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The mycovirome of a fungal collection from the sea cucumber *Holothuria polii*

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ABSTRACT

Holothuria polii is a marine animal with an important ecological and economic impact. In the present study we analysed the presence of mycoviruses associated to fungi that were isolated from different *H. polii* tissues. Among the 48 fungal isolates analysed we identified 10 viruses in 8 strains belonging to 7 fungal species. Five out of nine viruses have a dsRNA genome: three of them belong to the *Partitiviridae* family, one belongs to a still undefined clade of bipartite viruses and the last one belongs to the *Chrysoviridae* family. We also identified two viruses belonging to a recently proposed new mycovirus taxon named polymycovirus. Two viruses belong to the positive single stranded RNA clade: one falls into the new *Botourmiaviridae* family, specifically in the *Magoulivirus* genus, and the other one falls into a still undefined clade phylogenetically related to tombusviruses. Finally, we also identified a virus with a negative stranded RNA genome showing similarity to a group of viruses recently proposed as a new family of mycoviruses in the order *Bunyavirales*. A bioinformatics approach comparing two datasets of contigs containing two closely related mycobunyaviruses allowed us to identify putative nucleocapsids (Nc) and non-structural (Ns) associated proteins.

The GenBank/eMBL/DDBJ accession numbers of the sequences reported in this paper are: PRJNA432529, MG913290, MG913291, MG887747, MG887748, MG887749, MG887750, MG887751, MG887752, MG887753, MG887754, MG887755, MG887756, MG887757, MG887758, MG887759, MG887760, MG887761, MG887762, MG887763, MG887764, MG887765, MG887766, MG887767, MH271211, MN163273, MN163274.

1. Introduction

Since mycoviruses were discovered as pathogens of cultivated mushrooms (Gandy, 1960; Hollings, 1962), many studies on the importance of these microorganisms were published. The best studied phenotype given by mycoviruses to their fungal host is hypovirulence as in the case of CHV1- *Cryphonectria parasitica* interaction (Chen et al., 1994; Turina and Rostagno, 2007), but cases of mycovirus-caused hypervirulence were also reported (Chun et al., 2018; Lau et al., 2018; Jian et al., 1997; Okada et al., 2018). Nevertheless, more recently, after the seminal work of Marquez and coauthors (Marquez et al., 2007) reporting the role of a mycovirus infecting an endophytic fungus which results in adaptive advantages to the plant host in high temperature soils, several studies on the possible ecological significance of mycoviruses have been carried out (Marzano and Domier, 2016; Nerva et al., 2016). An example of an adaptive ecological advantage provided by a mycovirus was recently shown for an oomycetes interacting with Phytophtora infestans RNA virus 2 (Cai et al., 2019).

The next generation sequencing (NGS) approaches applied to the characterization of the virome from environmental samples (or from specific complex substrates) has greatly added to the known biodiversity of mycoviruses (Marzano et al., 2016; Roossinck, 2015). If from one hand a growing number of new viral genomes are reported, on the other hand some new insights in the biological and ecological relevance of mycovirus-host interaction are now being elucidated. For example, the first single-stranded DNA (ssDNA) mycovirus reported, able to induce hypovirulence in the fungal host *Sclerotinia sclerotiorum* (Yu et al., 2010), displayed also the ability to be infectious as virus particle (Yu et al., 2013), which is a new feature for mycoviruses.

Another important aspect resulting from the complex characterization of viromes associated to classes of organisms is the possibility to better understand the phylogenetic and evolutionary relationship

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among viruses. An example comes from a wide analysis of invertebrate viromes which reported more than 1400 new viral RNA genomes from double-stranded RNA (dsRNA), positive stranded RNA (+sRNA) and the negative-stranded RNA (-sRNA) lineages (Shi et al., 2016). The results coming from this work filled some of the gaps present in the phylogenetic tree and allowed the proposition of new viral families, and more recently of new higher rank taxa that will result in a phylogeny based new taxonomical framework including almost the totality of the RNA viruses (Wolf et al., 2018).

Moreover, the evolutionary history of viruses is an additional fascinating aspect resulting from these studies. There are some viral families, like the *Betaflexiviridae*, the *Partitiviridae*, the *Chrysoviridae* and the *Totiviridae* (Chen et al., 2016; Ghabrial et al., 2008; Li et al., 2016; Roossinck, 2012) which show a mosaic of hosts: some species are able to infect fungi some other are able to infect plants. The promiscuity of these families seems to suggest the possibility of a relatively easy plant-fungal host switch, an intriguing idea if correlated with the fact that very often fungi grow in close proximity with their hosts (both in case of pathogenic and symbiotic fungi) (Roossinck, 2012). This proximity can bring to casual cytosolic exchanges in which viruses can pass form a host belonging to a kingdom to a new host belonging to a different kingdom. We recently demonstrated this possibility, at least from the experimental point of view, showing that some viruses belonging to the *Partitiviridae* and *Totiviridae* families isolated from fungi were able to replicate in different plant cell systems (Nerva et al., 2017b). Plant viruses can also infect fungi naturally as the recently reported case of CMV infecting *Rhizoctonia solani* (Andika et al., 2017).

In a previous work we reported the mycovirome of 91 fungi isolated from the sea grass *Posidonia oceanica*, identifying for the first time the virome from marine fungi (Nerva et al., 2016). Here we wanted to characterize the virome associated to fungi isolated from the marine invertebrate *Holothuria polii*. *H. polii* is a soft-bodied echinoderm displaying a flexible, elongated, worm-like body, with a leathery skin and gelatinous body, looking like a cucumber and this is the reason for its alternative name “sea cucumber”. It is an important animal for the marine ecosystem because it takes part into organic matter recycling present in the sea floor sediments (Bakus, 1968). Moreover, due to its importance as human food source, *H. polii* has a high commercial value: it is particularly appreciated in some parts of Asia where it is eaten raw or cooked (Preston, 1993). Taxonomical and evolutionary aspects of identified viruses are here discussed.

2. Materials and methods

2.1. Fungal isolates and maintenance

Fungal isolates were collected and identified by the *Mycotheca Universitatis Taurinensis* (MUT). Forty-nine fungal isolates were obtained from different tissues of *H. polii*; 39 of them were selected and analyzed with RNAseq to detect possible viral infections (Supplementary Table 1). Isolates that were found to be infected were maintained in liquid or solid cultures at 25 °C using ACM media: 10 g/L glucose, 6 g/L sodium nitrate, 2 g/L peptone, 1 g/L yeast extract, 1.5 g/L casein hydrolysate, 10 ml/L salts solution (140 g/L KH_2PO_4, 90 g/l K2HPO4, 10 g/L KCl, 10 g/L $MgSO_4$), and 1 ml/L trace element solution (ingredients for 100 ml: 0.04 g $Na_2B_4O_7$, 0.4 g $CuSO_4$, 0.8 g $FeSO_4$, 0.8 g $MnSO_4$, 0.8 g $NaMoO_4$, 8 g $ZnSO_4$).

2.2. Total RNA extraction and RNAseq

Total RNA from each fungal isolate was extracted using Total Spectrum RNA reagents (Sigma–Aldrich, Saint Louis, MO, USA). RNAs were quantified with a NanoDrop 2000 Spectrophotometer (Thermoscientific, Waltham, MA, USA). To reduce sequencing costs, we pooled different RNA samples, sequencing more than one isolate per library. Pooled samples are listed in Supplementary Table 2 and were obtained by mixing 1 μg of RNA from each fungal sample. Eight μg from each pool were sent to sequencing facilities (Macrogen, Seoul, Rep. of Korea): ribosomal RNA (rRNA) were depleted (Ribo-ZeroTM Gold Kit, Epicentre, Madison, USA), cDNA libraries were built (TrueSeq total RNA sample kit, Illumina) and sequencing were performed by an Illumina HiSeq4000 system generating paired-end sequences.

2.3. Transcriptome assembly

Bioinformatics analysis of RNAseq raw reads was performed as previously detailed (Matsumura et al., 2017; Nerva et al., 2018b). Briefly, de novo assembly was made with Trinity (2.0.2) (Haas et al., 2013), and assembled contigs were filtered with BLAST (ver. 2.2.30) (Altschul et al., 1997) to search for homology with viral sequences against a custom viral proteins database. BWA (0.5.9) (Li and Durbin, 2009) and SAMtools (0.1.19) (Li et al., 2009) were used to map reads on assembled contigs and visualized with IGV (Thorvaldsdottir et al., 2013); open reading frames were searched with ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/).

