saccharomyces cerevisiae virus L-A was used as an outgroup. The red circles (●) indicate the totiviruses discovered in this study a. Denotes totiviruses which have not yet been formally classified into a genus by the ICTV (https://ictv.global/taxonomy)., but which exhibit genetic characteristics and phylogenetic relationships suggest their classification within the genus *Victorivirus*.

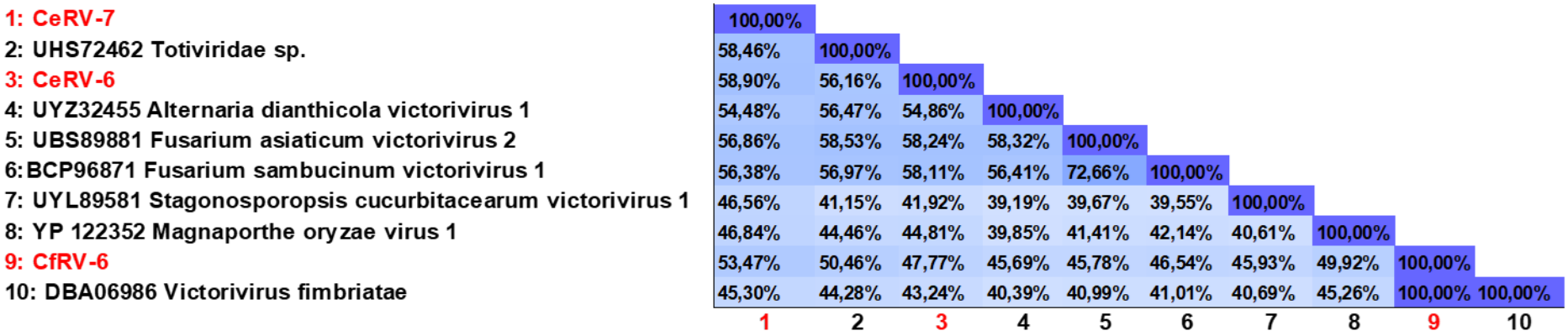


Fig. 11 Percent Identity Matrix of the totiviruses from this study, and other viral members from *Totiviridae*, generated using Clustal Omega. The colour gradients represent percent identities, with darker blue shades indicating higher identities and lighter shades indicating lower identities. The totiviruses are numbered from 1 to 10, and their corresponding labels are displayed at the bottom of each column. Totiviruses from this study are highlighted in red for better readability.

Discussion

The rapid advancements in Next-Generation Sequencing (NGS) technologies have significantly enhanced our ability to identify and understand mycoviruses. It is now widely acknowledged that fungal viruses are widespread across the fungal kingdom. These viruses have been found not only in the more commonly studied fungal groups like Ascomycota and Basidiomycota but also in some of the earlier diverging lineages, including Zygomycota, Blastocladiomycota, Chytridiomycota, and Neocallimastigomycota [40, 59]. However, despite the increasing awareness of mycoviral prevalence, some fungal families and genera have not received sufficient attention in this context. The current study aims to address part of this knowledge gap by exploring the diversity of mycoviruses within *Ceratocystis*, a genus belonging to the Ceratocystidaceae family.

A limited number of fungal members of *Ceratocystis* have been evaluated for the presence of mycoviruses. Species which have previously been examined within this group include *C. plantani* (chapter 2), *C. cacaofunesta*, and *C. fimbriata* [44]. In this study, three additional species were analysed, namely *C. albifundus* CMW 4068, *C. manginecans* CMW 46461, and *C. eucalypticola* CMW 53160. It is worth noting that *C. fimbriata* CMW 14799 had previously been investigated for mycoviruses by Espinal et al. [44]. However, this study relied on a publicly available transcriptome from NCBI SRA for mycovirus detection, which was generated with the use of a poly-A selection step during library preparation. This selection process may exclude mycoviruses lacking a poly-A tail [60] and may potentially result in truncated genomes for certain viruses. To address this concern and minimize possible biases, our current study generated an additional transcriptome for the same *C. fimbriata* isolate. This time, a ribo-depletion step was employed during library selection.

Most known mycoviruses have either dsRNA, ssRNA, or more rarely ssDNA genomes [6166]. While the majority of known mycoviruses have historically been characterized by dsRNA genomes [59], the present investigation has revealed an overarching prevalence of mycoviruses featuring ssRNA genomes. In this study, mycoviruses with +ssRNA genomes belonged to the families *Mitoviridae*, *Endornaviridae*, and *Virgaviridae*. Additionally, a mycovirus with a ssRNA genome showing similarity to members of *Mymonaviridae* was discovered in the transcriptomic datasets from *C. albifundus*. Consequently, this study underscores the suitability of leveraging RNA sequencing (RNA-seq) for the detection of +ssRNA mycoviruses, which have been underrepresented in the past.

The *Mitoviridae* viral family includes mitochondrially replicating viruses with +ssRNA genomes, typically ranging from 2.0 to 4.5 kbp in size [59]. These viruses also have simple genomes with a single ORF, which encodes for an RdRp protein [57]. This RdRp protein typically exhibits a molecular weight ranging from approximately 80 to 97 kDa and includes six conserved amino acid domains [57, 67-69]. The ORF is also flanked by 5' and 3' untranslated regions (UTRs), with varying lengths observed among different mitoviruses [7072]. Seven mitoviruses exhibiting these distinctive characteristics were identified within the transcriptomic datasets analysed in this study. Among these, three were isolated from *C. manginecans*, two from *C. eucalypticola*, and two from *C. fimbriata*.

The occurrence of multiple stem-loop structures within both the 3' and 5' UTRs represents another prevalent feature among mitoviruses [71, 73-75]. Moreover, these UTRs frequently exhibit reverse complementarity, thereby facilitating the formation of a panhandle structure [71, 73, 75]. Notably, in the context of this investigation, most of the surveyed mitoviruses adhered to this distinctive trait. However, one notable exception was observed in the case of CmRV-3, where the 5' and 3' UTRs displayed limited complementarity, consisting of only two base pairs, and possessing a free energy value of -1.10 kcal/mol. Studies suggest that these secondary RNA structures may serve as recognition sites for RdRps, which are pivotal components in viral replication and translation [67]. Additionally, they may play a role in safeguarding viral RNA from ribonucleases [67]. However, the absence of a panhandle structure has been documented for some mitoviruses [76-78], indicating that the stem-loop structures may carry greater significance in this context.

Another prominent feature of mitoviruses is their unique capability to make use of the mitochondrial genetic code. Given that mitoviruses replicate within the mitochondria of their fungal hosts, they have evolved to adapt to this specific environment. Within this context, the universal stop codon, UGA, assumes the role of encoding tryptophan [79]. The mitoviruses examined in this study also utilized this code, as predictions using the conventional genetic code yielded truncated and fragmented ORFs, whereas the mitochondrial genetic code produced intact and uninterrupted ORFs.

The viral genus previously recognized as '*Mitovirus*' has recently undergone reclassification as an independent family, a decision made by the International Committee on Taxonomy of iruses (ICTV). This revision has given rise to the establishment of a distinct taxonomic family referred to as *Mitoviridae*, which has been separated from the *Narnaviridae* family. This taxonomic

restructuring has further led to the delineation of four newly designated genera within the *Mitoviridae* family, namely *Duamitovirus*, *Kvaramitovirus*, *Triamitovirus*, and *Unuamitovirus* (https://ictv.global/taxonomy). Phylogenetic analysis of the mitoviruses investigated in this study has revealed their clustering with two of these newly defined genera, specifically *Unuamitovirus* and *Duamitovirus*.

Precise species demarcation criteria within the family *Mitoviridae* have not yet been definitively defined. Nonetheless, the ICTV guidelines stipulate that putative RdRp protein amino acid sequence identities between variants belonging to the same mitovirus species should exceed a threshold of 90%. To evaluate these viruses according to these criteria, an amino acid percent identity matrix was constructed, comparing the mitoviruses of interest with a select group of their closest relatives. As anticipated, it was observed that CfRV-4 and CfRV5 exhibited an amino acid percent identity exceeding or very close to 90% when compared to the RdRp proteins of UMVcefi1 and MVcefi1, respectively. This finding suggested that CfRV4 and CfRV-5 were either the same virus or two distinct variants of the same virus, a conclusion consistent with the characterization of these viruses in the study by Espinal et al. [44]. It is noteworthy that, in terms of genome size, CfRV-4 and CfRV-5 displayed only slightly larger genomes than the endornaviruses described by Espinal et al. [44]. Notably, the incorporation of a ribo-depletion step during the library selection process did not appear to yield discernible differences, at least concerning the mitoviruses investigated within the scope of this study. Nevertheless, this marks the first report of mitoviruses in *C. eucalypticola* and *C. manginecans*.

Intriguingly, the percentage identities between CeRV-2 and CmRV-2 exceeded 90% when compared to CfRV-5 and to each other. Likewise, CeRV-1 exhibited a percent identity exceeding 90% when compared to CfRV-4. This observation suggests the possibility of these viruses being either identical or representing different strains of the same viral species. Such an observation is unusual, given that these fungi are not known to share a common plant host or ecological niche, thus minimizing opportunities for cross-species interaction.

A plausible explanation for these findings might be linked to the laboratory environment and the utilization of techniques such as carrot baiting. Carrot baiting is a widely used method for the isolation of *Ceratocystis* spp. from environmental samples [80-82]. Worth mentioning is the fact that mitoviruses, historically confined to filamentous fungi, have, until recently, remained exclusively within this fungal domain. However, recent investigations have unveiled the presence of mitoviral-like sequences within the nuclear genomes of plants, and, in some

instances, nearly complete mitoviral sequences have been identified in the transcriptomes of various plant species [83, 84]. For example, Nibert et al. [84] reported the discovery of multiple nearly complete mitoviral genomes within the transcriptomes of various land plants, including agriculturally significant crops such as beet and hemp.

