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REPORT

Prevalent and persistent viral infection in cultures of the coral algal endosymbiont *Symbiodinium*

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Abstract Reef corals are under threat from bleaching and disease outbreaks that target both the host animal and the algal symbionts within the coral holobiont. A viral origin for coral bleaching has been hypothesized, but direct evidence has remained elusive. Using a multifaceted approach incorporating flow cytometry, transmission electron microscopy, DNA and RNA virome sequencing, we show that type C1 *Symbiodinium* cultures host a nucleocytoplasmic large double-stranded DNA virus (NCLDV) related to *Phycodnaviridae* and *Mimiviridae*, a novel filamentous virus of unknown phylogenetic affiliation, and a single-stranded RNA virus related to retroviruses. We discuss implications of these findings for laboratory-based experiments using *Symbiodinium* cultures.

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Introduction

Reef-building corals form a multi-partite symbiosis with photosynthetic dinoflagellate algae and a diverse resident microbial community comprising bacteria, archaea, fungi, protists, and viruses, collectively termed the coral holobiont (Rohwer et al. 2002). Some of these coral–microbe associations are obligate, including the symbiosis with dinoflagellate algae in the genus *Symbiodinium*. The coral relies on photosynthate translocated from *Symbiodinium* cells into its tissues to meet most of its nutritional requirements (Muscatine and Porter 1977). Stress commonly results in disruption of this symbiosis, manifested as the loss of *Symbiodinium* cells and/or *Symbiodinium* photosynthetic pigments from the coral tissues, called coral bleaching (Hoegh-Guldberg 1999). Mass coral bleaching events mostly occur due to a combination of high

temperature and irradiance, including ultraviolet light (UV) exposure (Ferrier-Pagès et al. 2007; Weis 2008) commonly associated with summer doldrums (Eakin et al. 2009). A range of other environmental stressors are also known to cause bleaching, including low salinity, sedimentation, pollution, herbicides, and decreased temperature (Brown 1997; Baker et al. 2008). The primary mechanism thought to drive coral bleaching caused by increasing temperature is the formation of excess reactive oxygen species (ROS) that cause oxidative damage to photosynthetic membranes and host tissues, resulting in loss of symbionts and, ultimately, bleaching of the coral (Lesser and Farrell 2004; Weis 2008; Baird et al. 2009).

The microbial bleaching hypothesis posits that high temperature acts on the coral-associated microorganisms, causing a change in the microbial community structure that may either directly or indirectly contribute to bleaching (Rosenberg et al. 2009). This hypothesis was supported by the observation of bleaching caused by *Vibrio shiloi* and *V. coralliilyticus* in the corals *Oculina patagonica* and *Pocillopora damicornis*, respectively (reviewed in Rosenberg and Kushmaro 2011). Lysis of *Symbiodinium* by proviruses has been suggested as an alternative cause of some instances of coral bleaching (Wilson et al. 2001; Lohr et al. 2007; Correa et al. 2016), although this has yet to be experimentally confirmed. For example, *Symbiodinium* cells isolated from the temperate sea anemone, *Anemonia viridis,* were subjected to heat shock and subsequently seen to undergo lysis linked to latent viruses (Wilson et al. 2001), as were dinoflagellate symbionts in tissues of the tropical reef-building corals *Pavona danai, Acropora formosa*, and *Stylophora pistillata* (Wilson et al. 2005a; Davy et al. 2006). Furthermore, following a 31 °C heat shock, expression of a eukaryotic viral protein increased >100-fold in a *Symbiodinium*-enriched fraction of *Stylophora pistillata* tissue (Weston et al. 2012). Interestingly, viral infection in the marine alga *Emiliania huxleyi* leads to the production of ROS (Evans et al. 2006), which triggers caspase activity and programmed cell death (PCD) in host cells (Bidle and Vardi 2011), both of which are known to be involved in coral bleaching (Weis 2008). A role for intracellular protein phosphorylation in bleaching has also been proposed (Sawyer and Muscatine 2001), following discovery that host phosphorylated protein levels were altered during temperature stress. Although protein phosphorylation is important for host cells, viruses are also known to hijack the host kinase machinery to enable phosphorylation of viral proteins to help establish viral replication and a productive infection (Walsh and Mohr 2011). These observations support the hypothesis that viral lysis may be contributing to some instances of coral bleaching.