2.4. Identification of virus-infected fungal isolates

Quantitative RT-PCR (qRT-PCR) analyses with virus specific primers (Supplementary Table 3) were performed to associate any specific virus assembled *in silico* with the original specific RNA sample. DNA copy was produced using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher scientific, Waltham, MA, USA) following manufacturer instructions. Quantitative RT-PCR were performed using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and iTaq™ Universal Probes Supermix (Bio-Rad Laboratories, Hercules, CA, USA) was used for primers designed with the internal TaqMan probe while iTaq™ Universal SYBR® Green Supermix was chosen where the TaqMan probe was not available.

Infected isolates where used to clone viral genomic cDNA fragments of an average length between 300 and 400 base pairs in order to obtain run off transcripts for strand specific hybridization experiments. For this purpose, cDNA was synthetized using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher scientific, Waltham, MA, USA) with custom designed primers (Supplementary Table 4) and then amplified in a PCR reaction. PCR products were isolated from agarose gel after electrophoresis and purified using Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA, USA). Purified PCR fragments were ligated in pGEM®-T Easy Vector Systems (Promega, Madison, WI, USA). Positive clones with the predicted insert (checked by Sanger sequencing) were used to purify plasmids; the same plasmids were linearized and used as template to synthesize radioactive probes.

2.5. Northern blot analysis

Total RNA was extracted from virus-infected isolates using Total Spectrum RNA reagents (Sigma–Aldrich, Saint Louis, MO, USA). Two μg of total RNA were denatured with glyoxal and then separated on 1% agarose gel in HEPES-EDTA buffer (Sambrook et al., 1989). Separated RNAs were transferred onto a nylon membrane (Immobilon-Ny + Membrane, Merck KGaA, Darmstadt, Germany) and hybridized with a radio-labeled RNA probe as previously detailed (Bertran et al., 2016). RNA probes were prepared through linearization of plasmids containing cDNA clones of portions of the viral genome fragments coding for RdRp. Linearized plasmids were transcribed using Maxiscript T7 kit reagents (Thermo Fisher Scientific Inc., Waltham, MA, USA) following manufacturer instructions.

2.6. Virus particle purification and electron microscopy

Two approaches were used to purify viruses: for most fungal isolates containing ssRNA viruses, dsRNA viruses and minus strand RNA viruses

Table 1
List of the identified viruses with the reference fungal isolate and derived acronym.

Host(s)	Viral name	Genome segment	Accession number	Mapping reads	Av. depth	Contig length
MUT1071 & MUT1105 *Penicillium citrinum*	Penicillium citrinum ourmia-like virus 1 (PcOLV1)	RNA1 (RdRp)	MG887747	2203	87	2528
MUT1443 *Myriodontium keratinophilum*	Myriodontium keratinophilum bipartite virus 1 (MkBV1)	RNA1 (RdRp)	MG887758	14320	677	2113
		RNA2	MG887759	15703	820	1915
MUT1097 *Penicillium brevicompactum*	Penicillium brevicompactum polymycovirus 1 (PbPMV1)	RNA1 (RdRp)	MG887750	14281	601	2400
		RNA2 (Serin protease)	MG887753	10772	481	2259
		RNA3 (MT)	MG887752	7821	405	1930
		RNA4 (PAS rich)	MG887751	39720	3326	1206
	Penicillium brevicompactum ssRNA virus 1 (PbSSRV1)	ORF1 + ORF2 (RdRp)	MG887748	363586	11661	3149
	Penicillium brevicompactum partitivirus 1 (PbPV1)	RNA1 (RdRp)	MG887763	254894	14674	1737
		RNA2 (CP)	MG887764	254323	16530	1538
MUT1993 *Aspergillus spelaeus*	Aspergillus spelaeus polymycovirus 1 (AsPMV1)	RNA1 (RdRp)	MG887754	3443	145	2384
		RNA2 (Serin protease)	MG887757	21828	993	2219
		RNA3 (MT)	MG887756	9408	482	1968
		RNA4 (PAS rich)	MG887755	49843	4216	1194
MUT2096 *Aspergillus ochraceus*	Aspergillus ochraceus virus (AoV)	RNA1 (RdRp)	MG887765	104985	5378	1952
		RNA2 (CP)	MG887766	126307	7899	1599
		RNA3	MG887767	110548	8445	1309
MUT2120 *Penicillium stoloniferum*	Penicillium stoloniferum virus S (PsV-S)	RNA1 (RdRp)	MG913290	97603	5564	1754
		RNA2 (CP)	MG913291	98857	6248	1582
MUT2146 *Penicillium roseopurpureum*	Penicillium roseopurpureum chrysovirus 1 (PrCV1)	RNA1 (RdRp)	MG887760	85039	2049	3565
		RNA2 (CP)	MG887761	68858	2432	2859
		RNA3	MG887762	47345	1639	2917
		RNA4	MH271211	44873	1583	2834
	Penicillium roseopurpureum negative sense RNA virus 1 (PrNSRV1)	RdRp	MG887749	348053	5404	6506

a general particle purification approach was performed as previously described (Nerva et al., 2017a). Briefly, 300 mg of lyophilized mycelia were homogenized in a bead beater (FastPrep24, M.P. Biomedicals, Irvine, CA, USA) with glass and iron beads; after a first run, 10 ml of extraction buffer (0.25 M potassium–phosphate pH 7, 10 mM EDTA, 0.5% thioglycolic acid) were added. Samples were centrifuged at 10,000 *g* for 10 min, the supernatant was added with 1% Triton X-100, 10% PEG8000, and 0.1% sodium chloride. Samples were stirred for one hour (4 °C) and centrifuged for 10 min at 10,000 x *g*, pellets obtained were resuspended in extraction buffer and centrifuged at 180,000 × g on a 20% sucrose layer. Final pellets were resuspended in 300 µl of extraction buffer and stored at −80 °C.

For the two fungal isolates containing the putative polymycoviruses found in our analysis, purification was attempted following a method previously described (Kanhayuwa et al., 2015). Briefly, 50 g of frozen mycelia were homogenized in 100 ml of TE buffer; the lysate was filtered through Miracloth and the liquid was then centrifuged at 10,000 *g* for 20 min. PEG-6000 (final concentration 10%) and NaCl (0.6 M final concentration) were added to supernatant and stirred over-night at 4 °C. The mixture was then centrifuged to collect colloidal material (10,000 *g* for 20 min), pellet was resuspended in TE buffer and clarified with a further low-speed centrifugation step. Colloidal material present in the clarified supernatant was pelleted by ultracentrifugation (105,000 *g* for 90 min), resuspended in 1 ml TE buffer and clarified by a further low-speed centrifugation (10,000 *g* for 20 min). Gradient centrifugation was performed through a cushion of CsCl (density 1.45 g/cm^3) at 55,000 *g* for 90 min. Visible colloidal material was collected and re-pelleted by ultracentrifugation (105,000 *g* for 90 min).

Purified virus particles were observed with a TEM (CM 10 electron microscope, Philips, Eindhoven, The Netherlands). Ten µl of sample were let to adsorb on carbon and formvar-coated grids; the grids were then washed with water and negatively stained with 0.5% uranyl acetate.

2.7. Phylogenetic analysis of viral sequences

Genome segments encoding for RNA dependent RNA polymerase (RdRp) were used to obtain the predicted amino acid sequences and then used for multiple alignments using ClustalW implemented in MEGA 6 (Tamura et al., 2013). Aligned amino acid sequences were used to estimate the best substitution rate models, by using the implemented algorithm in MEGA6: for ourmia-like viruses Le-Gascuel model (Le and Gascuel, 2008) (+G + I) was used; for dsRNA viruses Le-Gascuel model (+Gamma + Freq) was chosen; for -ssRNA viruses Dimmic reverse transcriptase model (Dimmic et al., 2002) (+Gamma + Freq) was used; for + ssRNA and the polymycovirus Le-Gascuel LG model (+G + I) was chosen. Maximum likelihood method (1000 bootstrap replicates) was used to infer phylogenetic relationships. A list of the accession

numbers of the sequences included in all the different trees is shown in Supplementary Table 5.

