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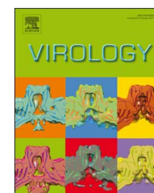

The challenges of using high-throughput sequencing to track multiple bipartite mycoviruses of wild orchid-fungus partnerships over consecutive years

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ABSTRACT

The bipartite alpha- and betapartitiviruses are recorded from a wide range of fungi and plants. Using a combination of dsRNA-enrichment, high-throughput shotgun sequencing and informatics, we report the occurrence of multiple new partitiviruses associated with mycorrhizal *Ceratobasidium* fungi, themselves symbiotically associated with a small wild population of *Pterostylis sanguinea* orchids in Australia, over two consecutive years. Twenty-one partial or near-complete sequences representing 16 definitive alpha- and betapartitivirus species, and further possible species, were detected from two fungal isolates. The majority of partitiviruses occurred in fungal isolates from both years. Two of the partitiviruses represent phylogenetically divergent forms of *Alphapartitivirus*, suggesting that they may have evolved under long geographical isolation there. We address the challenge of pairing the two genomic segments of partitiviruses to identify species when multiple partitiviruses co-infect a single host.

1. Introduction

Members of the family *Partitiviridae* are classified into five genera: *Alphapartitivirus*, *Betapartitivirus*, *Deltapartitivirus*, *Gammapartitivirus* and *Cryspovirus* (Nibert et al., 2014). Their host ranges include plants, fungi and protozoa (Ghabrial et al., 2012). Members of this family are characterised by having isometric particles ranging from 25 to 40 nm in diameter and a bipartite genome that encodes for an RNA-dependent RNA polymerase (RdRp) on one segment and a coat protein (CP) on the second segment (Ghabrial et al., 2012; Nibert et al., 2014). Infection by these viruses is often persistent and latent (Roossinck, 2010; Ghabrial et al., 2012; Nibert et al., 2014).

Alphapartitivirus and *Betapartitivirus* contain both plant-infecting and fungus-infecting species (Nibert et al., 2014). Their genetic relatedness suggests that partitiviruses have transmitted among and between plants and fungi (Roossinck, 2010; Nibert et al., 2014). Orchids rely on partnerships with compatible mycorrhizal fungi, whose hyphae are ingested by the plants to provide nutrients required for germination and growth (Ramsay et al., 1986; Swarts and Dixon, 2009). Such close interactions may provide opportunities for partitiviruses to transmit between plants and fungi. Currently, *Diuris pendunculata* cryptic virus (DPCV), isolated from an *ex-situ* population of *D. pendunculata*, is the only proposed partitivirus reported in Australia and from orchids (Wylie et al., 2013). The only two plant viruses described from *Pterostylis* orchids have both been potyviruses (family *Potyviridae*, genus *Potyvirus*) – bean yellow mosaic virus and *Ornithogalum* mosaic virus (*syn Pterostylis* virus Y) (Gibbs et al., 2000). Seven mycorrhizae-derived endornaviruses (family *Endornaviridae*, genus *Endornavirus*) were identified from fungal pelotons in related orchid species *Pterostylis* sp. (Ong et al., 2016). In this study, a high-throughput sequencing approach was used to identify partitiviruses infecting mycorrhizal fungi associated with a small population of *Pterostylis sanguinea* orchids (dark banded greenhood orchid) growing in a natural habitat. We discuss the challenges in identifying and pairing co-occurring, novel and closely-related bipartite viruses.

2. Materials and methods

2.1. Sample collection

Leaves and underground stems (Fig. 1) were collected from a small

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Fig. 1. *Pterostylis sanguinea* (A) whole plant (B) labella (C) leaves (D) underground stem and (E) old (brown) and new (white) tubers. Scale bar: (A) 5 cm (B-E) 2 cm.

natural population of *P. sanguinea* orchid plants located on the Murdoch University campus, Western Australia (GPS coordinates −32° 3' 54.9714", 115° 50' 26.448") in 2012 and 2013. The population consisted of three (in 2012) and four (in 2013) orchid shoots growing within a one square metre area in natural bushland. Because orchid tubers may germinate unevenly (Brundrett, 2014), it was impossible to definitively select leaf material from the same plants in both years of the study. Leaf material was combined from three plants in 2012 (sample P-2012) and four plants in 2013 (sample P-2013) for nucleic acids extraction and sequencing. In each of the years, a fungal culture was established from one peloton isolated from the underground stem (fungal isolates F-2012 and F-2013) of one of the plants sampled. Collection of plant tissues, including the underground stem, did not cause the death of plants because the new tubers remained undisturbed.