It is plausible, then, that a mitovirus might have been inadvertently transferred from the carrot used as bait to the different *Ceratocystis* isolates used in this study. Intriguingly, CmRV-1, CfRV-4, and CeRV-1 also exhibit close genetic relationships to Thielaviopsis basicola mitovirus, which was initially isolated from a strain of this fungus found on carrot roots [85]. The proposition that mitoviruses may be capable of crossing from plants to fungi, potentially serving as a plant defense mechanism against fungal pathogens, holds particular relevance [79]. This notion gains credence, considering that a significant number of mitoviruses are able to induce hypovirulence in their fungal hosts [72, 78, 85-88]. Moreover, the discovery of fungal mitoviruses which are related to plant mitochondrial genomes, lends further support to this concept [72, 86]. To confirm the presence of these viruses in the original cultures, a confirmation PCR will be essential. Subsequent studies should also investigate whether these viruses may have originated from the carrots, an inquiry that holds crucial implications for the use of such techniques in laboratory experiments.

CeRV-3, a +ssRNA virus identified in *C. eucalypticola*, was found to exhibit a close relationship with virga-like viruses and other members of the *Virgaviridae* family. Members of *Virgaviridae* are characterized by genomes typically spanning from 6.3 to 13 kb in length, with some genera exhibiting multiple genome segments [89]. The genomes of virgaviruses consistently features a large ORF with key functional domains, notably the Methyltransferase (Mtr) and Hel domains [90-93]. In addition, RdRp proteins are a common feature in these viruses, and they may be encoded on separate genomic segments or on the same segment as the aforementioned ORF [90-94]. The genomes of some virgaviruses also encode for a movement protein (MP) or CP [89]. While *Virgaviridae* members primarily infect plants, certain species within this family can also be transmitted by nematode or plasmodiophorid vectors [89, 95-98]. Notably, recent research has also unveiled the presence of virgaviruses in fungal transcriptomes [43, 99-102]. For example, the virga-like viruses Armillaria borealis mycovirgavirus 1 and Auricularia heimuer mycovirgavirus 1 was discovered in the fungal transcriptomes of *Armillaria borealis* and *Auricularia heimuer*, respectively [101, 102].

The vast majority of virga-like viruses found in fungi share striking similarities in genome organization with members of the *Tobamovirus* genus, encoding essential components such as Mtr, Hel, RdRp, and a MP [101, 102]. In contrast, CeRV-3 has a distinct genomic structure. It consists of a single ORF, encoding Hel and RdRp domains but lacking the Mtr domain. Furthermore, CeRV-3 does not encode either a MP or a CP, which deviates from the genomic features observed in other virgaviruses associated with fungi. As suggested by Linnakoski, Sutela, Coetzee, Duong, Pavlov, Litovka, Hantula, Wingfield and Vainio [102], the presence of these genes in fungal virga-like viruses could signify vestiges of their evolution from plant virgaviruses. In the context of plant viruses, the MP and CP are critical facilitators of viral movement within and between plant cells [103, 104]. Conversely, the majority of mycoviruses lack these proteins, since their primary mode of transmission occurs intracellularly through hyphal anastomosis and sporulation, thereby rendering these genes superfluous [30]. Consequently, it is possible that CeRV-3 lost these genes over time, as they were unnecessary for the replication and maintenance of this virus.

Phylogenetic analysis revealed that CeRV-3 forms a cluster with an unclassified virga-like virus, Plasmopara viticola lesion-associated virus, and other +ssRNA viruses. Notably, this virga-like virus does not demonstrate phylogenetic clustering with members of either the *Pomovirus* or *Tobamovirus* genera, precluding its classification within any pre-existing genus within *Virgaviridae*. Furthermore, CeRV-3 does not exhibit substantial sequence similarity to other closely related virga or virga-like viruses. CeRV-3 is thus a putatively novel virga-like virus which may belong to an unclassified genus in this family, or potentially to an unclassified viral family which may be related to *Virgaviridae*.

Six +ssRNA mycoviruses belonged to the family *Endornaviridae* and were identified in the transcriptomes associated with *C. fimbriata*, *C. manginecans*, and *C. albifundus*. This family is characterized by mycoviruses with relatively long genomes, ranging in size from 9.7-17.6 kb [58]. Typically, the genomes of endornaviruses feature a single large ORF, in which an RdRp domain is omnipresent [58]. Additionally, the ORF of some endornaviruses contain Gtf, Hel, Mtf, capsular polysaccharide synthase, and phytoreo_S7 domains, though the presence of these domains is not universal among all endornaviruses [105-109]. Furthermore, a series of poly (C) nucleotide sequences is a common feature within the genomes of the majority of endornaviruses [110, 111]. The endornaviruses CaRV-2, CmRV-4, CfRV-1, CfRV-2, and CfRV3 collectively displayed these common characteristics. Although Hel, Gtf and RdRp domains

appears to be common in mycoviral genomes [105, 106, 112, 113], the presence of an AAA domain in CfRV-1 is rare but has been reported in certain endornaviruses. This domain constitutes a part of a RecD helicase, a member of the DEAD-like helicase superfamily. This helicase has been identified in other endornaviruses such as members of the species *Chalara elegans endornavirus 1*, and other mycoviruses such as Rhizoctonia cerealis alphaendornavirus1084−7 (RcEV-1084−7), RcEV-10125−1, and RcEV-0942−1 [114, 115].

Phylogenetic analysis revealed that all endornaviruses evaluated in this study clustered with the *Alphaendornavirus* genus. In this genus, species differentiation necessitates that members of distinct species exhibit an overall nucleotide identity of less than 75% [58]. Notably, two of the endornaviruses, namely CaRV-2 and CmRV4, meet these criteria, designating them as putatively novel members of the *Endornaviridae* family, likely belonging to the *Alphaendornavirus* genus. Conversely, the endornaviruses found in the datasets of *C. fimbriata*, namely CfRV-1, CfRV-2, and CfRV-3, do not meet this species differentiation criterion and share identical characteristics with the viruses previously documented by Espinal, et al. [44]. However, it's worth noting that the endornaviruses identified by these researchers were all significantly truncated, with much shorter genomes than the same endornaviruses which were characterized in this study. This discrepancy raises the possibility that the datasets used by Espinal, et al.[44], which involved a poly-(A) selection step during library preparation, mostly excluded endornavirus polyadenylated genomes, resulting in truncated contigs. In contrast, the utilization of a ribodepletion step during library creation, as employed in the *C. fimbriata* datasets used in this study, likely led to the assembly of complete genomes for these viruses. This is attributed to the selective removal of ribosomal RNA molecules, which constitute a substantial proportion of total RNA, from the sample prior to NGS. This method effectively accommodates the sequencing of ssRNA viruses lacking a poly(A) tail, as demonstrated by the findings of Visser, et al. [116]. As part of this study, an amino acid percent identity matrix was also constructed to compare the amino acid identities of all endornaviruses identified in this study to other cognate viruses. This revealed that there was no significant similarity between the closely related endornaviruses and the endornaviruses from this study. It is important to note however, that the endornaviruses discovered by Espinal, et al. [44] were not incorporated in this analysis due to their truncation.

To comply with the ICTV guidelines for the official taxonomic placement of these viruses, it is essential to provide the complete genome sequence of the virus in question [117]. In this

study, the completeness of the CfRV-1, CfRV-2, and CfRV-3 genomes were determined by employing a 3' and 5' RACE PCR. These methods are widely recognized and commonly employed for uncovering the terminal ends of viral genomes, especially when dealing with novel viruses or incomplete viral sequences [118-121]. For CfRV-1, CfRV-2, and CfRV-3, RACE PCRs did not result in the extension of the viral genome, but rather confirmed their completeness.

It's important to note that RACE PCRs can only reveal the terminal ends of the genome. For viruses with severely truncated genomes, such as CaRV-1, CaRV-4, and the dsRNA mycoviruses analysed in this study, alternative methods are required. In the case of CaRV-1 and CaRV-4, the truncation of these viruses may have occurred due to a variety of reasons, including low viral abundance in host cells and the presence of regions in the genome with high GC content, which may introduce biases during sequencing and may lead to truncation. The truncation of viruses like CaRV-1 and CaRV-4 may be attributed to various factors, including low viral abundance within host cells and the presence of genomic regions with high GC content [122]. The latter can introduce biases during sequencing, potentially leading to genome truncation. To obtain more comprehensive genomes for such viruses, it may prove beneficial to enhance the coverage by incorporating multiple fungal transcriptomic datasets for individual isolates into the assembly. For addressing genomes with elevated GC content, the use of library preparation kits specifically optimized for high GC samples.

As previously mentioned, all totiviruses from this study were significantly truncated. The *Totiviridae* family typically consists of genomes ranging from 4.0 to 7.6 kb in length and includes two major ORFs [59, 123]. The first ORF typically encodes for a CP, while the second ORF encodes for an RdRp protein [123]. All three totiviruses identified in this study had genomic lengths shorter than those expected for members of this family. Although these three totiviruses contained an ORF encoding a complete RdRp protein, they lacked the ORF responsible for encoding a CP. Phylogenetic analysis revealed that these three viruses clustered with members of the *Victorivirus* genus within the *Totiviridae* family, suggesting a potential association with this genus. The close relationship to the previously mentioned victoriviruses was further affirmed through a multiple RdRP sequence alignment. Notably, this protein exhibited eight conserved domains, akin to those found in other victoriviruses [124, 125].

It's worth noting that a pseudoknot, typically located upstream of the RdRp-containing ORF, plays a role in the stop/restart translation of most victoriviruses [126]. Given that complex

secondary structures can sometimes hinder the reverse transcription step during library preparation [127, 128], it's plausible that the separation of the CP and RdRp-containing ORF occurred during this process. Supporting this hypothesis, genome segments encoding for a CP were also identified in these datasets. These segments may belong to the aforementioned three totiviruses and could have become separated during library preparation. To address this issue, potential solutions include optimizing the reaction conditions for the reverse transcription step. This involves utilizing higher temperatures to denature these secondary structures before reverse transcription and employing thermostable reverse transcriptase to aid in resolving these structures [128].