2.8. Bioinformatics analysis for the identification of possible nucleocapsids associated to bunya-like mycovirus

A bioinformatics strategy was developed with the aim of identifying a putative nucleocapsid associated to bunya-like (-) sRNA viruses. The rationale of our search relies on the possibility that some level of conservation between nucleocapsids (Nc) of closely related bunya-like mycoviruses can be detected by homology search, since no homology can be detected with existing/annotated nucleocapsids from *Bunyavirales* present in databases. We have the advantage of two closely related RdRP present in two distinct datasets: one identified in this paper (cfr. below, result section) and one in a dataset that we recently published (BioProject PRJNA509609, BioSample SAMN10585389, SRA Accession: SRR8303990) containing several mycoviruses, including the phylogenetically related (-) strand RNA virus Coniothyrium diploidella negative-stranded RNAvirus 1 (Accession number MK584854) (Nerva et al., 2019). As a first step, we disposed from both datasets all the assembled contigs matching NCBI nr database (Sep. 2018) in order to discard contigs that are highly conserved with sequences present in the database using DIAMOND (Buchfink et al., 2015). As a second step a tblastx among all the unmatched contigs of the two libraries was performed keeping all the contigs that found a hit using the default parameters. We manually inspected the list keeping only contigs that encoded for protein between 10 and 100 kDa using ORFfinder (https://www.ncbi.nlm.nih.gov/orffinder/). Finally, we submitted possible hits for a blastx search of the nr database (July 8th 2019) to further confirm absence of homology with other protein sequences. Presence of the putative candidates in the mycobunyavirus-infected fungal strains and absence from the other strains in the same library pool was confirmed by qRT-PCR using specific oligonucleotides (Supplementary on line Table 3).

3. Results

3.1. Virome analysis

Raw sequences from each of the RNA pool were analyzed to obtain putative contiguous viral transcripts (contigs). When putative virus sequences were identified, a protocol of quantitative retro transcription PCR (qRT-PCR) was used to detect viral RNA in the infected isolate(s). With this approach we identified 8 fungal isolates harboring viruses: two isolates of *Penicillium citrinum* (MUT1071 and MUT1105), one isolate of *Myriodontium keratinophilum* (MUT1443), one isolate of *P. brevicompactum* (MUT1097), one isolate of *Aspergillus spelaeus* (MUT1993), one isolate of *A. ochraceous* (MUT2096), one isolate of *P. stoloniferum* (MUT2120) and one isolate of *P. roseopurpureum* (MUT2146). Fungal host, main virus features, sequencing results and accession number of identified viruses are summarized in Table 1, Fig.1, Fig.2 and Fig. 3, and Supplementary on line Fig. 1.

Isolates MUT1071 and MUT1105 are infected by a virus related to ourmia-like viruses: the assembled genome is 2528 nucleotides in length, it displays a single ORF from nucleotide 33 to nucleotide 2105 encoding for a putative RNA-dependent RNA polymerase (RdRP) with a predicted molecular weight of 77.6 kDa. The virus is tentatively named Penicillium citrinum ourmia-like virus 1 (PcOLV1) (Fig. 1).

In isolate MUT1443 we identified a virus belonging to a previously characterized group of bipartite mycoviruses (Nerva et al., 2016): RNA1 is 2113 nucleotides in length and displays a single open reading frame (ORF) coding for a putative RdRP with a predicted molecular weight of 74.8 kDa; RNA2 is 1915 nucleotides in length and displays two distinct ORFs in the same frame coding for a protein of 40.2 kDa and 23.8 kDa respectively. These proteins only display limited conservation with homologous proteins in very closely related viruses. For this virus we propose the name Myriodontium keratinophilum bipartite virus 1 (MkBV1) (Fig. 2).

Isolate MUT1097 is infected by three distinct viruses: a virus related to the recently described group named polymycoviruses (Kanhayuwa et al., 2015; Kotta-Loizou and Coutts, 2017; Nerva et al., 2019), a + sRNA virus and a partitivirus. The virus with high similarity to other polymycoviruses is composed by four genomic fragments, each coding for a single ORF: RNA1 is 2400 nucleotides in length and encodes a putative RdRP of 86.1 kDa; RNA2 is 2259 nucleotides in length and it encodes a protein of 77.2 kDa displaying a putative serine protease domain; RNA3 is 1930 nucleotides in length and codes for a protein of 66.6 kDa with a methyl transferase domain; finally, RNA4 is 1206 nucleotides in length and codes for a protein of 29.1 kDa with a proline-alanine-serine (PAS) rich domain (Fig. 1). The virus is named Penicillium brevicompactum polymycovirus 1 (PbPMV1). The + sRNA genome virus found in *Penicillium brevicompactum* MUT1097 displays the highest similarity with Magnaporthe oryzae RNA virus, it is 3149 nucleotides in length and contains two ORFs: ORF1 encodes a hypothetical protein of unknown function of 28.3 kDa whereas ORF2 codes for a putative RdRP of 57.7 kDa (Fig. 1). The virus is named as Penicillium brevicompactum sigle-stranded RNA virus 1 (PbSSRV1). The third virus belongs to the *Partitiviridae* family and is composed of two RNAs each encoding for a single ORF: RNA1 is 1737 nucleotides in length and it codes for a protein of 62.1 kDa a putative RdRP; RNA2 is 1538 nucleotides in length and it codes for a protein of 47.1 kDa, the putative coat protein (CP). For this virus we propose the name Penicillium brevicompactum partitivirus 1 (PbPV1) (Fig. 2).

Isolate MUT1993 is infected by a polymycovirus, distinct from the one identified in MUT1097. The four genome segments display the following characteristics: RNA1 is 2384 nucleotides in length and encodes a putative RdRP of 83.5 kDa, RNA2 is 2219 nucleotides in length and it codes for a protein of 75.6 kDa displaying a putative serine protease domain, RNA3 is 1968 nucleotides in length and codes for a protein of 68.5 kDa with a methyl transferase domain and RNA4 is 1206 nucleotides in length and codes for a protein of 31.6 kDa with a proline-alanine-serine (PAS) rich domain. For this virus we propose the name Aspergillus spelaeus polymycovirus 1 (AsPMV1) (Fig. 1).

Isolate MUT2096 is infected by a member of the species *Aspergillus ochraceus virus*, belonging to the *Partitiviridae* family. It has three genome segments each encoding for a single protein: RNA1 encodes an RdRP, RNA2 encodes a CP and RNA3 codes for a protein with unknown function (Liu et al., 2008) (Supplementary on line Fig. 1).

Similarly, MUT2120, a *Penicillium stoloniferum* isolate, is infected by another partitivirus already described and belonging to the species *Penicillium stoloniferum partitivirus 1* (Supplementary on line Fig. 1). It has two genome segments, the first coding for an the RdRP and the RNA2 coding for the CP (Bozarth et al., 1971).

Isolate MUT2146 hosts two different viruses: a chrysovirus and a virus with a –sRNA genome. The first is composed of 4 genome segments: RNA1 is 3565 nucleotides in length and encodes a protein with a predicted molecular weight of 130.4 kDa with a conserved RdRP domain, RNA2 is 2859 nucleotides in length with a single ORF coding for a putative CP of 99.6 kDa, RNA3 is 2918 nucleotide in length and encodes a single protein, with unknown function, of 103.1 kDa, and RNA4 is 2833 nucleotides in length and codes for a protein of unknown function with a predicted molecular weight of 94.8 kDa. The virus is named Penicillium roseopurpureum chrysovirus 1 (PrCV1) (Fig. 2). Regarding the –sRNA virus, our first approach with our bioinformatic pipeline only identified a contig that is 6505 nucleotides in length, with the presence of a single ORF encoding for a protein with a conserved RdRP domain with a putative molecular weight of 245.3 kDa; for this virus we propose the name Penicillium roseopurpureum negative strand RNA virus 1 (PrNSRV1) (Fig. 3); other segments associated to this genome will be described below in a specific section.