Although virus-like particles (VLPs) associated with *Symbiodinium* have previously been described (Wilson et al. 2001; Lohr et al. 2007; Lawrence et al. 2014), confirmed viruses infecting dinoflagellates are currently limited to just two host species: *Gymnodinium mikimotoi* (Onji et al. 2003) and *Heterocapsa circularisquama* (Tomaru et al. 2004). The latter is infected by two viruses, a positive-sense ssRNA virus (30-nm-diameter capsid), HcRNAV (Tomaru et al. 2004) and a dsDNA virus (200-nm-diameter capsid) (Tarutani et al. 2001), indicating dinoflagellate species may be susceptible to infection by more than one virus family. Recently, the genome of a novel ssRNA virus with similarities in its major capsid protein (MCP) gene sequence to HcRNAV was assembled from transcriptomic data generated from a heat-stressed *Symbiodinium* type C1 culture isolated from the coral *Acropora tenuis* (Levin et al. 2016).

Here we explore viruses associated with four cultures of *Symbiodinium* type C1 isolated from the scleractinian coral *Acropora tenuis* on the Great Barrier Reef (GBR) and provide evidence for the persistent presence of at least three distinct viruses in all cultures. We initially set out to test the hypothesis that the coral algal symbiont *Symbiodinium* harbours a lysogenic virus within its genome that switches to an active lytic infection under stress, such as UV exposure or elevated temperature. We identified a number of morphologically distinct viruses in the *Symbiodinium* cultures and present genomic information regarding the potential taxonomy and functionality of a novel retrovirus and dsDNA virus.

Materials and methods

Growth and maintenance of *Symbiodinium* culture

Four *Symbiodinium* type C1 monoclonal strains (Australian Institute of Marine Science [AIMS] Symbiont Culture Facility strain codes: SCF055-A2; SCF055-B4; SCF055-B5; SCF055-B6) were cultured from a single colony of *Acropora tenuis* located at Magnetic Island (MI) on the GBR, Australia (19°9′6″S, 146°51′56″E), as described in Howells et al. (2012). Cultured cells (non-axenic) were grown in Daigo's IMK medium (Wako Pure Chemical Industries, Japan) with antibiotics for 1 month initially and were subcultured monthly. Algal cultures were grown under a 14:10 h light/dark cycle at 26 °C, with a light intensity of 50 µmol quanta m^{-2} s^{-1} .

Experimental treatments

Triplicate exponentially growing monoclonal cultures (50 mL; approximately 10^5 cells mL^{-1}) were exposed for

different times (10; 30; 60; 90 s) to UVA (350 nm) and UVB (300 nm) light from a transilluminator placed 10 cm directly above the open petri dishes. After exposure, cultures were transferred into 50-mL flasks and incubated under control conditions. To induce potential lysogens using heat shock, temperature-stressed samples were exposed to 36 °C for 24; 48; 72; or 96 h and then maintained at 26 °C. Control and treated samples were monitored daily using flow cytometry to assess viral and bacterial populations.

Flow cytometry

Flow cytometric analyses were conducted using the protocol described by Brussaard (2004). A blank sample of TE buffer was analysed to ensure there was no background contamination in the diluted samples (Fig. 1a). For flow cytometry, virus population gates were formed based on two purified viral lysates of the algal viruses *Ostreococcus tauri* virus-1 (OtV-1) (Weynberg et al. 2009) (Fig. 1b) and *Emiliania huxleyi* virus-86 (EhV-86) (Wilson et al. 2005b) (Fig. 1c). OtV-1 viruses have a capsid diameter of 100–120 nm and a genome size of 190 kb, and EhV-86 viruses have a capsid size of ~170 nm and a genome size of 410 kb. For quantitative analysis, samples were viewed in a BD FACSVerse flow cytometer (Becton–Dickinson, California, USA) equipped with a 488-nm argon-ion laser. All flow cytometry data were analysed using FlowJo software v.10.09.8r1 (FlowJo LLC, Oregon, USA).