Nonetheless, it's important to note that all the totiviruses examined in this study retained an intact RdRp domain, enabling a comparison of their RdRp sequences with those of closely related viruses. This comparative analysis was accomplished through the construction of a percent identity matrix. The delineation of species within *Victorivirus* lacks precise definition, but it typically necessitates a percent identity of less than 60% to the RdRp of closely related viruses to be considered a novel species. As expected, CfRV-6 displayed 100% identity with a victorivirus referred to as VVf, which had been previously characterized by Espinal, et al. [44]. The victorivirus from this study also exhibited truncation, with separate RdRp and CP components. Thus, the inclusion of a ribo-depletion step during library preparation in the present study did not appear to have a significant impact on the assembly of totivirus genomes in the present study. The other totiviruses discovered in this study, CeRV-6 and CeRV-7, exhibited percent identities that correspond to the maximum 60% identity criterion established for distinguishing different species within the genus *Victorivirus*. Therefore, they are considered novel members of the family *Totiviridae* and likely belong to the genus *Victorivirus*.

The investigation of all four fungal genera in this study unveiled the presence of co-infecting viruses. Co-infections of mycoviruses within a single fungal host result in a spectrum of effects on the host fungus. Remarkably, certain co-infecting viruses exhibit synergy, intensifying their impact on the fungal host. In specific cases, this synergy leads to a reduction in host pathogenicity, thereby mitigating the harm caused to the plant host, as documented in certain studies [129, 130]. Moreover, research has shown that co-infections can influence the transmission dynamics of mycoviruses. For instance, a study by Wu, et al. [131] showed that Sclerotinia sclerotiorum mycoreovirus 4 (SsMYR4) downregulates cellular activities and pathways associated with vegetative incompatibility-mediated programmed cell death between

incompatible fungi. This downregulation, in turn, facilitates the horizontal transmission of other hypovirulent co-infecting viruses to the incompatible fungus. Mycoviruses with these traits hold immense potential as biocontrol agents, however, more research is required to fully understand the mechanisms behind these phenomena. It is plausible that mycoviruses identified in this study may similarly influence their fungal hosts. However, it is important to underscore that more extensive research is warranted to fully elucidate the potential effects of the mycoviruses on their fungal host, their interactions, and their mechanisms of action.

Taken together, the present study has revealed the presence of co-infecting ss-and-dsRNA viruses in *C. albifundus*, *C. fimbriata*, *C. manginecans*, and *C. eucalypticola* and the first identification of mycoviruses in the latter three species. While prior studies have already identified some mycoviruses in *C. fimbriata*, our research has contributed to a comprehensive characterization of three previously discovered endornaviruses within *C. fimbriata*, thus deepening our insights into their genome organization. As a result, this study has illuminated the extent of mycoviral diversity within *Ceratocystis*. Moreover, this investigation has provided valuable insights into the broader implications of mycoviral diversity in the *Ceratocystidaceae* family, demonstrating its association with a wide range of mycoviruses. These findings carry significant implications for the study of fungal pathogens within this family and offer the potential for developing strategies to manage or harness these viruses for various applications, spanning from biotechnology to agriculture.

Conclusion

In conclusion, the rapid evolution of NGS technologies has led to an exponential increase in the discovery and genomic characterization of mycoviruses. This investigation was primarily dedicated to the identification and characterization of mycoviruses within the fungal genus *Ceratocystis*, situated within the Ceratocystidaceae family. This research led to the identification a variety of co-infecting mycoviruses, mainly ssRNA viruses from families *Mymonaviridae*, *Mitoviridae* (*Unuamitovirus* and *Duamitovirus* genera), and *Endornaviridae* (*Alphaendornavirus* genus). Additionally, a putative +ssRNA virga-like virus was discovered but could not be definitively classified into a family or genus. A limited number of dsRNA viruses were also detected, belonging to the *Totiviridae* family. An in-depth analysis of three endornaviruses also revealed larger genomes compared to identical viruses described in other research, highlighting the effectiveness of ribo-depletion methods for studying mycoviruses with large genomes. Interestingly, identical mitoviruses were found in the *C. fimbriata*, *C.*

manginecans, and *C. eucalypticola* datasets, possibly indicating cross-species transmission during laboratory procedures like carrot baiting. This underscores the importance of evaluating viral sources in experimental settings. Overall, this study enhances our understanding of mycoviral diversity in *Ceratocystis* and raises questions about their origins.

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Supplementary Information

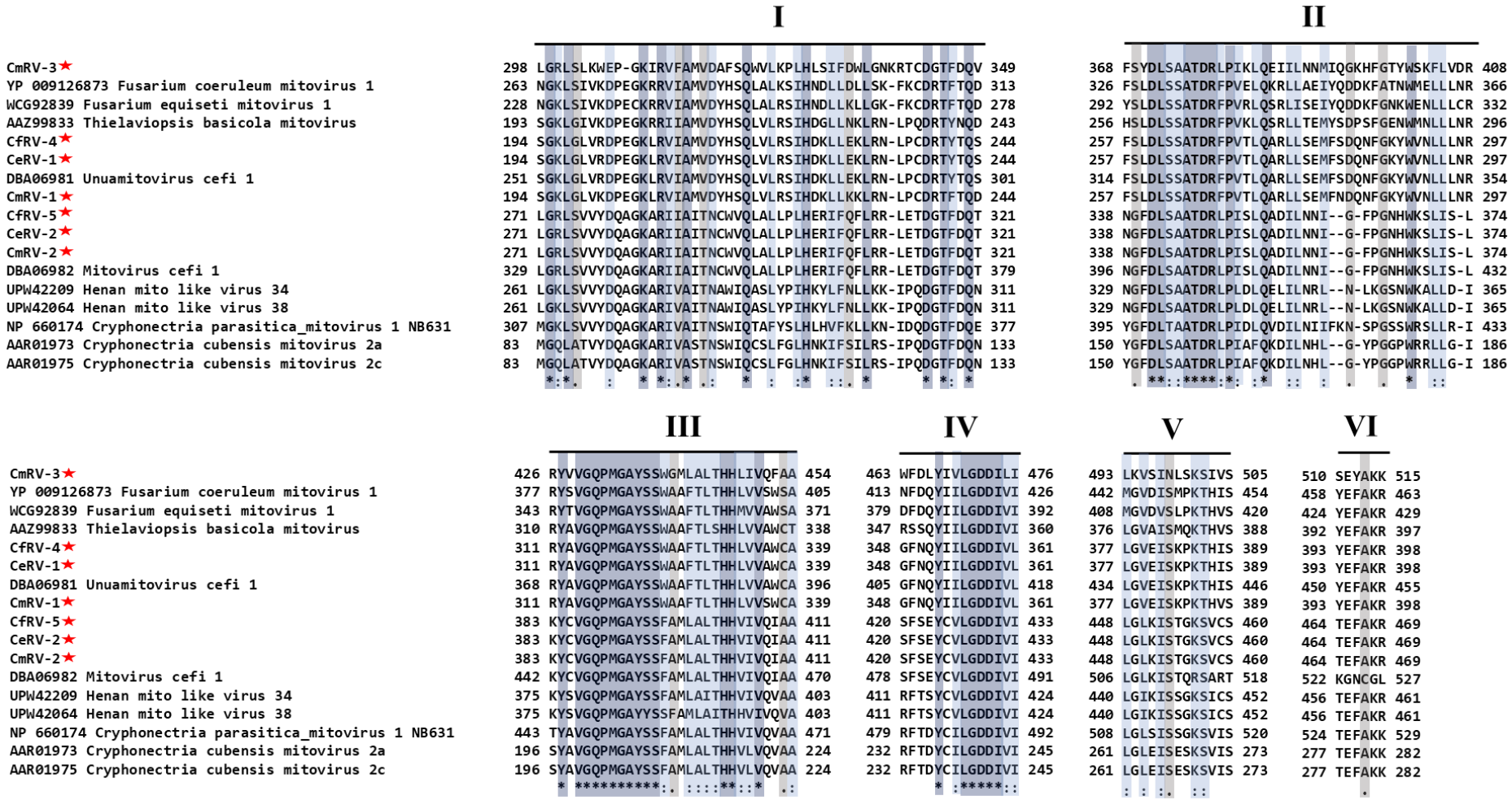


Fig S1. Multiple sequence alignment of the RdRp amino acid Sequences from the mitoviruses investigated in this study (indicated with red stars), and other selected viruses from *Mitoviridae*. The alignment was generated using Clustal Omega 1.2.1 with default parameters. Conserved residues are denoted by an asterisk (*) and are highlighted in dark blue. Colons (:) and dots (.) signify conserved and semi-conserved residues, respectively, and are coloured light blue and grey. There were 6 conserved motifs in total, and these are numbered from I to VI.