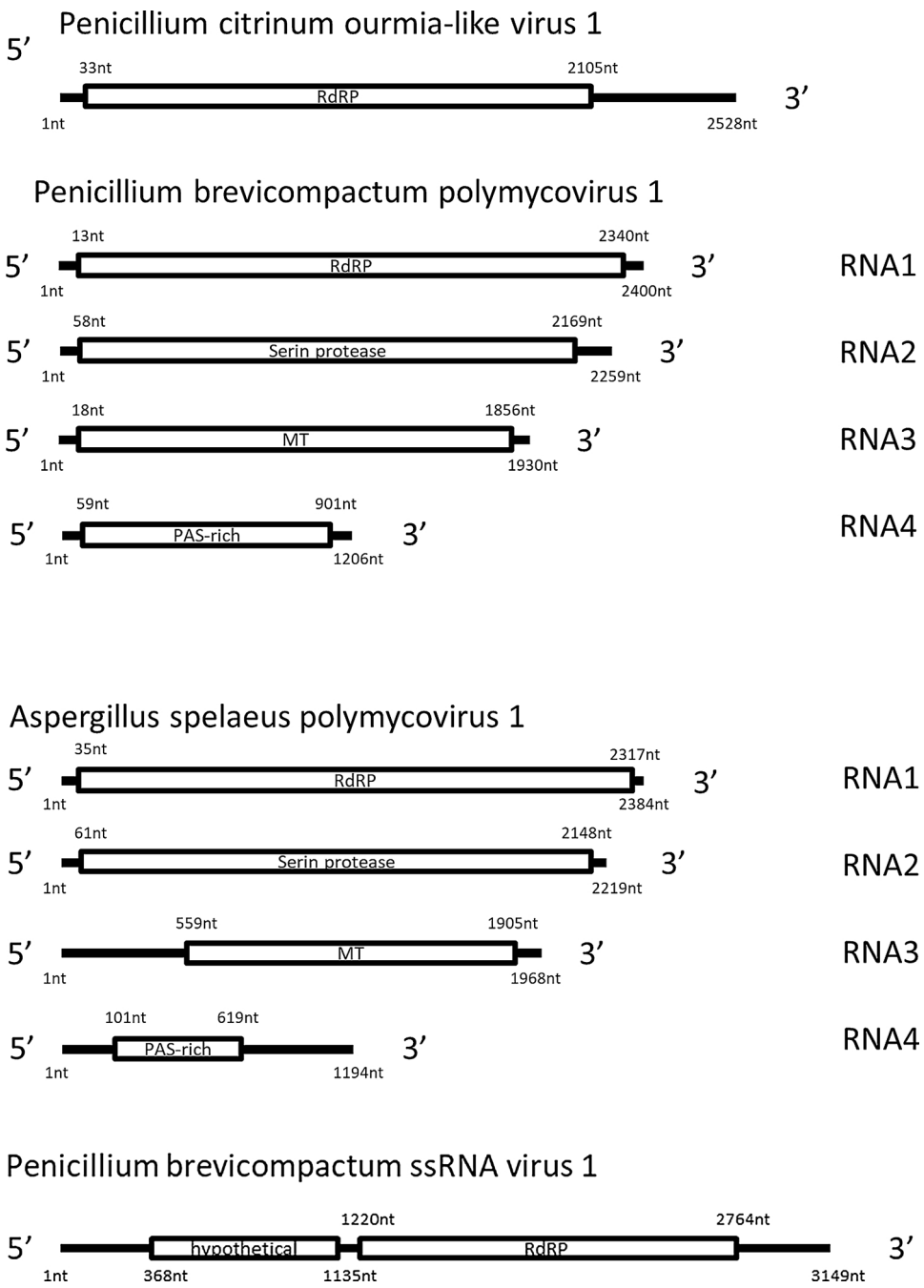

Fig. 1. Schematic representation of the genome organization of the polymycoviruses and single stranded RNA viruses characterized in this study. Open rectangles represent the open reading frame. Black lines represent the genome segments. RdRP = RNA dependent RNA polymerase; MT = methyl transferase domain; PAS = proline-alanine-serine rich domain; nt = nucleotides.

3.2. In vivo validation of assembled viral genomes

In order to confirm the viral sequences assembled in-silico, and to assess their nature of self-replicating RNA viruses we used different approaches. Initially we used a qRT-PCR protocol to confirm the presence of each virus in each fungal subculture. Results confirmed viral infection in all the different subcultures (data not shown). Then we performed PCRs on total RNA without reverse transcription: in this way we wanted to exclude that the viral sequences come from transcripts of DNA integrated into the host fungal genome or as recently shown for mitoviruses of *Gigaspora margarita*, DNA fragments not endogenized (Turina et al., 2018). None of the viruses gave us amplification without retrotranscription (data not shown). We decided then to amplify short fragments (about 400 nucleotides in length) from each of the RdRP sequences which were cloned and sub-sequentially used to derive riboprobes for northern blot analysis. We obtained cloned sequences for MkBV1, AoV, PrCV1, PrNSRV1, PbSSRV1, PbPV1 and PbPMV1 which were used for northern hybridization as shown in Fig. 4.

Northern blot analysis on MkBV1 AoV, PrCV1, PrNSRV1, PbPV1, PbSSRV1, PbTMV1 RdRp sequences confirmed the presence of each virus in the expected sample. Molecular size observed in northern hybridization confirmed the assembled length of each segment and no signal was observed in each negative control. No putative subgenomics bands were observed for any of the virus tested.

A further confirmation of viral infections was obtained through partially purified virus particles for viruses producing virions. In

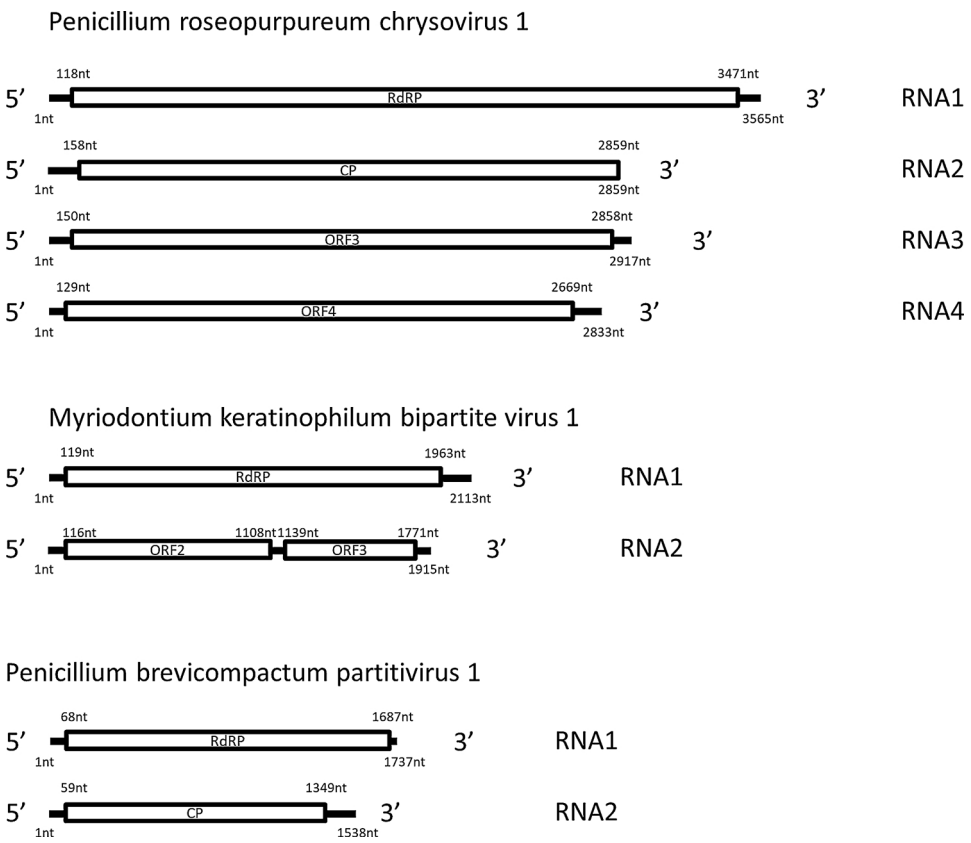

Fig. 2. Schematic representation of the genome organization of the double stranded RNA viruses characterized in this study. Open rectangles represent the open reading frame (ORF). Black lines represent the genome segments. RdRP = RNA dependent RNA polymerase; CP = coat protein; nt = nucleotides.

specific, for AoV and PbPV1 we observed icosahedral virus particles of about 30–37 nm in diameter (Fig. 5A-B) as expected for *Partitiviridae*. For PsV-S we observed particles that appear polygonal in profile, with diameter of about 40 nm (Fig. 5C). Virus particles from MUT2146 revealed the presence of filamentous snake-like particles of about 12 nm in diameter (Fig. 5D) and polygonal particles of about 42 nm attributable to PrCV1 (Fig. 5E). A specific virus purification approach on PbPMV1 and AsPMV1 did not reveal filamentous virions as previously observed with closely related viruses (Kanhayuwa et al., 2015).