2.2. Fungal isolation from underground stems

Each underground stem was surface-sterilised by immersion in 2% (w/v) sodium hypochlorite solution for 3 min, dipped in 70% ethanol for 10 s, followed by two rinses in sterile distilled water. The stem was then transferred to a 1.5 mL centrifuge tube with sterile water and ground with a pestle to produce a suspension of pelotons (fungal coils located within the underground stem) and plant debris. Under a compound microscope, individual pelotons were located and transferred onto fungal isolation medium (FIM) agar plates ($0.3\ g\ L^{-1}$ $NaNO_3$, $0.2\ g\ L^{-1}$ KH_2PO_4, $0.1\ g\ L^{-1}$ $MgSO_4 \cdot 7H_2O$, $0.1\ g\ L^{-1}$ KCl, $0.1\ g\ L^{-1}$ yeast extract, $2.5\ g\ L^{-1}$ sucrose and $8\ g\ L^{-1}$ agar; $100\ mg\ L^{-1}$ filter-sterilised streptomycin sulphate) (Clements and Ellyard, 1979). Fungal isolates were left to incubate in the dark at 24 °C for 5–7 days. Mycelium was subcultured onto fresh FIM plates and into 100 mL FIM liquid medium (FIM without agar). Liquid cultures were incubated on a shaker at 24 °C in the dark until 80–100 mg fungal biomass could be harvested.

2.3. Nucleic acids extraction, cDNA synthesis and amplification

DNA and RNA extraction was from 80 to 100 mg of plant or fungal tissue using a cellulose-based method that enriched the sample for double-stranded RNA (dsRNA) (Morris and Dodds, 1979). The aqueous phase following phenol-chloroform processing was mixed with Whatman CF-11 cellulose powder, centrifuged and resulting supernatant containing DNA was collected.

cDNA synthesis was carried out in a 20 µL volume containing 1× GoScript™ RT buffer (Promega), 3 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 mM of random primer (5' CGTACAGTTAGCAGGCNNNNNNNNNNNN 3', where N is any nucleotide), 160 units of GoScript™ and 4 µL of heat-denatured RNA (50–100 ng). cDNA synthesis occurred after an initial incubation at 25 °C for 5 min, incubation at 42 °C for 60 min and enzyme denaturation at 70 °C for 15 min.

PCR amplification was done in a 20 µL reaction volume consisting of 1× GoTaq® Green Master Mix (Promega), 1 mM barcode primer (5' XXXXCGTACAGTTAGCAGGC 3') and 2 µL of cDNA. Each barcode primer was tagged with a unique 4-nt barcode at the 5' terminus of a 16-nt adaptor sequence that was complementary to the 5' end of the cDNA synthesis primer. The cycling reaction was carried out with an initial incubation of 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C, and a final extension of 10 min at 72 °C.

Amplicons were pooled in equimolar amounts and purified using a Qiagen QIAquick PCR Purification Kit. Ten micrograms of pooled amplicons were submitted to the Australian Genome Research Facility (Melbourne, Australia) or Macrogen Inc (Seoul, South Korea) for library construction and high-throughput sequencing of paired ends over 100 cycles on a HiSeq. 2000 (Illumina).

2.4. Identification of fungi

The 5.8S ribosomal gene and flanking internally transcribed spacer (ITS) regions were amplified using fungal universal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990). Amplified PCR products were purified using QIAquick (Qiagen) columns and sequenced using the Sanger method (BigDye® version 3.1 terminator mix; Applied Biosystems). Sequences were edited and pairwise aligned using the alignment tool in Geneious v7.0.6 (Biomatters). Blastn (Altschul et al., 1990) searches identified the fungal matches.

2.5. Sequencing data analysis

CLC Genomic Workbench v6.5.1 (Qiagen) software was used for *de novo* assembly of reads to form contigs. Settings used for assembly

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