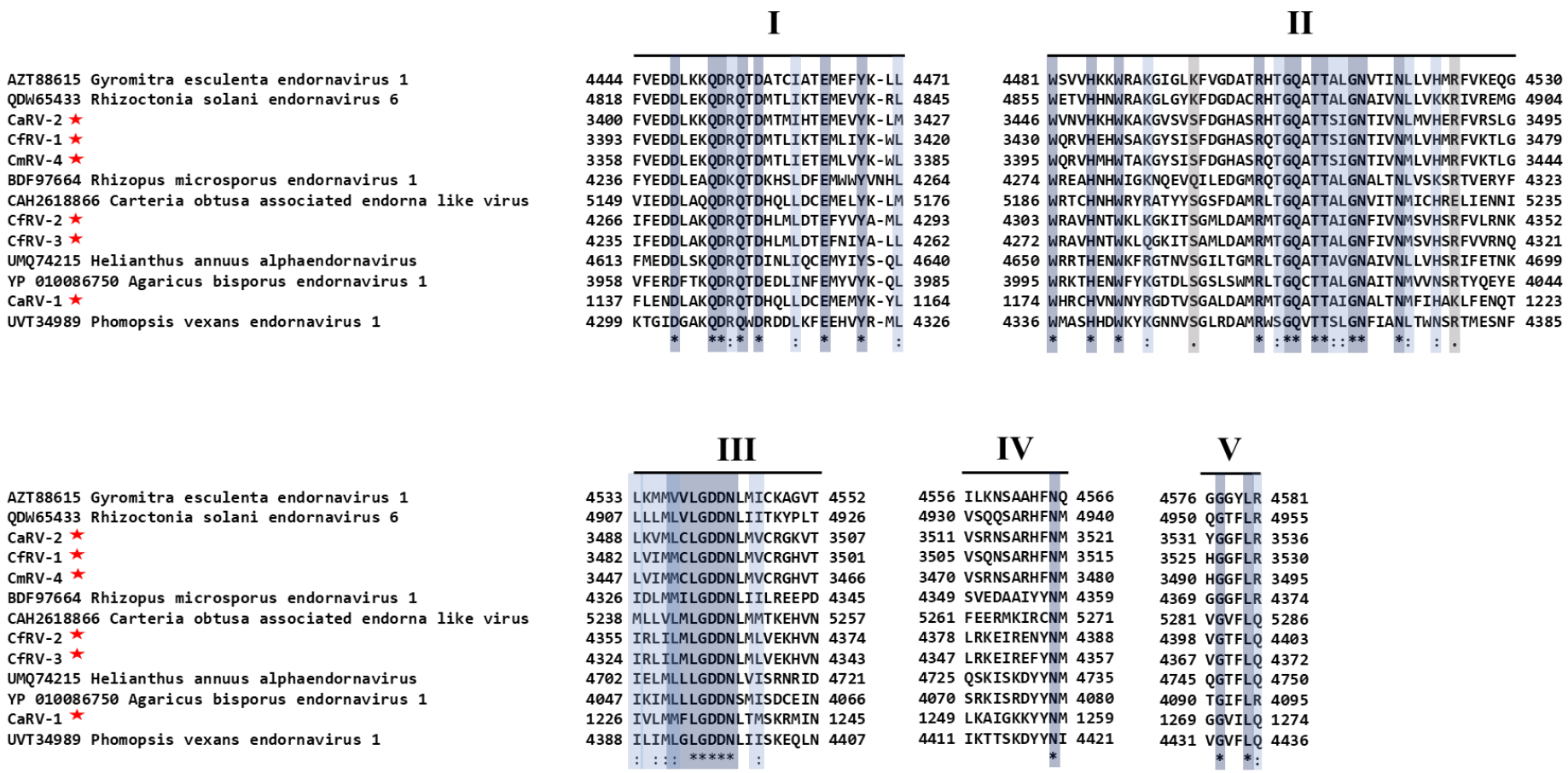


Fig S2. Multiple sequence alignment of the RdRp amino acid Sequences from the endornaviruses (indicated with red stars) investigated in this study, and other selected viruses from *Endornaviridae*. The alignment was generated using Clustal Omega 1.2.1 with default parameters. Conserved residues are denoted by an asterisk (*) and are highlighted in dark blue. Colons (:) and dots (.) signify conserved and semi-conserved residues, respectively, and are coloured light blue and grey. There were 5 conserved motifs in total, and these are numbered from I to V.

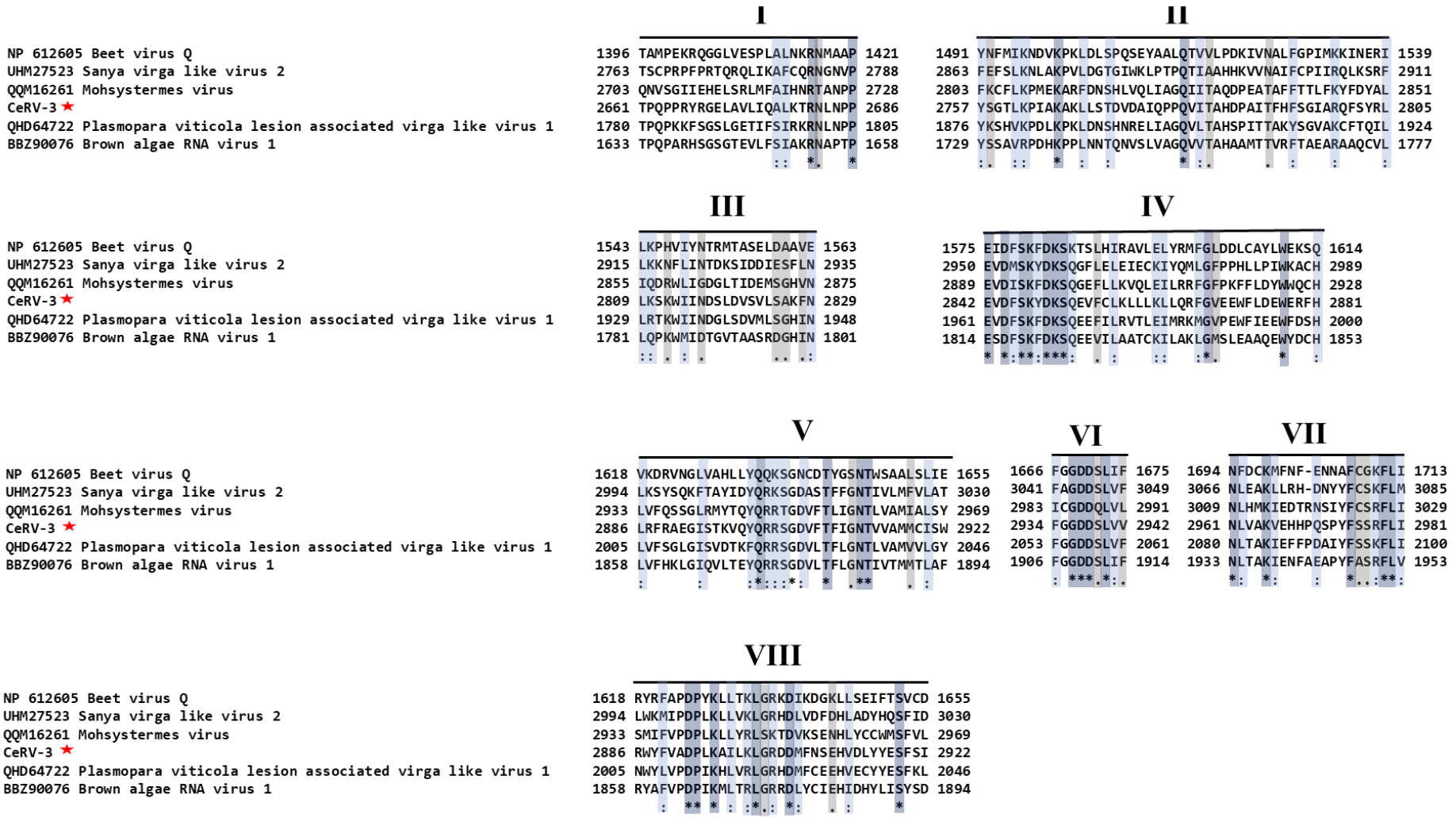


Fig S3. Multiple sequence alignment of the RdRp amino acid Sequences from the virgavirus (indicated with a red star) investigated in this study, and other selected viruses from *Virgaviridae*. The alignment was generated using Clustal Omega 1.2.1 with default parameters. Conserved residues are denoted by an asterisk (*) and are highlighted in dark blue. Colons (:) and dots (.) signify conserved and semi-conserved residues, respectively, and are coloured light blue and grey. There were 8 conserved motifs in total, and these are numbered from I to VIII.