3.3. Phylogenetic analysis

Conserved regions of the identified viral polymerases were aligned to similar sequences found in the databases in order to provide a proper taxonomical placement and, secondarily, infer evolutionary relationships. From the aligned amino acid sequences, we derived several phylogenetic trees. In Fig. 6 we can observe the phylogenetic placement of PcOLV1: this virus groups with members of the *Magoulivirus* genus in the recently new accepted family *Botourmiaviridae*. In Fig. 7 the maximum likelihood phylogeny for dsRNA viruses shows that the viruses in this lineage fall in already established group of viruses (*Chrysoviridae*, *Partitiviridae*) with the exception of MkBV1 which groups with a fairly

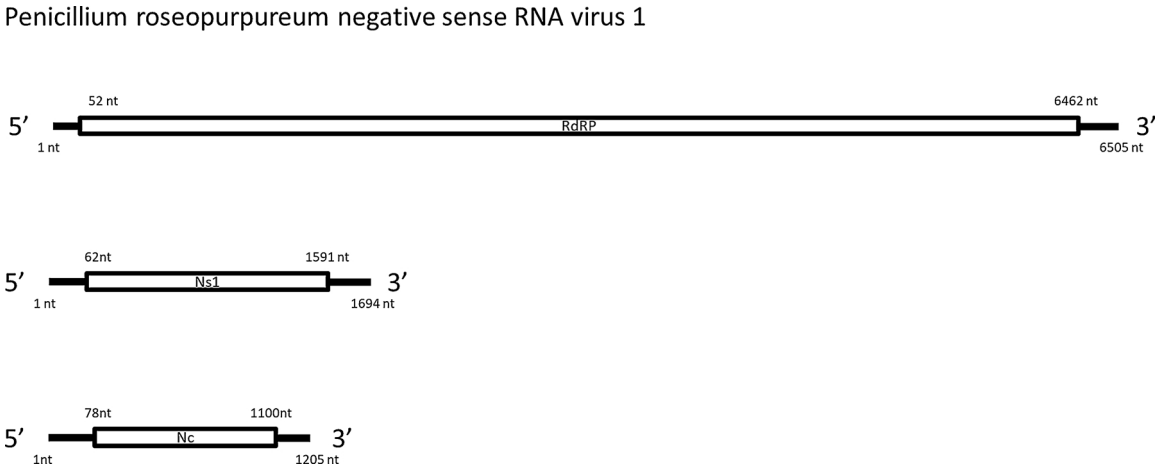

Fig. 3. Schematic representation of the genome organization of Penicillium roseopurpureum negative sense RNA virus 1 (PrNSRV1). RdRP = RNA dependent RNA polymerase; Ns1 = non-structural protein 1; Nc = Nucleocapsid; nt = nucleotides.

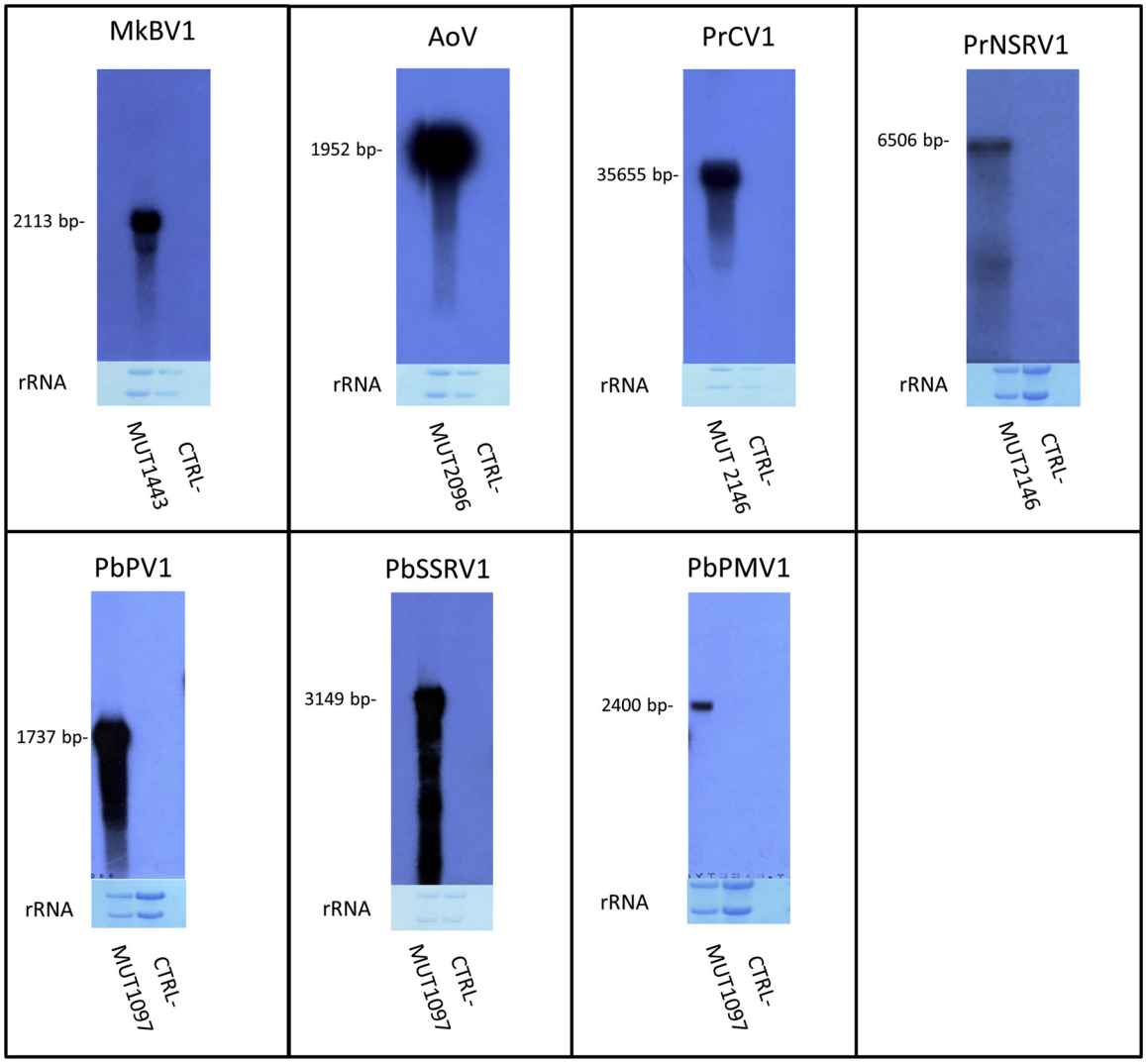

Fig. 4. Northern blot analysis of total RNA extracted from fungal isolates (labels at the bottom of each panel). Specific probes were used to detect the viral genomes (labels on top of each panel representing viral acronyms: Myriodontium keratinophilum bipartite virus 1 (MkBV1); Penicillium brevicompactum polymycovirus 1 (PbPMV1); Penicillium brevicompactum ssRNA virus 1 (PbSSRV1); Penicillium brevicompactum partitivirus 1 (PbPV1); Aspergillus ochraceus virus (AoV); Penicillium roseopurpureum chrysovirus 1 (PrCV1); Penicillium roseopurpureum negative sense RNA virus 1 (PrNSRV1)). Under each panel a methylene blue stain of the membrane is reported to show ribosomal RNA loadings. CTRL-= RNA extract from *Cryphonectria parasitica* EP67.

large and well characterized clades which is still unrecognized taxonomically, previously suggested as a new family (Nerva et al., 2016). Regarding PbSSRV1 phylogenetic analysis (Fig. 8), we here confirm and highlight that a new clade (likely a new viral family) is well supported statistically and contains a number of mycoviruses with conserved genomic features for which we propose the name “Mycotombusviridae”.

Finally, the phylogenetic placement of PrNSRV1 is displayed in Fig. 9 inside the order *Bunyavirales*, where we can observe that it falls in new recently proposed clade (Betamycobunyaviridae) together with Fusarium poae negative strand virus 1 and the recently characterized bunya-like virus Coniothyrium diploidella negative-stranded RNA virus 1 (Nerva et al., 2019).