Transmission electron microscopy

Thin-sectioning

For preparation of thin sections, 1 mL of untreated *Symbiodinium* cells from all four algal strains was pelleted by centrifugation at 500 rpm (Eppendorf 5424, USA Scientific) for 1 min. The pellets were resuspended, washed, and repelleted twice in 0.02-µm filtered (i.e. virus-free) seawater and fixed in 0.5% glutaraldehyde. A 1% osmium tetroxide solution mixed 1:1 with filtered seawater was added to the samples. Samples were processed under vacuum in a microwave (PELCO biowave) for 6 min at 80 W (2 min on, 2 min off, 2 min on), followed by three washes in filtered seawater (pore size = 0.02 µm). Samples were subsequently dehydrated through a graded ethanol series into acetone, and finally embedded in Procure/Araldite resin. Cured (24 h, 60 °C) blocks were sectioned using a diamond knife, with 100-nm-thick unstained sections mounted on copper grids. The sections were imaged unstained.

Negative staining

The four strains of untreated *Symbiodinium* monoclonal cultures were centrifuged at 500 rpm for 10 min and filtered through a 0.2-µm Durapore filter. The resulting filtrate was concentrated using Centricon centrifugal devices. Concentrated samples for transmission electron microscopy (TEM) observation were negatively stained by placing 10 µL of fixed (0.5% glutaraldehyde) sample on a continuous carbon-filmed copper grid for 1 h, without drying. Excess liquid was then wicked off with filter paper, and each preparation was stained with uranyl acetate (2% [w/v] in water) for 60 s before washing for 60 s in distilled water and drying. All TEM imaging was conducted at 120 kV (JEM2100 JEOL Ltd, Tokyo, Japan) with data recorded on an 11-megapixel digital camera (Gatan ORIUS). Digitally acquired images were analysed using ImageJ software.

Virome sequencing

Three litres of cultured, untreated *Symbiodinium* strain SCF055-A2 was centrifuged at 500 rpm for 10 min and filtered through a 0.2-µm Durapore filter. The resulting filtrate was concentrated using Centricon centrifugal devices (Millipore). Viromes from the culture medium were prepared for sequencing following the protocol described by Weynberg et al. (2014). Briefly, concentrated 0.2-µm culture filtrates were purified on a caesium chloride (CsCl) step density gradient and 1-mL fractions along the gradient were removed. The targeted fraction (density ~1.2–1.3 g cm^{-3}) was then subjected to buffer exchange using Amicon Ultra 100-kD centrifugal devices (Millipore) to remove CsCl salts, treated with DNase and RNase, and extracted for DNA and RNA as previously described (Weynberg et al. 2014). Extracted nucleic acids were amplified using sequence-independent single primer amplification (SISPA) (Culley et al. 2010; Weynberg et al. 2014), libraries were constructed using Nextera XT, and sequencing was performed on a MiSeq (2 × 300 bp paired-end) at the Ramaciotti Centre (University of NSW, Sydney, Australia).

Quality control of reads and assembly

Raw sequence reads were trimmed and filtered for quality by CLC Genomics Workbench package v8.0 (CLC Bio, Aarhus, Denmark). A PHRED score of 20 and a minimum sequence size cut-off of 100 base pairs (bp) were adopted for all individual reads. Paired reads were merged and combined with unmerged orphan sequences, before a final sequence length cut-off of 200 bp was applied. Single read metagenomic analysis of the RNA virome and predicted