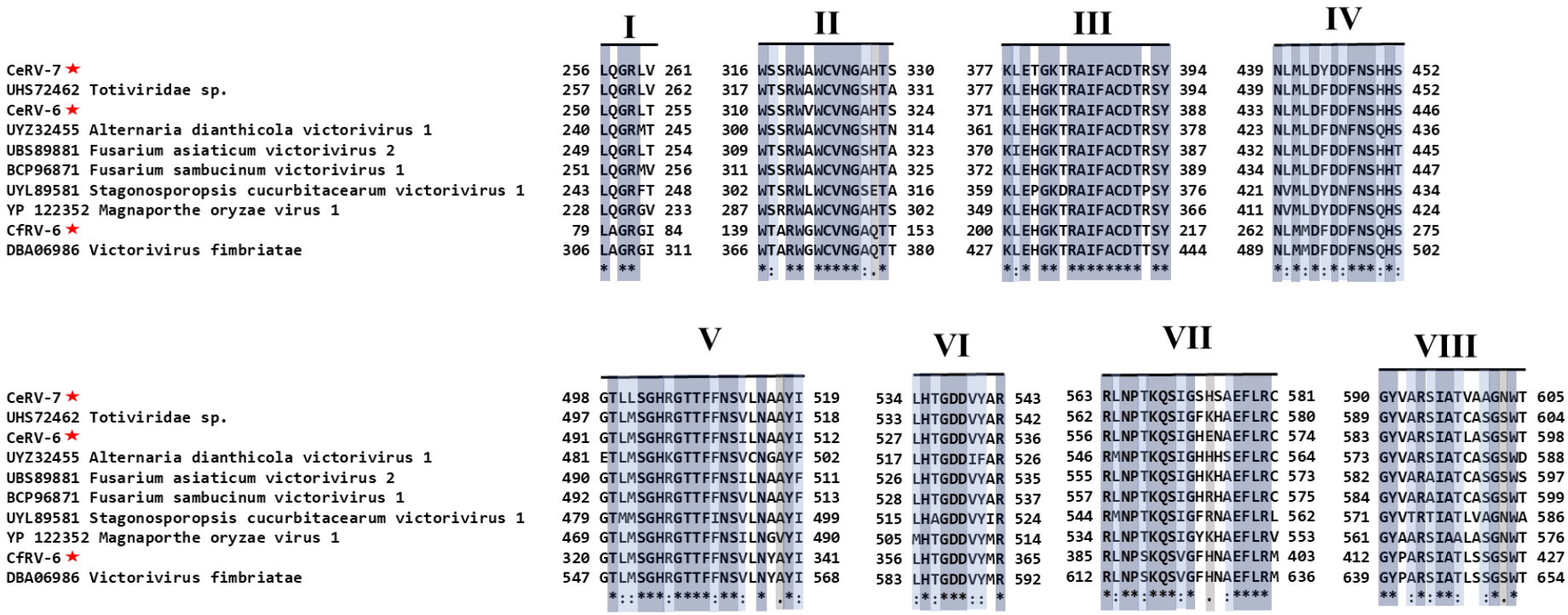
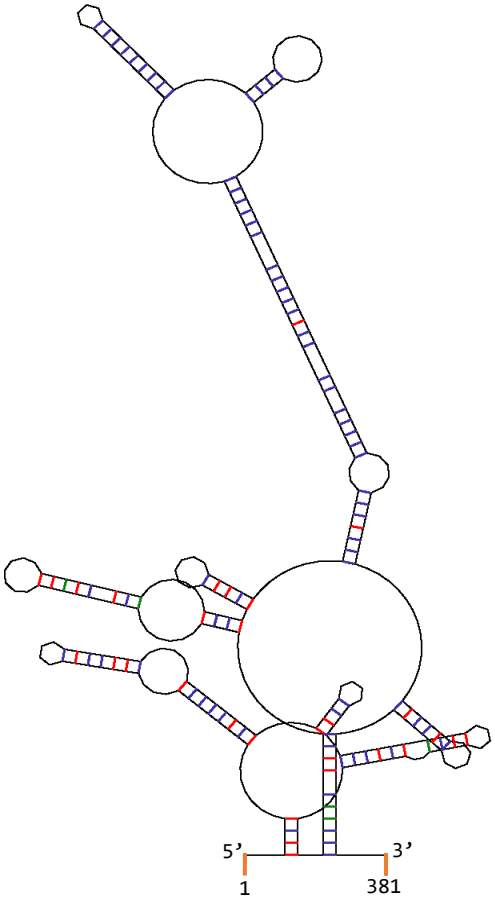
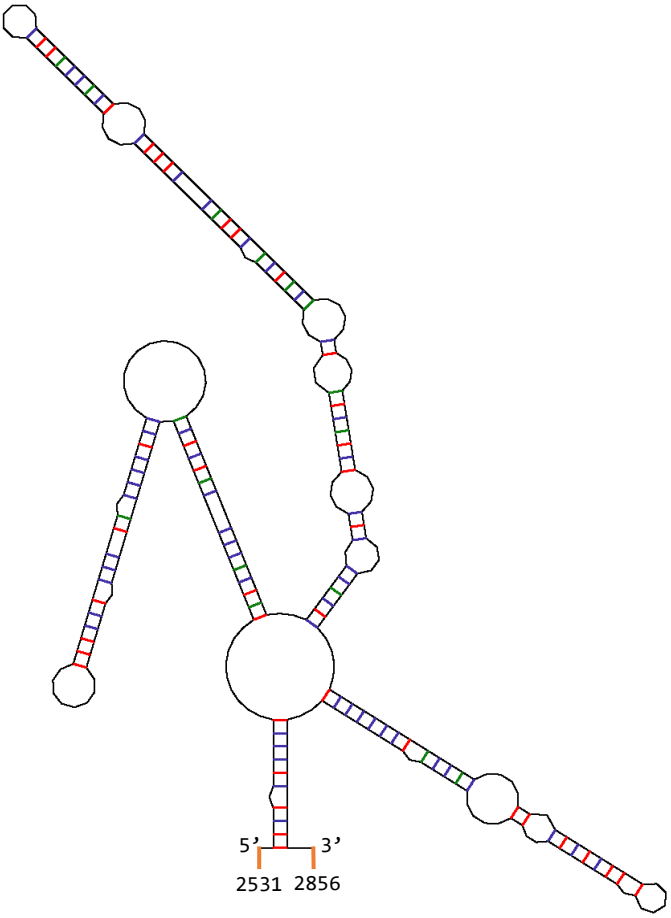


Fig S4. Multiple sequence alignment of the RdRp amino acid Sequences from the totiviruses (indicated with red stars) investigated in this study, and other selected viruses from the genus *Victorivirus*. The alignment was generated using Clustal Omega 1.2.1 with default parameters. Conserved residues are denoted by an asterisk (*) and are highlighted in dark blue. Colons (:) and dots (.) signify conserved and semi-conserved residues, respectively, and are coloured light blue and grey. There were 8 conserved motifs in total, and these are numbered from I to VIII.

A: E = -73.73 kcal/mol



B: E = -101.45



C: E = -4.00 kcal/mol

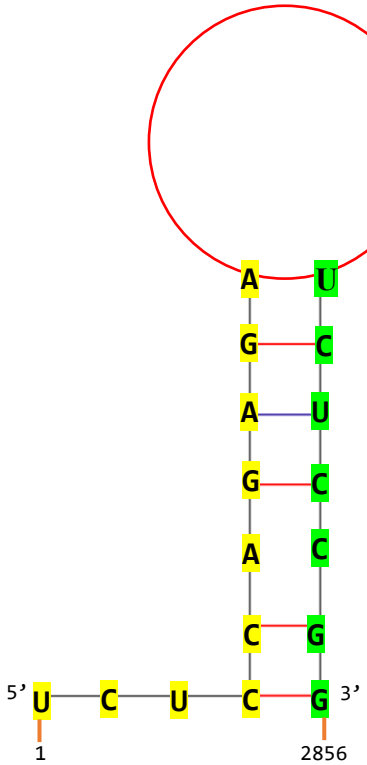


Fig. S5 Predicted secondary structures of the CmRV-1 5' (A) and 3' (B) terminal UTRs, as well as the predicted panhandle structure (C) formed through complementary pairing between the two UTRs. Nucleotide positions 1 and 2856 represent the first and last nucleotide in the 5’ and 3’ UTRs, respectively. In the panhandle structure, complementary bases between sections of the 5' and 3' UTRs are highlighted: yellow for the 5' UTR and green for the 3' UTR. For the 3’ UTR, complementary base pairing starts at position 2850, and ends at position 2856. Complementary base pairing for the 5’ UTR starts at position 4 and ends at position 10. The red circle (Not drawn to scale) represents the inner RNA Molecule. The secondary structures were predicted using Mfold.

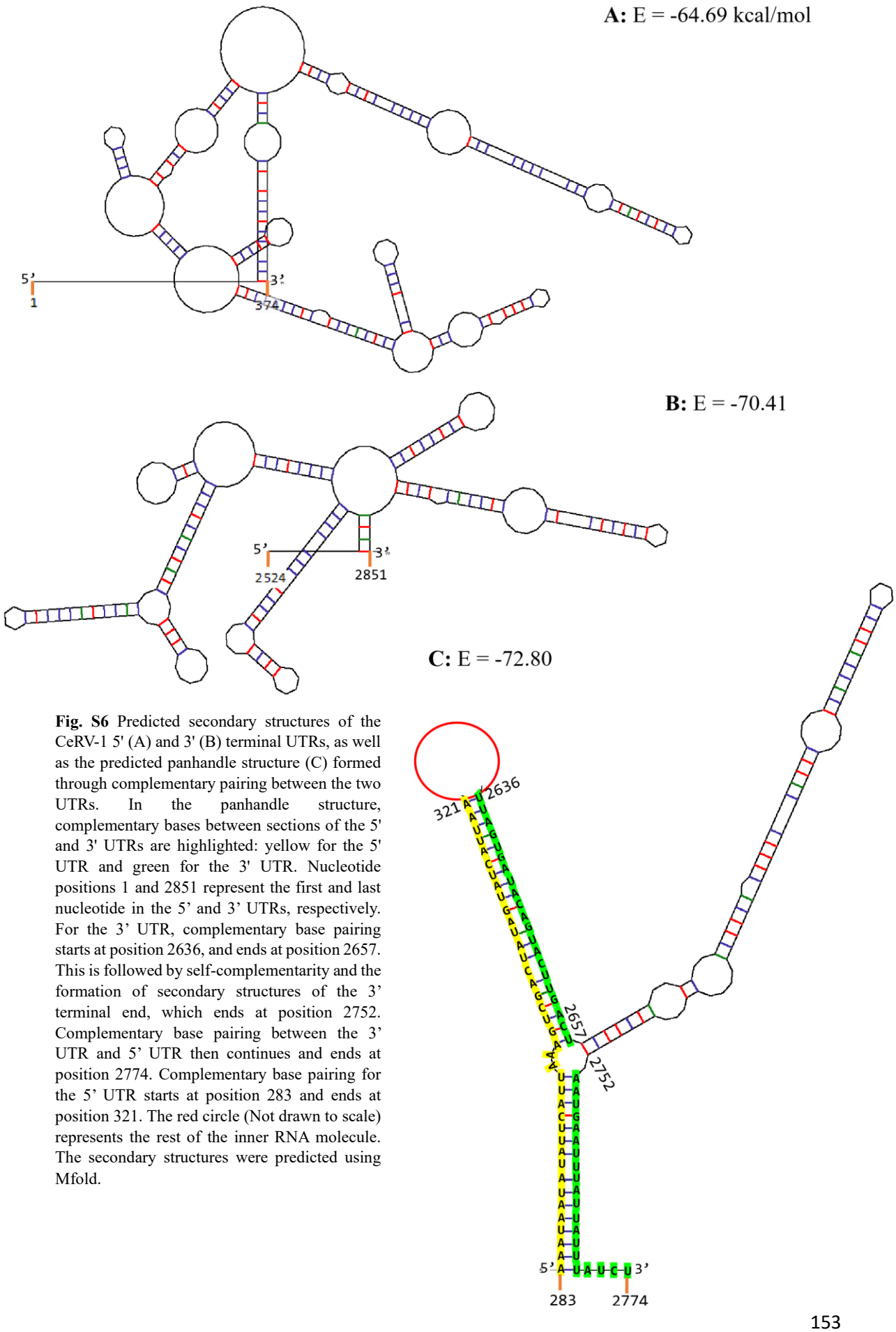


Fig. S6 Predicted secondary structures of the CeRV-1 5' (A) and 3' (B) terminal UTRs, as well as the predicted panhandle structure (C) formed through complementary pairing between the two UTRs. In the panhandle structure, complementary bases between sections of the 5' and 3' UTRs are highlighted: yellow for the 5' UTR and green for the 3' UTR. Nucleotide positions 1 and 2851 represent the first and last nucleotide in the 5' and 3' UTRs, respectively. For the 3' UTR, complementary base pairing starts at position 2636, and ends at position 2657. This is followed by self-complementarity and the formation of secondary structures of the 3' terminal end, which ends at position 2752. Complementary base pairing between the 3' UTR and 5' UTR then continues and ends at position 2774. Complementary base pairing for the 5' UTR starts at position 283 and ends at position 321. The red circle (Not drawn to scale) represents the rest of the inner RNA molecule. The secondary structures were predicted using Mfold.