The phylogenetic placement of AsPMV1 and PbPMV1 confirms that these two viruses belong to an already characterized yet not recognized group of viruses called polymycoviruses, due to multipartite nature of their genomes (Supplementary on line Fig. 2).

3.4. Search for RNA sequences associated to PrNSRV1

Given that we had access to libraries containing two closely related bunya-like mycoviruses (See Fig. 9), we decided to proceed with the approach described in detail in the materials and methods to obtain possible nucleocapsid encoding segments. When we submitted our candidates for the final check to NCBI nr database (in order to exclude the pair had some homology with existing already characterized proteins), surprisingly an identified pair of contigs (one for each of the two closely related bunya-like viruses) matched a putative Nc of a Penicillium discovirus (MF 142460.1), a sequence that is neither present in the viral database nor referenced in a publication at this time. A second pair of contigs also matched another protein from Penicillium discovirus, named Ns1. We then designed primers to detect the contigs encoding for the putative Nc and Ns1 in *H. polii* cDNAs, confirming the presence of the two contigs in sample MUT2146, and their absence from the other samples (not shown). We then also used the Nc we retrieved to analyze another dataset that we recently published containing another closely related my**co-**bunyavirus (Rhizoctonia solani bunya/phlebo-like virus 1) (Picarelli et al., 2019). However, in this case we failed to associate any new virus segment, showing that Nc and Ns1 proteins are likely conserved only among closely related viruses (possibly defining new virus families inside the order *Bunyavirales*). The feature of the new virus segments associated to PrNSRV1 are detailed in

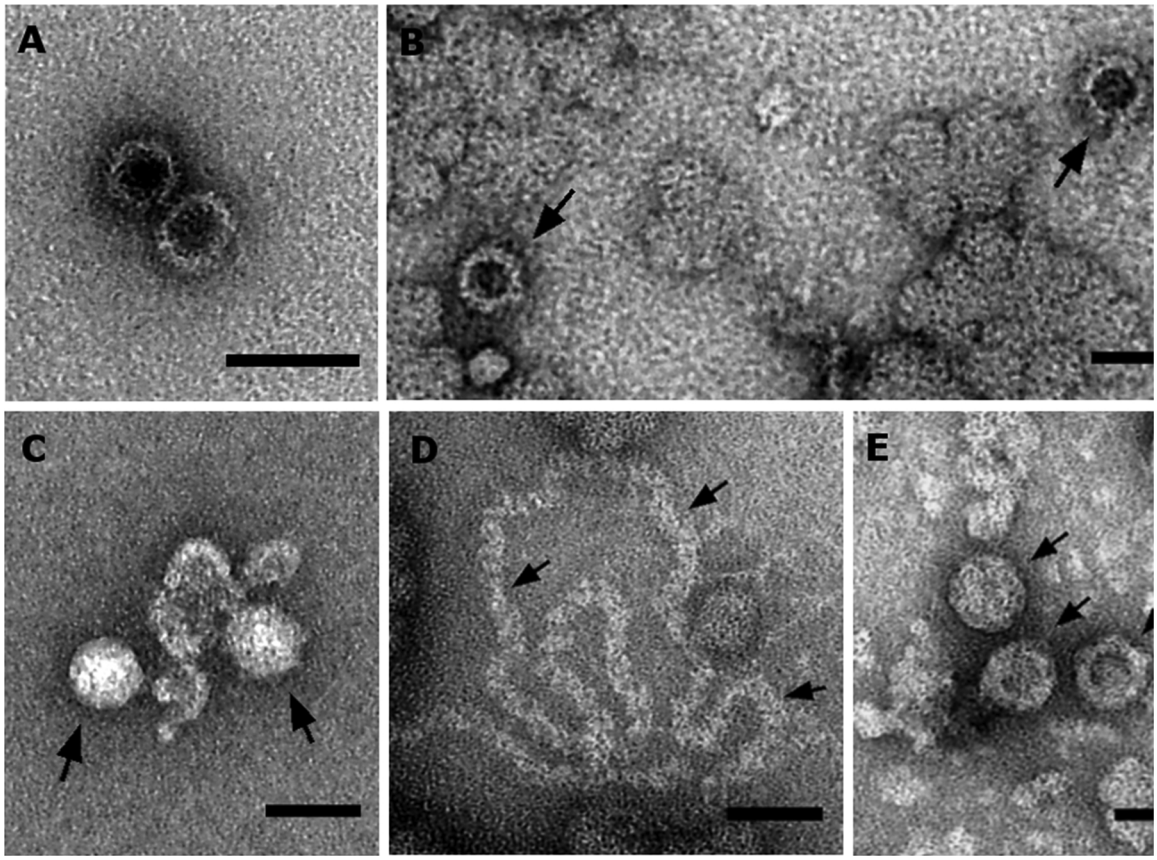

Fig. 5. Micrograph of negative stained viral particles from partially purified fungal extracts. In isolate MUT 1097 (A) and MUT 2096 (B) particles looked rounded in profile and penetrated by the stain, with a mean diameter of about 30 nm and 37 nm respectively. Isolate MUT 2120 (C) showed particles not penetrated by stain, with polygonal shape and diameter of 40 nm. In isolate MUT 2146 (D, E), particles with two different morphologies were observed: filamentous snake-like particles of about 12 nm in diameter (D) and polygonal particles of about 42 nm, which were sometimes penetrated by the stain (E). Arrows point to viral particles. Scale bars represent 50 nm.

Fig. 3. The RNA2 encodes the putative Ns1 protein of 58.1 kDa and pI of 6.8; the RNA3 encodes the putative Nc protein with a molecular mass of 37.1 kDa and a pI of 9.1. No specific domain could be identified in either of the two proteins. The alignments of the three available Ns1 and Nc belonging to this new clade of mycobunyaviruses is displayed in Supplementary on line Fig. 3.

4. Discussion

Sea cucumbers are key components for the marine environment: they are deposit feeders playing an important role in recycling organic matter, mixing the sediment layer and stimulating the growth of many microalgae (MacTavish et al., 2012). More than 60 different species are fished and considered a delicacy in Asian countries (Purcell et al., 2010). Therefore, these animals result in an important resource from both the ecological and the socio-economical perspective.

We decided to investigate the virome associated to *H. polii* fungal collection because we were looking for possible multitrophic interactions. Although the biological and ecological role that mycoviruses play in multitrophic interactions is largely unknown and complicated by frequent multiple infections (Khalifa and Pearson, 2013; Nerva et al., 2016), some hints of the importance of virome on fungal behavior were reported. For example the ability to modulate effects of pathogenic fungi on the host (i.e. hypovirulence and hypervirulence) (Ahn and Lee, 2001; Du et al., 2014; Potgieter et al., 2013; Turina and Rostagno, 2007; Xu et al., 2015; Chun et al., 2018; Lau et al., 2018; Jian et al., 1997), or the ability to induce thermal tolerance in a three partite interaction were reported (Marquez et al., 2007). In the last few years, the way to look at viruses is rapidly changing: in fact, the concept of "good viruses" is now widely accepted (Roosinck et al. 2011). More in details, in the case of mycoviruses, it seems that partitiviruses and chrysoviruses can often have a positive effect on their fungal hosts, as reported for *Trichoderma herzianum* (Chun et al., 2018) and for *Alternaria alternata* (Okada et al., 2018). Moreover in a recent report, we demonstrated that AoV, a partitivirus reported in a strain of *A. ochraceus* unable to produce ochratoxin A (OTA), is instead able to induce an overproduction of OTA in a different isolate of *A. ochraceus* (Nerva et al., 2018a).

In this study, we identified 10 mycoviral genomes, and a total of 24 genome segments, using a metatranscriptomics approach on total RNA extracted from 39 fungal isolates. Interestingly, among the 10 virus strains two of them (AoV and PsV-S) belong to previously described species within the family *Partitiviridae* (Bozarth et al., 1971; Liu et al., 2008). This finding is interesting, because studies through the analysis of metagenomes of environmental samples show that less than 1% of the viral diversity has been described so far (Marzano and Domier, 2016; Mokili et al., 2012).