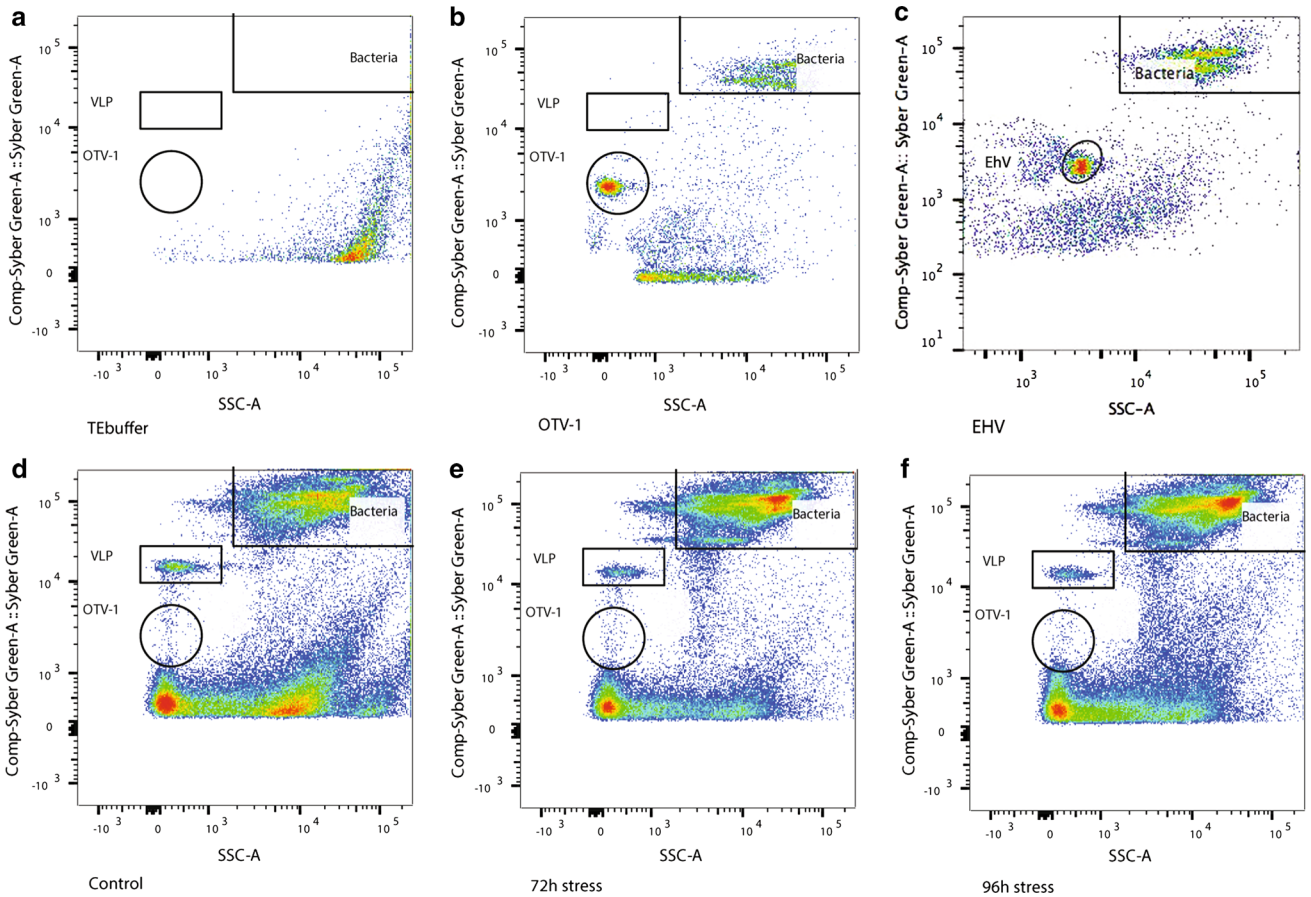

Fig. 1 Flow cytograms showing the presence of virus-like particles in purified algal lysates for *Symbiodinium* strain SCF055-A2 control cultures and 24 h after exposure to elevated temperature stress. **a** TE buffer control sample. **b** Purified algal lysate culture of *Ostreococcus tauri* virus-1 (OtV-1). **c** Purified algal lysate culture of *Emiliania huxleyi* virus-86 (EhV-86). **d** Control cytogram of *Symbiodinium* culture strain SCF055-A2 type C1. **e** Elevated temperature-exposed (36 °C for 72 h) *Symbiodinium* culture strain SCF055-A2 type C1 **f** Elevated temperature-exposed (36 °C for 96 h) *Symbiodinium* culture strain SCF055-A2 type C1. All other strains showed the same pattern (data not shown)

gene analysis of the assembled DNA virome were performed in Metavir2 (Roux et al. 2014) using Metavir's BLAST-based comparison (e-value $\leq 10^{-5}$) to the 19 January 2015 NCBI viral RefSeq database and normalized to genome length using the built-in genome-relative abundance and average size (GAAS) normalization tool (Angly et al. 2009). Assembly of DNA viromes was performed as follows: Illumina adapters were removed, and the reads were trimmed for quality using Trimmomatic v.0.33 (Bolger et al. 2014), cutting when the quality per base dropped below 20 and removing reads less than 150 bp. Trimmed reads were assembled using SPAdes v3.5.0 (Bankevich et al. 2012) with the single cell flag to account for coverage biases introduced during the SISPA procedure. Open reading frames were extracted from the contigs using prodigal v2.6.1 (Hyatt et al. 2010) and BLASTp was used against the National Center for Biotechnology Information (NCBI) non-redundant (nr) database for functional annotation. Reads were archived at the NCBI Sequence Read Archive (SRA): DNA virome data SRR2818102; RNA virome data SRR2818103.

