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Multiple approaches for the detection and characterization of viral and plasmid symbionts from a collection of marine fungi

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ABSTRACT

The number of reported mycoviruses is increasing exponentially due to the current ability to detect mycoviruses using next-generation sequencing (NGS) approaches, with a large number of viral genomes built *in-silico* using data from fungal transcriptome projects. We decided to screen a collection of fungi originating from a specific marine environment (associated with the seagrass *Posidonia oceanica*) for the presence of mycoviruses: our findings reveal a wealth of diversity among these symbionts and this complexity will require further studies to address their specific role in this ecological niche. In specific, we identified twelve new virus species belonging to nine distinct lineages: they are members of megabirnavirus, totivirus, chrysovirus, partitivirus and five still undefined clades. We showed evidence of an endogenized virus ORF, and evidence of accumulation of dsRNA from metaviridae retroviral elements. We applied different techniques for detecting the presence of mycoviruses including (i) dsRNA extraction and cDNA cloning, (ii) small and total RNA sequencing through NGS techniques, (iii) rolling circle amplification (RCA) and total DNA extraction analyses, (iv) virus purifications and electron microscopy. We tried also to critically evaluate the intrinsic value and limitations of each of these techniques. Based on the samples we could compare directly, RNAseq analysis is superior to sRNA for *de novo* assembly of mycoviruses. To our knowledge this is the first report on the virome of fungi isolated from marine environment.

The GenBank/eMBL/DDBJ accession numbers of the sequences reported in this paper are: KT601099–KT601110; KT601114–KT601120; KT592305; KT950836–KT950841.

1. Introduction

More than half a century has passed since the discovery of the first mycovirus, and the interest in these symbionts has grown steadily, particularly in the last decade as many new viral species are reported every year. Although initially reported as diseases of cultivated mushroom (Gandy, 1960; Hollings, 1962), to date the majority of the described mycoviruses are studied for their ability to change the virulence (if the host is a pathogen) or more generally the phenotype of their hosts. Another interest in mycovirus research comes from early work on the use of fungi in biotechnology (yeast fermentation, antibiotic production, and interferon purification) and their possible interference with fermentation processes or their added value given by the presence of double-stranded RNA (dsRNA) in culture filtrates (Banks et al., 1968; Woods and Bevan, 1968). Less is known about the ecological role of these fungal symbionts in nature and how they can change or drive the evolution of their hosts. From an ecological point of view, an interest for these symbionts has grown after the description of a three-way symbiotic system where a mycovirus harbored in the endophytic fungus *Curvularia protuberata* is able to confer thermal tolerance to *Dichanthelium lanuginosum*, a grass plant species (Marquez et al., 2007). Mycoviruses are widely distributed in all the major fungal taxonomic groups (Goeker et al., 2011), including saprophytes and symbionts. The majority of mycoviruses present in databases have dsRNA genomes and are transmitted among different individuals through cellular fusion (anastomosis) without an extracellular phase. However, in the last few years, mycoviruses possessing single-stranded RNA (ssRNA) positive (+) and negative (−) sense genomes as well as a circular ssDNA genome have been reported (Yu et al., 2010, 2013; Du et al., 2014). In particular the characterized mycovirus with ssDNA genome seems to

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be acquired externally without the occurrence of fungal anastomoses (Du et al., 2014). The classic method for the detection of mycoviruses consists of the purification of dsRNA using CF11 cellulose: this easy and convenient technique is able to detect mostly virus with dsRNA genomes (and to some extent also dsRNA replication intermediates from ssRNA virus genomes) and this is likely the reason why the majority of the mycoviruses described so far have a dsRNA genome. Another common way to detect mycoviruses is through virus particle purification using differential centrifugation protocols combined with observation by transmission electron microscopy (TEM). This approach allows for the detection of viruses with different kinds of genomes, but requires traditional virological expertise and expensive equipment, which are not always available in fungal and molecular biology laboratories. Furthermore, this technique is unsatisfactory for "naked" viruses, a fairly common feature for mycoviruses. Therefore, identification of new mycovirus taxonomic groups requires new detection approaches.

A new impulse to mycovirus research comes from the wide availability of next-generation sequencing (NGS) protocols. As demonstrated in a large number of scientific works, NGS techniques are able to detect the presence of viral sequences in samples where the viral titer is very low. An example of the usefulness of these techniques comes from two previous works (Al Rwahnih et al., 2011; Espach, 2013), where the authors analyzed a plant total RNA extract using NGS techniques and not only were they able to detect plant viruses but they also detected the complex virome of the fungal endophytes. NGS applied to total RNA extracts allows for the detection of all the different viral taxa because, independently from the DNA or RNA genome, all viruses must produce mRNA during their replication cycle.

Another way to detect viral sequences is to apply the NGS technique to sequencing small RNA (sRNA) libraries. This idea comes from the work of Kreuze et al. 2009 where the authors showed the possibility to assemble new and unknown viral sequences from sRNA libraries obtained from plants. To our knowledge, such an approach has only very recently been used for *de novo* assembly of viral genomes from fungi (Vainio et al., 2015).