Our results suggest that some limitation in our approach to characterize the virome associated to fungi inhabiting *H. polii* still exists: first of all the use of high throughput sequencing is based on the identification of specific conserved domains (i.e. the RNA-dependent RNA polymerase) (Mokili et al., 2012; Roossinck, 2015), hence new viral species with non-conserved domains can go undetected while following this approach. A second limiting factor is due to the fact that we performed the analysis on fungi that were isolated and cultured in axenic condition, hence all the unculturable fungi were excluded from the analysis. In our opinion, metatranscriptomes analyses from one hand can unveil the presence of all the viral genomes associated to a

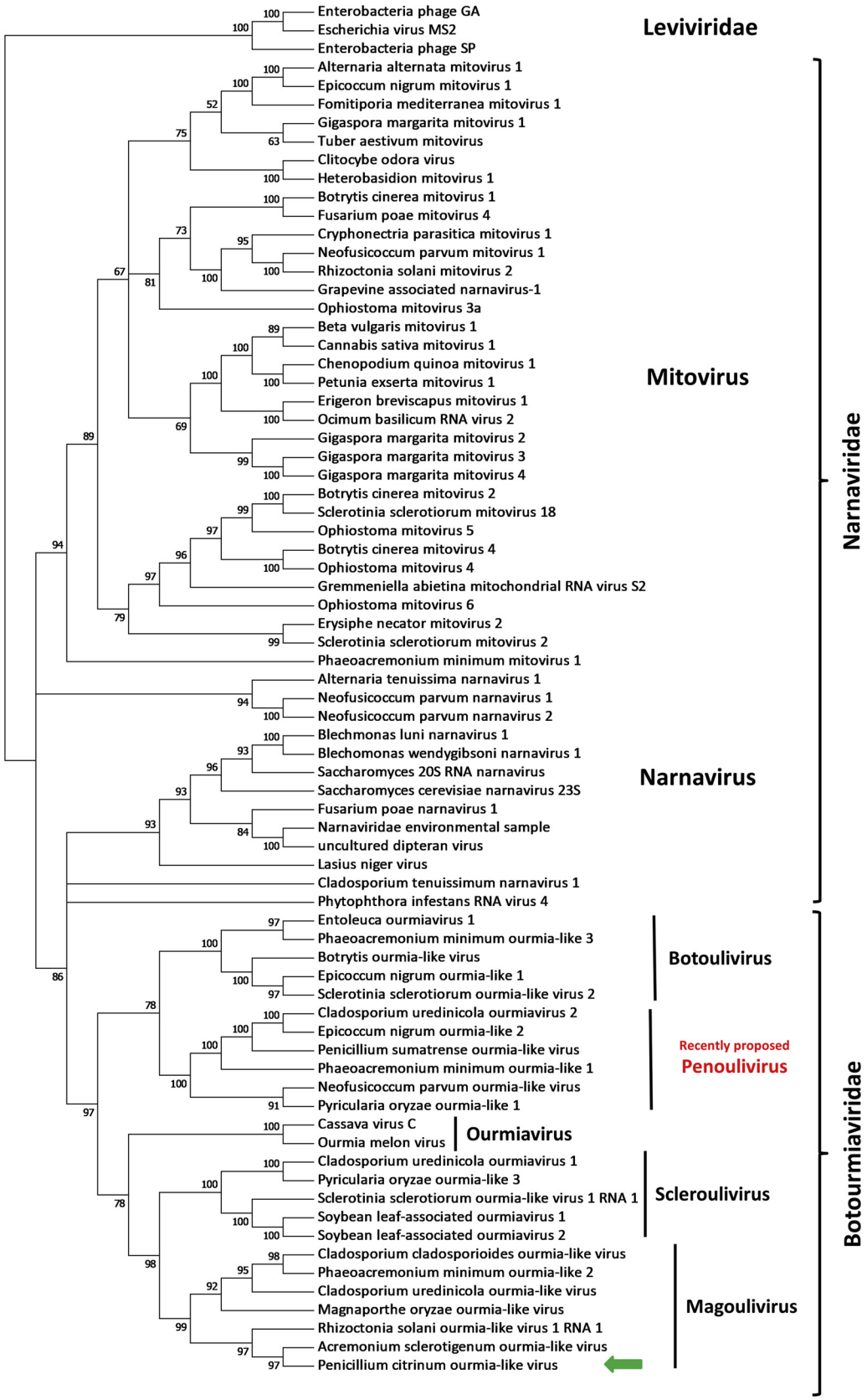

Fig. 6. Phylogenetic placement of putative viruses belonging to the narna, mito, and ourmia-like viruses. Amino acids sequences of RNA-dependent RNA polymerases (RdRPs) were aligned with ClustalW and then phylogeny was derived using the maximum likelihood methodology in MEGA6. Numbers above branches represent statistical support based on bootstrap analysis (1000 replicates). The virus identified in this work is highlighted by a green arrow.

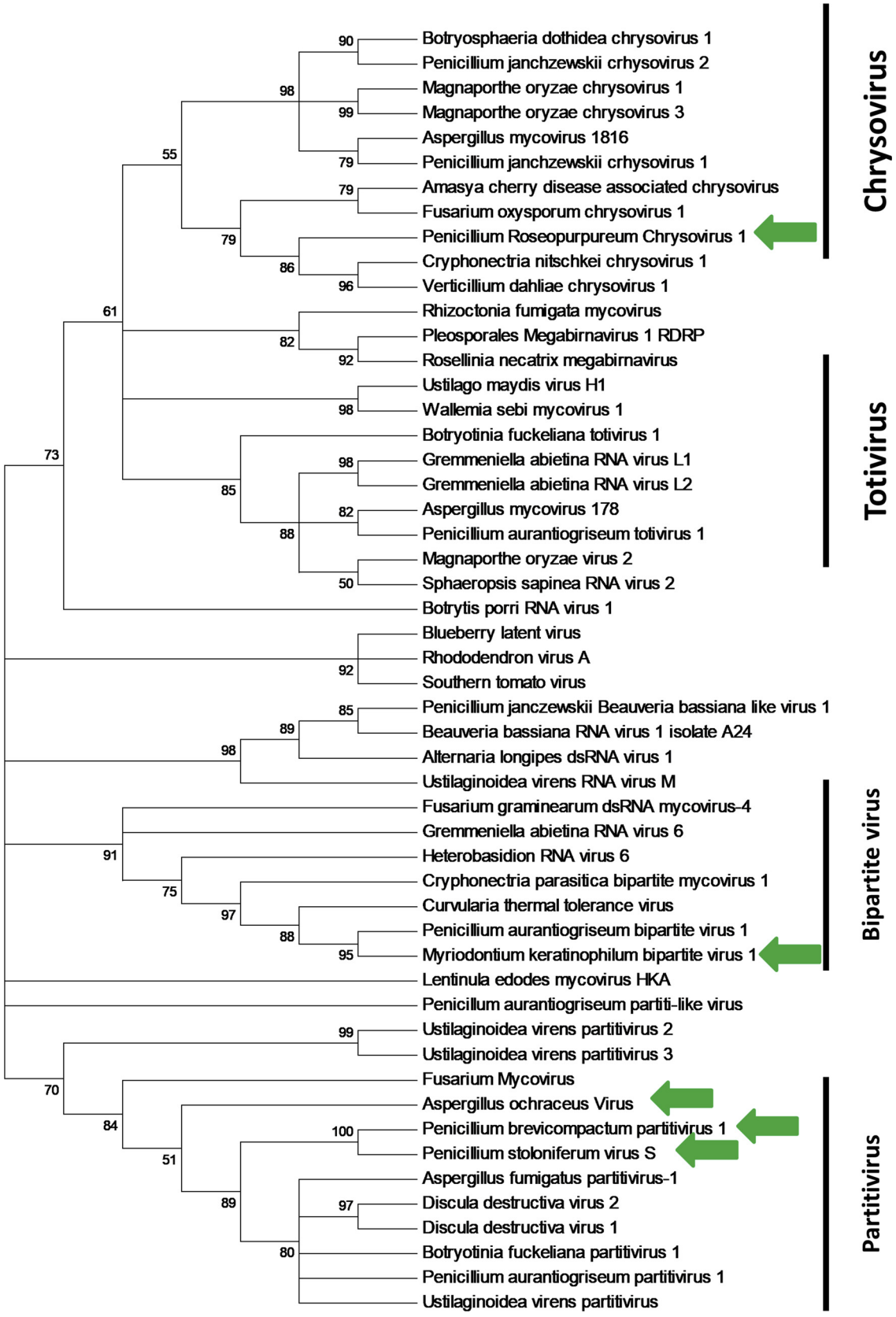

Fig. 7. Phylogenetic placement of putative viruses displaying dsRNA genomes. Amino acids sequences of RNA-dependent RNA polymerases (RdRPs) were aligned with ClustalW and then phylogeny was derived using the maximum likelihood methodology in MEGA6. Numbers above branches represent statistical support based on bootstrap analysis (1000 replicates). Viruses identified in this work are highlighted by green arrows.