Results

Flow cytometry

Initial flow cytometry analyses of monoclonal C1 cultures (SCF055-A2 shown as representative) without (control, Fig. 1d) and with UV and temperature stress exposure (Fig. 1e, f—only temperature stress data shown) revealed a high number of VLPs in both control and stressed *Symbiodinium* C1 cultures. No differences were detected between control and treatment when flow cytometry samples were visualized 24 h after elevated temperature exposure for 72 or 96 h (Fig. 1d–f). Flow cytograms showed the same virus population in all four strains: similar internal complexity or 'granularity' to OtV-1,

based on side scatter, and a larger genome size than OtV-1, based on green fluorescence (Fig. 1d–f). These four mono-cultured *Symbiodinium* C1 strains, held in the AIMS Symbiont Culture Facility for >3 yr, were non-axenic and had similar bacterial populations based on flow cytometry (Fig. 1d–f).

Virus morphology

TEM of untreated *Symbiodinium* cultures (controls) revealed VLPs with morphological similarities to filamentous and icosahedral VLPs (Fig. 2). Filamentous VLPs appeared flexuous and ranged from 1000 to 2500 nm in length and 20–30 nm in diameter (Fig. 2a–c). Icosahedral VLPs ranged in diameter from 50 to 80 nm (Fig. 3b, c) or 125–180 nm (Fig. 3a, d), with many VLPs displaying an electron dense core and lipid layer surrounding a capsid (Figs. 2, 3).

TEM analysis of thin sections of untreated *Symbiodinium* cultured cells revealed the presence of healthy nuclei in some cells (Fig. 4a), but others contained filamentous VLPs in the nuclei (Fig. 4b–d). Untreated *Symbiodinium* cells with more filamentous VLPs within their nuclei also had a concomitant decrease in the uniform condensed liquid crystalline appearance of chromosomes (Fig. 4a) as the integrity of the nuclei degraded (Fig. 4b–d). Other organelles, such as chloroplasts and mitochondria, remained intact until the filamentous particles had spread intracellularly and filled much of the cell (Fig. 4b–d).

DNA and RNA viromes

After quality control (QC), the DNA virome consisted of 591 095 raw reads. Assembly of all the DNA virome reads resulted in 1008 contigs of which 21.72% presented a significant hit to viruses and contained a total of 1785 predicted open reading frames. The majority of the predicted genes returned matches to hypothetical proteins. Thirty-four of the assembled contigs contained predicted genes with hits to eukaryotic viruses within the *Phycodnaviridae*, *Mimiviridae*, and *Retroviridae* (Table 1; Fig. 5). Following QC, the RNA viral fraction comprised 965 825 raw reads of which 0.13% of the virome sequences presented a significant match to viruses in Metavir2 (BLAST, bit score ≥50).

DNA virome taxonomic and functional composition of cultured *Symbiodinium*

Taxonomic BLASTx results of eukaryotic viruses in the *Symbiodinium* C1 DNA virome show similarities predominantly to dsDNA viruses within the *Phycodnaviridae*