After the description of a circular ssDNA mycoviruses with a strong impact on host virulence (Du et al., 2014), a relatively new branch of mycovirology (the one dealing with DNA genome viruses) seems to be born. A general protocol used for environmental samples and for higher eukaryotes to specifically detect ssDNA virus (the "circuloma" approach) relies on the aid of a new technique called rolling circle amplification (RCA) (Ali et al., 2014). RCA has the potential to detect also plasmid DNA and since the 1970s it has been shown that fungi can host DNA plasmids in their cytoplasm or in their mitochondria (Stahl et al., 1978). These plasmids seem to confer beneficial phenotypes to the fungi such as hypovirulence (Monteiro-Vitorello et al., 2000), drug resistance or resistance to abiotic stress (Griffiths, 1995).

The marine environment is characterized by the presence of high salinity and a recent study showed that the number of viruses in the sea is at least ten times higher than the number of any other microorganism (Breitbart, 2012; Suttle, 2007). Specifically, most marine viruses are cyanophages and have been shown to be major players in biogeochemical cycles and drivers of evolution of their algal hosts (Brussaard et al., 2008; Fuhrman, 1999) by influencing microbial population size through their lytic capacity, altering their metabolic output and providing an immensely diverse pool of genetic material available for horizontal gene transfer (Brum et al., 2015). Till now, no virus associated to marine fungi has been reported, probably because the presence and ecological role of fungi in marine environment has been neglected till few years ago. Hence, we decided to investigate viruses infecting fungi in marine environment, so far a much overlooked aspect of ocean microbiology (Richards et al., 2012).

Water samples are not fully representative of the marine environment, and fungi could actually play a major role associated to algae and marine plants. We decided, therefore, to screen for the presence of virus and plasmid symbionts in a collection of marine fungi isolated from the seagrass *Posidonia oceanica* (Panno et al., 2013; Gnavi et al., 2014). *P. oceanica* is a protected species of utmost importance for sea ecology as exemplified by specific European legislation addressing this issue (EEC 92/43, 1992). Characterizing the microbiological niche associated with this plant could help in monitoring and managing the health of our sea environment. We selected a subset of 91 fungal isolates representative of the biological diversity of the fungi isolated from *P. oceanica* and we applied the different techniques for mycovirus detection described above to compare the positive and negative aspects of each methodological approach.

2. Materials and methods

2.1. Fungal isolates

The fungal isolates used in this work come from a collection of marine fungi of the Mycoteca Universitatis Taurinensis (MUT) (Supplementary Table 1). The analyzed strains were isolated from different districts of the seagrass *P. oceanica* (Panno et al., 2013). All the fungi were grown in liquid cultures at 24 °C in malt extract broth with 3% of sea salts (Sigma–Aldrich, Saint Louis, MO, USA) producing a good amount of mycelia in 4 days, with the exception of *Wallemia sebi* isolate MUT4935 that grew better in malt yeast extracts supplemented with 40% sucrose (MY40) at 24 °C and was harvested after 5 days.

2.2. dsRNA purification

Lyophilized mycelium was first ground with iron beads, then 200 mg were transferred to a 15 ml tube with 4 ml of glass beads (0.5 mm diameter) and homogenized for 30 s in a bead beater (FastPrep24, M.P. Biomedicals, Irvine, CA, USA), added with 5 ml of STE 2X (200 mM NaCl, 10 mM Tris–HCl pH7, EDTA 2 mM pH 8) SDS 1%, and 5 ml of phenol (Phenol BioUltra, Sigma–Aldrich, Saint Louis, MO, USA), mixed again for 30 s in a bead beater and then centrifuged for 15 s at 1000 g. Five ml of the supernatant were transferred to a new tube, added with 5 ml of phenol-chlorofom (1:1), vortexed and then centrifuged for 15 min as above. The supernatant was transferred to a new 50 ml tube with 1 g of Whatman CF11 cellulose previously equilibrated with 15% Et-OH in 1X STE. After discarding the supernatant, the CF11 was re-suspended in 20 ml of 15% ethanol in 1X STE and loaded onto a chromatography column, washed with 20 ml of 15% ethanol in 1X STE, finally eluted with 10 ml of STE 1X. Two volumes of ethanol and 5% of 3 M Na-Acetate pH 5.2 were added to the eluate for precipitation. The dsRNA was kept at −80 °C for 30 min, centrifuged at 12,000 × *g* for 15 min, resuspended in 500 μl of nuclease S1 buffer (sodium acetate 40 mM, sodium chloride 300 mM, zinc sulfate 2 mM) and then added with 2 μl of nuclease S1, incubated at 37 °C for 20 min, added with the same volume of phenol-chloroform, centrifuged for 5 min; the supernatant was precipitated at −80 °C as described above. The pellet was recovered and resuspended in 40 μl of water and traces of DNA were removed by adding 1 μl of DNase I (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's protocol. The dsRNA was then analyzed by electrophoresis in 1% agarose gel in 1X TAE and each distinct single or group of dsRNA fragments was purified from agarose gel using the Zymoclean Gel DNA Recovery kit (Zymoresearch, CA, USA) following manufacturer instructions and eluting the samples in a final volume of 15 μl of water.