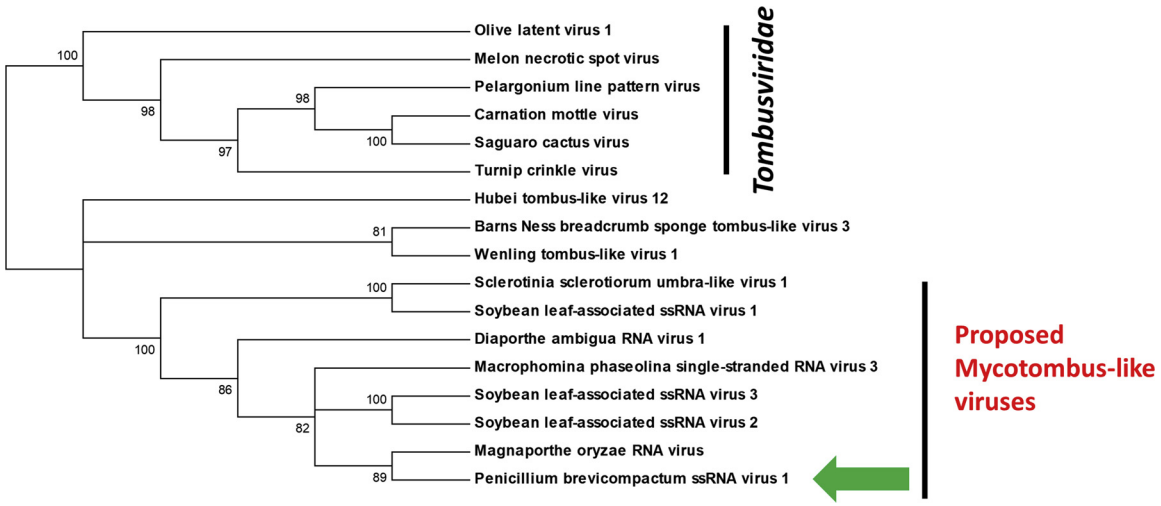

Fig. 8. Phylogenetic placement of Penicillium brevicompactum ssRNA virus 1 RdRP. The protein was aligned with ClustalW to closely related mycoviruses still not assigned taxonomically, and some representative of the family *Tombusviridae*. The phylogenetic tree was derived using the maximum likelihood methodology in MEGA6. Numbers above branches represent statistical support based on bootstrap analysis (1000 replicates). The virus identified in this work is highlighted by a green arrow.

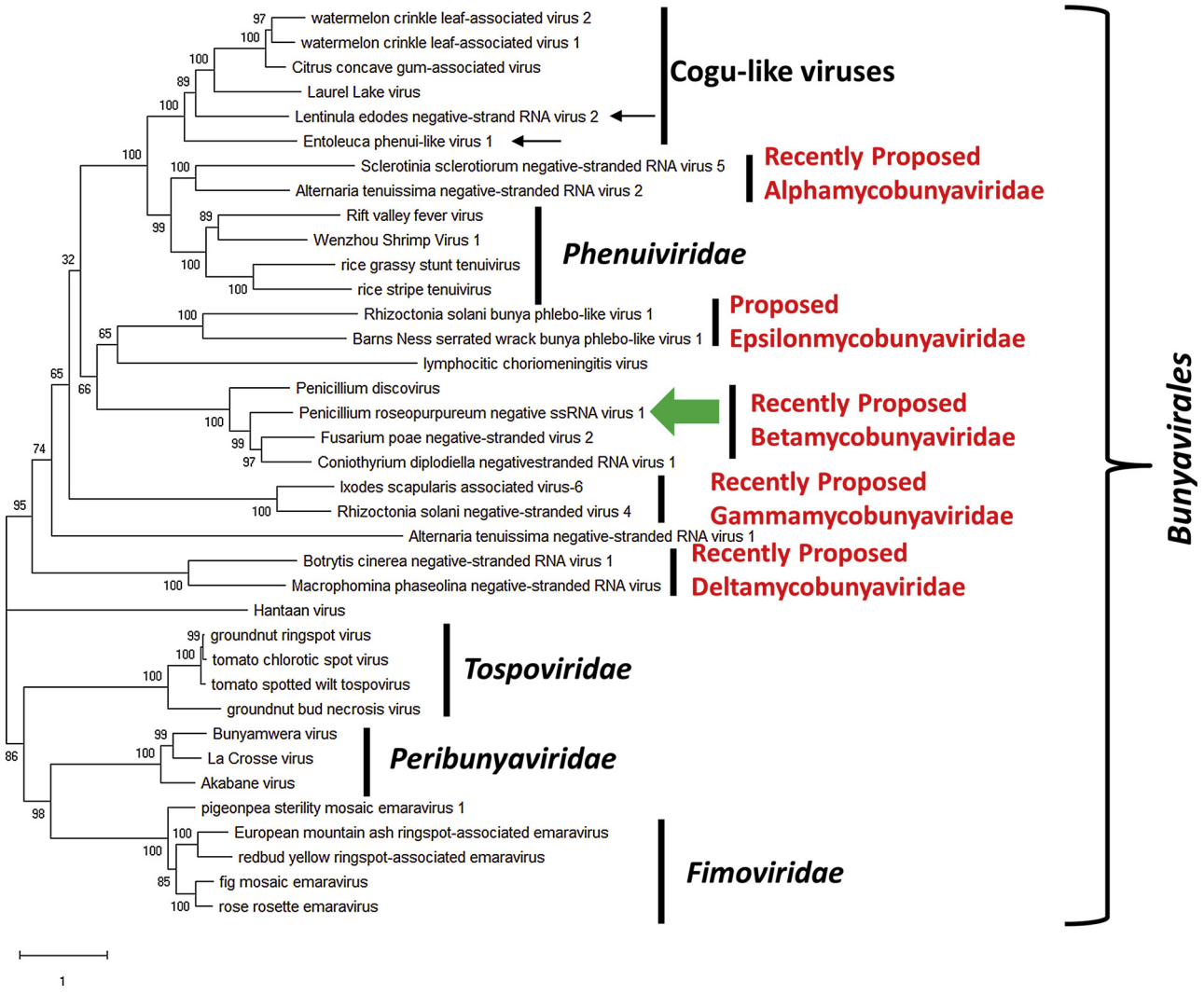

Fig. 9. Phylogenetic placement of putative viruses displaying negative single-stranded RNA (-ssRNA) genomes. Amino acids sequences of RNA-dependent RNA polymerases (RdRPs) were aligned with ClustalW and then phylogeny was derived using the maximum likelihood methodology in MEGA6. Numbers above branches represent statistical support based on bootstrap analysis (1000 replicates). Name of family still not recognized by the ICTV are written in red. The virus identified in this work is highlighted by a green arrow.

holobiont, also from the unculturable microorganisms, on the other hand often lose the information about the link of specific viruses to specific biological entities. Therefore, especially for viruses that need a specific host to replicate, data originated from in silico analysis remain unexplored from the biological point of view, limiting the discovery to a mere annotation of nucleotide sequences.

Interestingly, the viral sequence of PrNSRV1 shows the highest similarity with RdRPs of minus strand RNA multipartite viruses, and as reported in Fig. 5d we also identified possible virus-like particles originated by the virus. We attempted to identify other viral segments belonging to this virus using a comparative approach, in which another RNAseq library was used to identify conserved sequences shared by the two datasets, and we were able to identify two new segments putatively coding for a nucleocapsid and a non-structural protein. Recently two *bona fidae* mycoviruses from the *Bunyavirales* were characterized, including their putative Nc and Ns1 proteins being these the first reports of multi segmented bunyavirales from fungi (Lin et al., 2019; Velasco et al., 2019); neither the Nc or the Ns1 of these new viruses have a detectable homology with the Nc and Ns1 we describe in our work. Our data contributes to further characterize a second group of myco-bunyaviruses with at least three genomic segments, maintaining a detectable homology among their Nc and Ns1 proteins.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2019.197737.

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