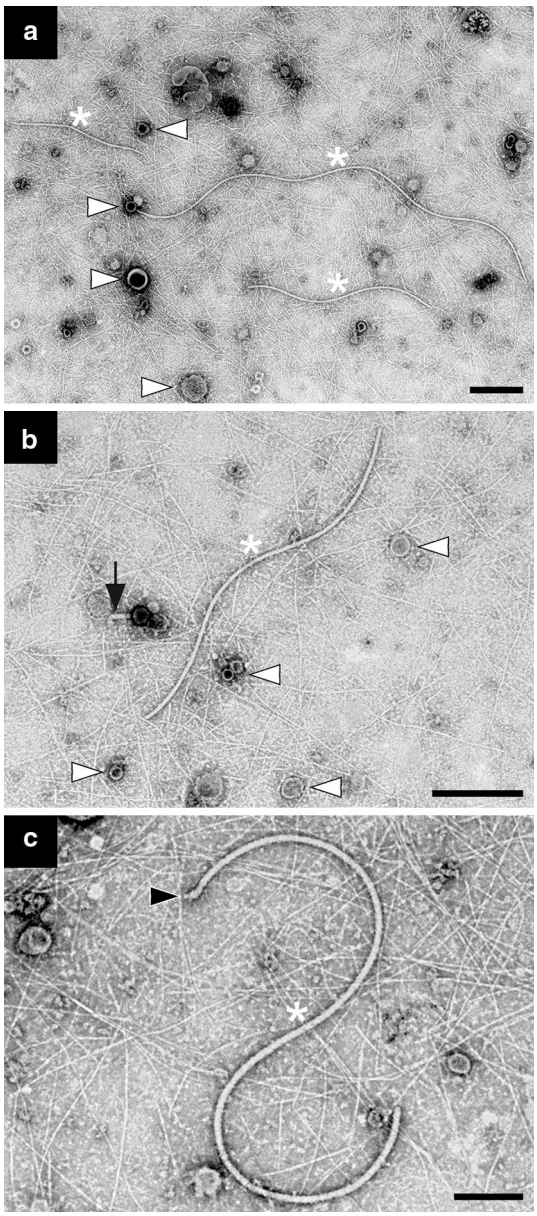

Fig. 2 Transmission electron micrographs (TEM) of negatively stained VLPs in cells of *Symbiodinium* culture strain SCF055-A2. **a–c** Filamentous flexuous virus-like particles (VLPs) are seen in all TEM images (*white star*). **a, b** *White triangles* indicate icosahedral VLPs, and **b** *black arrow* indicates a tailed bacteriophage. **c** Flexuous filamentous VLP, with a *black triangle* indicating possible attachment site at one end of capsid. *Scale bars*: **a**, **b** 300 nm, **c** 200 nm

and *Mimiviridae,* and to a lesser extent other members of the NCLDVs, including the *Marseilleviridae, Poxviridae*, and *Iridoviridae* (Electronic supplementary material, ESM, Table S1). In addition, sequence similarities to baculoviruses, and members of the *Herpesvirales, Nimaviridae*, and *Nudiviridae* were detected (ESM Table S1). The majority of functional gene predictions were highly similar (threshold of 50 on the BLAST bitscore) to phycodna- and mimiviruses (Table 1) returning BLAST hits to a number

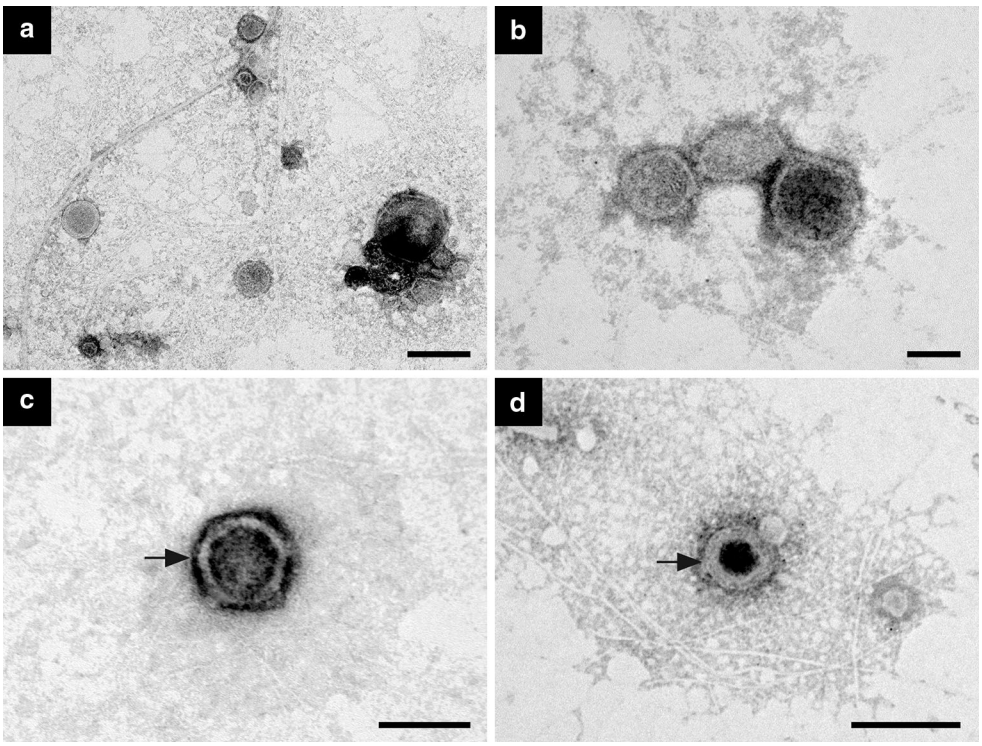

Fig. 3 Transmission electron micrographs of negatively stained icosahedral VLPs (**a**, **b**) from surrounding media of *Symbiodinium* type C1 strain SCF055-A2 cultures. **c**, **d** *Black arrows* indicate lipid layer membrane surrounding capsid. *Scale bars*: **a**, **d** 250 nm; **b**, **c** 50 nm