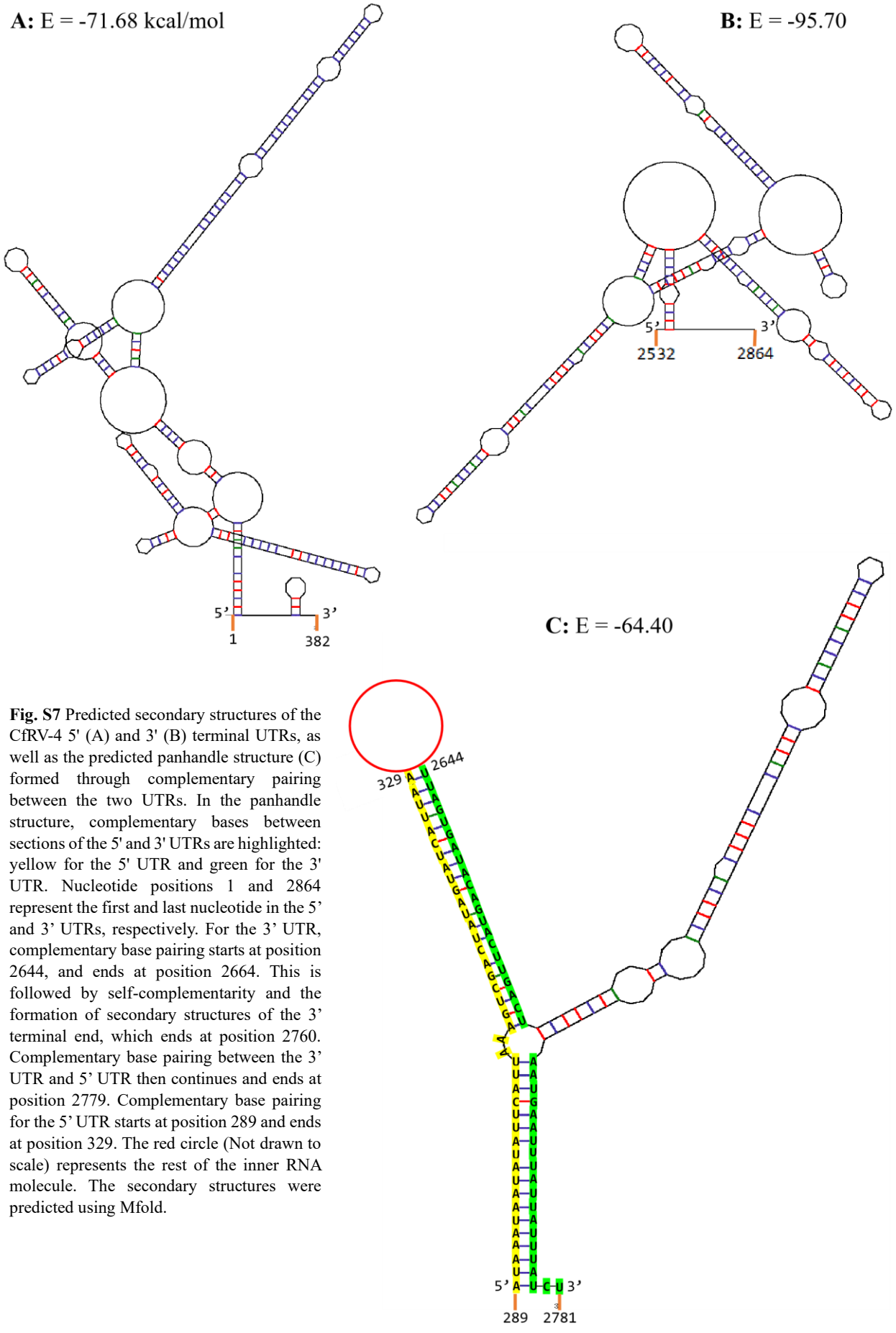
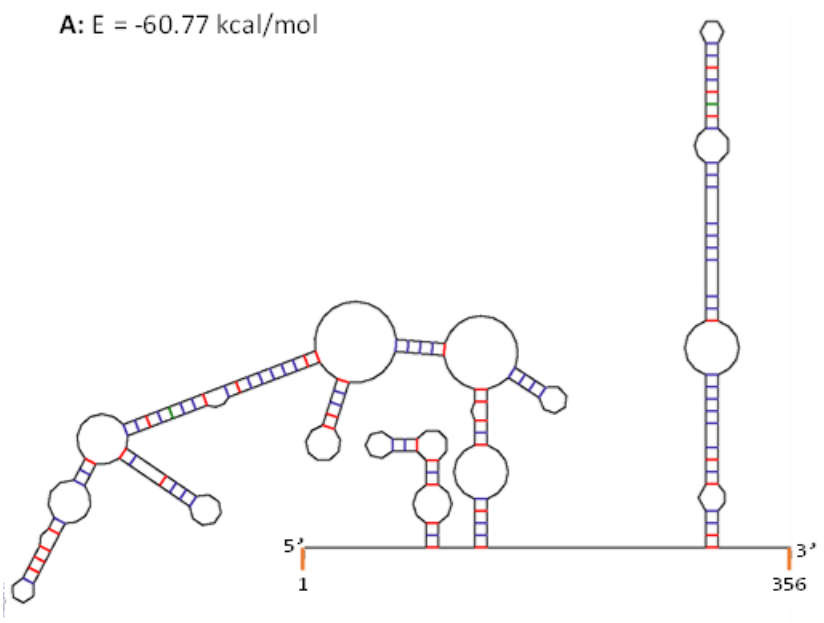
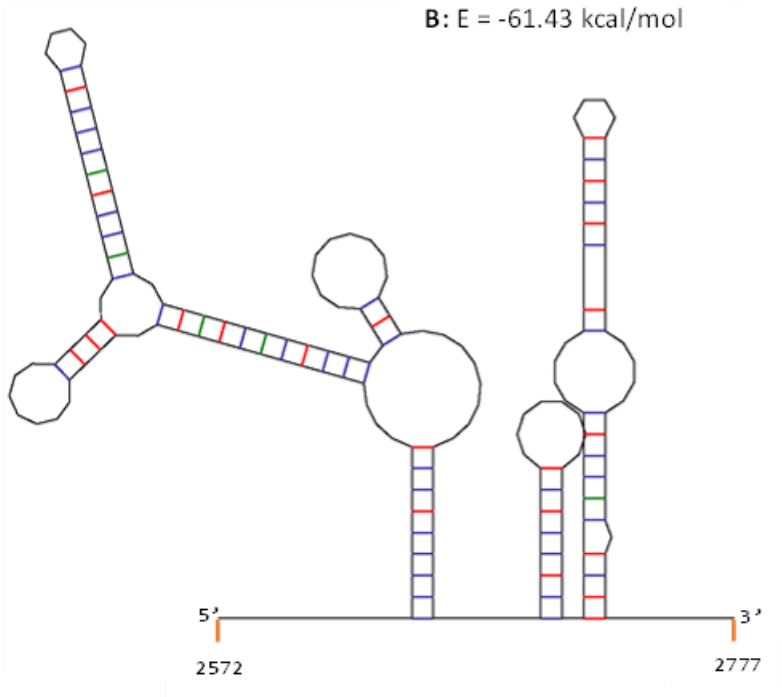


Fig. S7 Predicted secondary structures of the CfRV-4 5' (A) and 3' (B) terminal UTRs, as well as the predicted panhandle structure (C) formed through complementary pairing between the two UTRs. In the panhandle structure, complementary bases between sections of the 5' and 3' UTRs are highlighted: yellow for the 5' UTR and green for the 3' UTR. Nucleotide positions 1 and 2864 represent the first and last nucleotide in the 5' and 3' UTRs, respectively. For the 3' UTR, complementary base pairing starts at position 2644, and ends at position 2664. This is followed by self-complementarity and the formation of secondary structures of the 3' terminal end, which ends at position 2760. Complementary base pairing between the 3' UTR and 5' UTR then continues and ends at position 2779. Complementary base pairing for the 5' UTR starts at position 289 and ends at position 329. The red circle (Not drawn to scale) represents the rest of the inner RNA molecule. The secondary structures were predicted using Mfold.