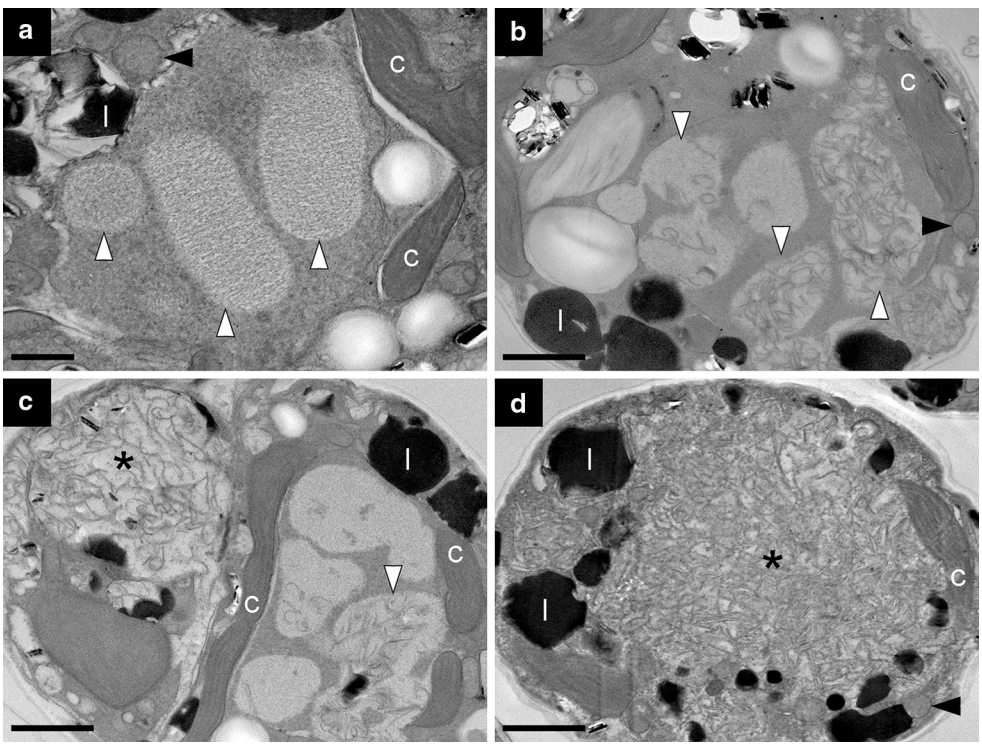

Fig. 4 Transmission electron micrographs of thin sectioned *Symbiodinium* cultured cells. **a** Healthy *Symbiodinium* cell from strain SCF055-B6 with intact chromosome (*white triangles*) packed inside nuclei and intact mitochondria (*black triangles*). **b** Initial breakdown of chromosome and nuclei structures (representative images from strain aims-aten-C1-MIa-cfu-A2). **c** Further progression of chromosome and nuclei degradation (representative image from strain SCF055-B4). **d** Complete degradation of cellular structures (representative image from strain SCF055-B6). *c* Chloroplast; *l* lipid; *asterisk* virus spreading throughout cell. *Scale bars*: **a** 500 nm, **b–d** 1 μm

of genes involved in synthesis and metabolism of pyrimidines and purines, components of nucleic acids, including a thymidylate synthase, uridine kinase, and aspartate transcarbamylase (Table 1). There were also similarities to genes encoding enzymes involved in amino acid biosynthesis, (e.g. pyridoxal phosphate-dependent transferase, tRNA synthetases, e.g. isoleucyl-tRNA synthetase) as well as a homologue of eukaryotic translation initiation/elongation factor eEF-3. The dsDNA virus identified here encodes genes implicated in virus-directed DNA repair mechanisms: pyrimidine DNA glycosylase; a virally encoded dUTPase (used in DNA repair by excising uracil);