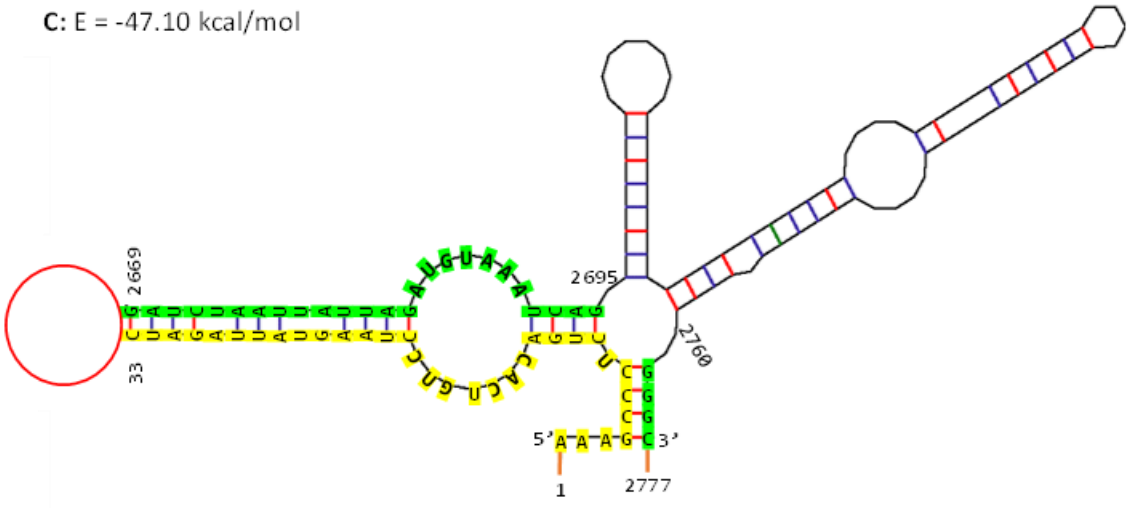
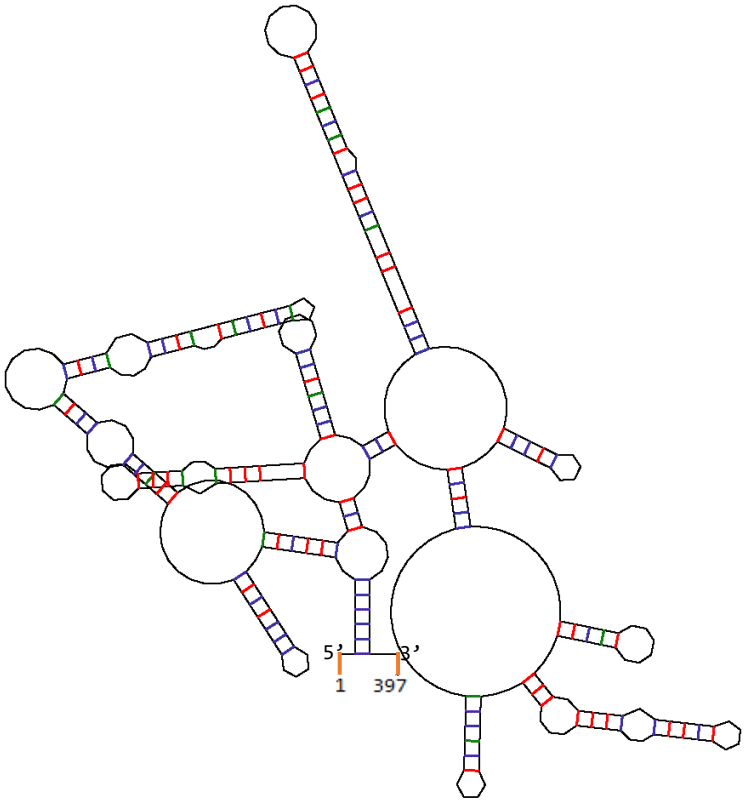
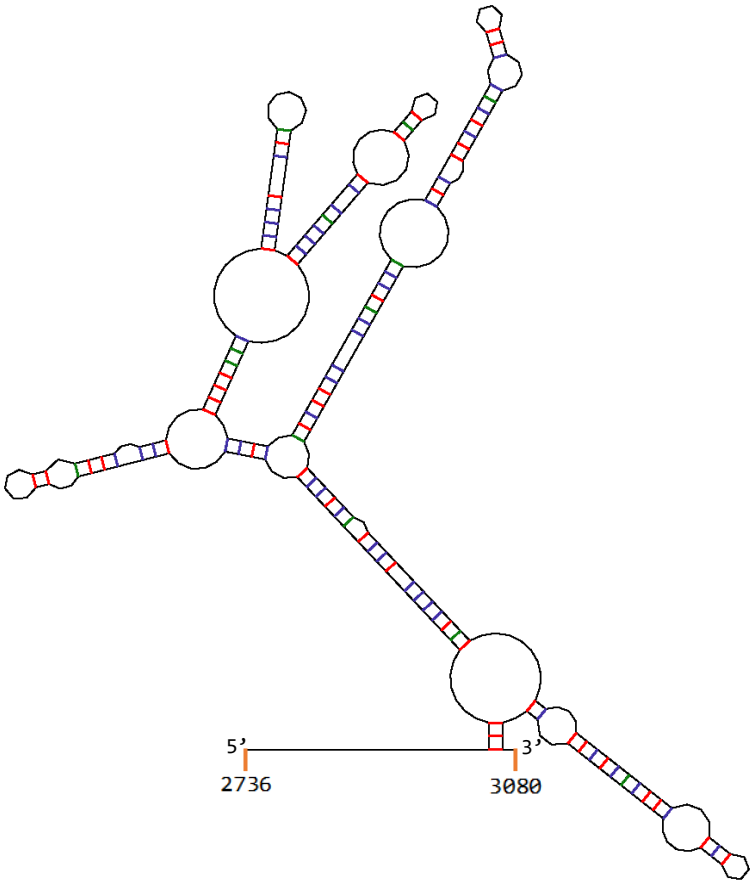


Fig. S8 Predicted secondary structures of the CmRV-2 5' (A) and 3' (B) terminal UTRs, as well as the predicted panhandle structure (C) formed through complementary pairing between the two UTRs. In the panhandle structure, complementary bases between sections of the 5' and 3' UTRs are highlighted: yellow for the 5' UTR and green for the 3' UTR. Nucleotide positions 1 and 2777 represent the first and last nucleotide in the 5' and 3' UTRs, respectively. For the 3' UTR, complementary base pairing starts at position 2669, and ends at position 2694. This is followed by self-complementarity and the formation of secondary structures of the 3' terminal end, which ends at position 2760. Complementary base pairing between the 3' UTR and 5' UTR then continues and ends at position 2772. Complementary base pairing for the 5' UTR starts at position 4 and ends at position 33. The red circle to the left (not drawn to scale) represents the rest of the inner RNA molecule. The secondary structures were predicted using Mfold.

A: E = -100.82 kcal/mol



B: E = -93.19 kcal/mol



C: E = -1.10 kcal/mol

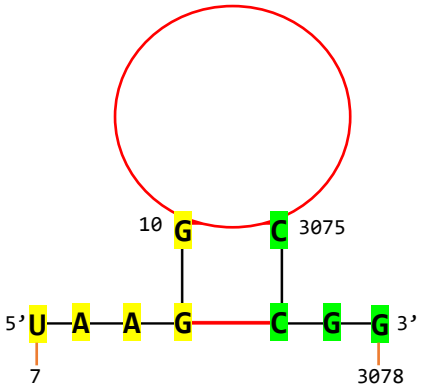


Fig. S9 Predicted secondary structures of the CmRV-3 5' (A) and 3' (B) terminal UTRs, as well as the predicted panhandle structure (C) formed through complementary pairing between the two UTRs. In the panhandle structure, complementary bases between sections of the 5' and 3' UTRs are highlighted: yellow for the 5' UTR and green for the 3' UTR. Nucleotide positions 1 and 3080 represent the first and last nucleotide in the 5’ and 3’ UTRs, respectively. For the 3’ UTR, complementary base pairing starts at position 3075, and ends at position 3076. Complementary base pairing for the 5’ UTR starts at position 9 and ends at position 10. The red circle (not drawn to scale) represents the rest of the inner RNA molecule. The secondary structures were predicted using Mfold.

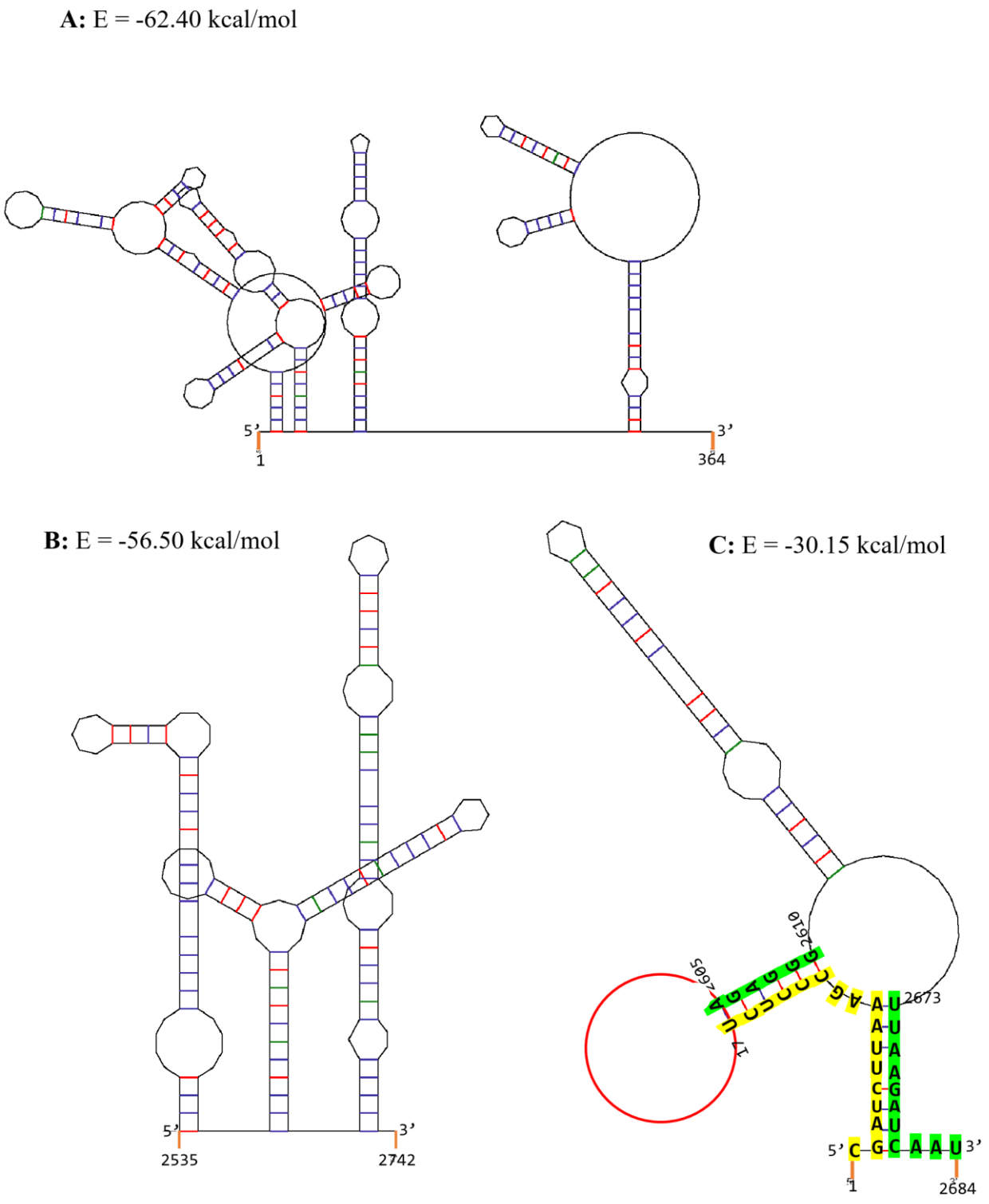


Fig. 10 Predicted secondary structures of the CeRV-2 5' (A) and 3' (B) terminal UTRs, as well as the predicted panhandle structure (C) formed through complementary pairing between the two UTRs. In the panhandle structure, complementary bases between sections of the 5' and 3' UTRs are highlighted: yellow for the 5' UTR and green for the 3' UTR. Nucleotide positions 1 and 2742 represent the first and last nucleotide in the 5' and 3' UTRs, respectively. For the 3' UTR, complementary base pairing starts at position 2605, and ends at position 2610. This is followed by self-complementarity and the formation of secondary structures of the 3' terminal end, which ends at position 2660. Complementary base pairing between the 3' UTR and 5' UTR then continues and ends at position 2684. Complementary base pairing for the 5' UTR starts at position 2 and ends at position 17. The red circle (not drawn to scale) represents the rest of the inner RNA molecule. The secondary structures were predicted using Mfold.

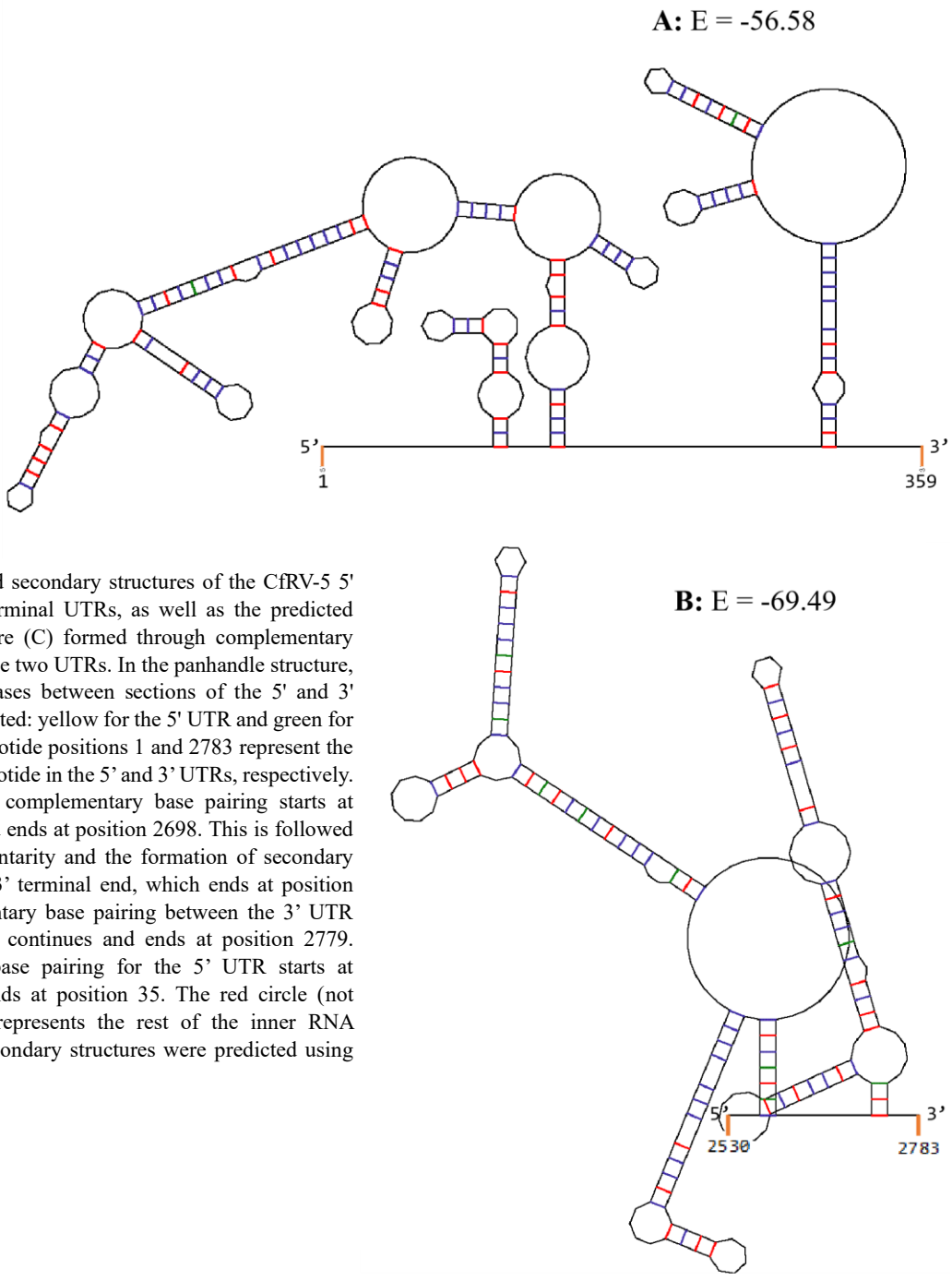


Fig. S11 Predicted secondary structures of the CfRV-5 5' (A) and 3' (B) terminal UTRs, as well as the predicted panhandle structure (C) formed through complementary pairing between the two UTRs. In the panhandle structure, complementary bases between sections of the 5' and 3' UTRs are highlighted: yellow for the 5' UTR and green for the 3' UTR. Nucleotide positions 1 and 2783 represent the first and last nucleotide in the 5' and 3' UTRs, respectively. For the 3' UTR, complementary base pairing starts at position 2672, and ends at position 2698. This is followed by self-complementarity and the formation of secondary structures of the 3' terminal end, which ends at position 2774. Complementary base pairing between the 3' UTR and 5' UTR then continues and ends at position 2779. Complementary base pairing for the 5' UTR starts at position 3 and ends at position 35. The red circle (not drawn to scale) represents the rest of the inner RNA molecule. The secondary structures were predicted using Mfold.

Summary

Plant pathogenic fungi represent a significant global threat, causing substantial economic losses in both agricultural crops and forests. Fungicide use, while common, faces challenges due to fungal resistance, as well as concerns over human and environmental health. Mycoviruses offer eco-friendly alternatives since some can attenuate fungal pathogenicity. However, despite their potential, mycoviruses remain underexplored in various fungal families and genera. This dissertation aimed to address this knowledge gap by focusing on the underrepresented fungal family Ceratocystidaceae. The first part of the study involved the identification and characterization of mycoviruses in publicly available fungal transcriptomes from this family. Datasets from public repositories often undergo poly-A selection during library preparation, resulting in the underrepresentation of mycoviruses lacking a poly-A tail, and potentially leading to truncation of some mycoviruses. To overcome this limitation, the second chapter of the study utilized ribo-depletion during library preparation to generate fungal transcriptomes for members of the genus *Ceratocystis*. This approach was able to resolve the complete genomes of three endornaviruses in *Ceratocystis fimbriata*, which had remained unresolved in earlier research. Overall, this investigation led to the identification of 28 mycoviruses across nine fungal species. The majority of these mycoviruses were single-stranded RNA viruses, and were tentatively classified into the viral families *Mitoviridae*, *Mymonaviridae*, and *Endornaviridae*, while one virga-like virus remains unclassified. The study also revealed double-stranded RNA mycoviruses in some fungal transcriptomes, which were putatively classified into the *Totiviridae* family. This research marks the first identification of mycoviruses in *Thielaviopsis ethacetica*, *Thielaviopsis paradoxa*, two distinct isolates of *Huntiella omanensis*, *Ceratocystis platani*, *Ceratocystis eucalypticola*, *Ceratocystis manginecans*, and *Ceratocystis albifundus*. Equally noteworthy is the discovery of identical mitoviruses in two fungal species from *Thielaviopsis*, as well as within three different fungal species from *Ceratocystis*, despite these occupying different ecological niches and plant hosts. While the transmission mechanism of these viruses remains uncertain, the data implies a need to assess isolation procedures, like carrot baiting, as potential sources of viral transmission in experiments. This dissertation significantly advances our knowledge of mycoviral diversity within the Ceratocystidaceae family and raises intriguing questions about the origins of these viruses. While more tests are needed to precisely determine the effects of these mycoviruses on their hosts, the insights obtained here provide a basis for future research into biocontrol strategies against these plant pathogenic fungi.