Table 1 Top BLASTp hits (against NCBI non-redundant database) for predicted genes in assembled contigs of the eukaryotic DNA virome of *Symbiodinium*

Function and protein	*E* value	Closest viral similarity	Function and protein	*E* value	Closest viral similarity
DNA, RNA, replication, repair, recombination			***Protein and lipid synthesis/modification***		
DNA ligase	**8e−44**	**Lausanne**	Serine palmitoyltransferase	1e−49	EhV
ATP-dependent metalloprotease FtsH	3e−129	MpV12T	DnaJ/Hsp40	8e−48	CroV
ATP-dependent DNA helicase	3e−32	CroV	DnaK/Hsp70	1e−70	CroV
ATP-dependent RNA helicase	**1e−34**	**Mimi**	Cys/met metabolism PLP dependent enzyme	4e−10	Pandora
DNA repair protein	5e−21	Moumou	***Transport***		
Exodeoxyribonuclease VII large subunit	2e−36	CroV	ABC2 type transporter superfamily	4e−13	CroV
eIF-2/eIF-5B	1e−21	CroV	P-aminobenzoic acid synthase	1e−12	Pandora
Superfamily II helicase/eIF-4AIII	1e−57	CroV	DNA-binding ferritin-like protein	6e−24	Pitho
Uvr/REP helicase	3e−07	Irido	Lon protease	3e−70	AaV
Pyrimidine dimer-specific glycosylase	3e−35	PBCV-1	***Nucleotide transport and metabolism***		
dUTPase	8e−55	Lausanne	**Thymidylate synthase**	**2e−60**	**Herpes**
DNA polymerase X family	2e−29	Mega	Deoxycytidylate deaminase	1e−21	EhV
Ribonucleotide-diphosphate reductase small subunit	**0.0**	**OlV5**	GMP reductase	1e−17	PgV
Ribonucleotide-diphosphate reductase small subunit	**1e−31**	**PBCV-1**	tRNA threonylcarbamoyl adenosine biosynthesis	6e−24	Pandora
Chromosome segregation protein	7e−16	TvV	NAD-dependent epimerase/ dehydratase	1e−28	Moumou
Translation			Uridine kinase	4e−10	Moumou
Isoleucyl-tRNA synthetase	4e−24	Mimi	Dihydrofolate reductase	3e−24	Iridovirus
Methionyl tRNA synthetase	4e−25	Mimi	Nucleoside diphosphate kinase	1e−09	PgV
tRNA threonylcarbamoyl adenosine synthase	3e−24	Pandora	***Sugar manipulation enzymes***		
Translation elongation factor eEF-3	1e−10	PgV	UDP-glucose glycosyltransferase	2e−06	PBCV-1
Aspartate transcarbamylase	1e−31	PBCV-1	Pyrimidine dimer-specific glycosylase	3e−35	PBCV-1
Cysteine desulfurase	6e−29	Mimi	***Signalling***		
Pyridoxal P-dependent transferase	3e−54	Mimi	Histidine kinase	1e−61	EsV
Glutamine synthetase	1e−18	Moumou	Serine/threonine−protein kinase/ receptor	1e−49	EhV
DNA restriction/methylation			Ankyrin repeat protein	2e−24	Mimi
Macrocin o-methyltransferase	3e−10	Mega	**Thioredoxin**	**2e−15**	**Lausanne**
Cytosine-N(4)-specific DNA-methyltransferase	2e−09	Pandora			

Predicted protein hits to core NCLDV genes are highlighted in bold

AaV: *Aureococcus anophagefferens* virus; CroV: *Cafeteria roenbergensis* virus; EsV: *Ectocarpus siliculosus* virus; EhV: *Emiliania huxleyi* virus; Herpes: Equid herpesvirus; Irido: Iridescent insect virus; Lausanne: Lausannevirus; Mega: Megavirus; Mimi: *Acanthamoeba polyphaga* mimivirus; Moumou: Moumouvirus; MpV12T: *Micromonas pusilla* virus 12T; OlV5: *Ostreococcus lucimarinus* virus 5; Pandora: Pandoravirus; PBCV-1: *Paramecium bursaria Chlorella* virus 1; PgV: *Phaeocystis globosa* virus; Pitho: Pithovirus; TvV: *Tetraselmis viridis* virus

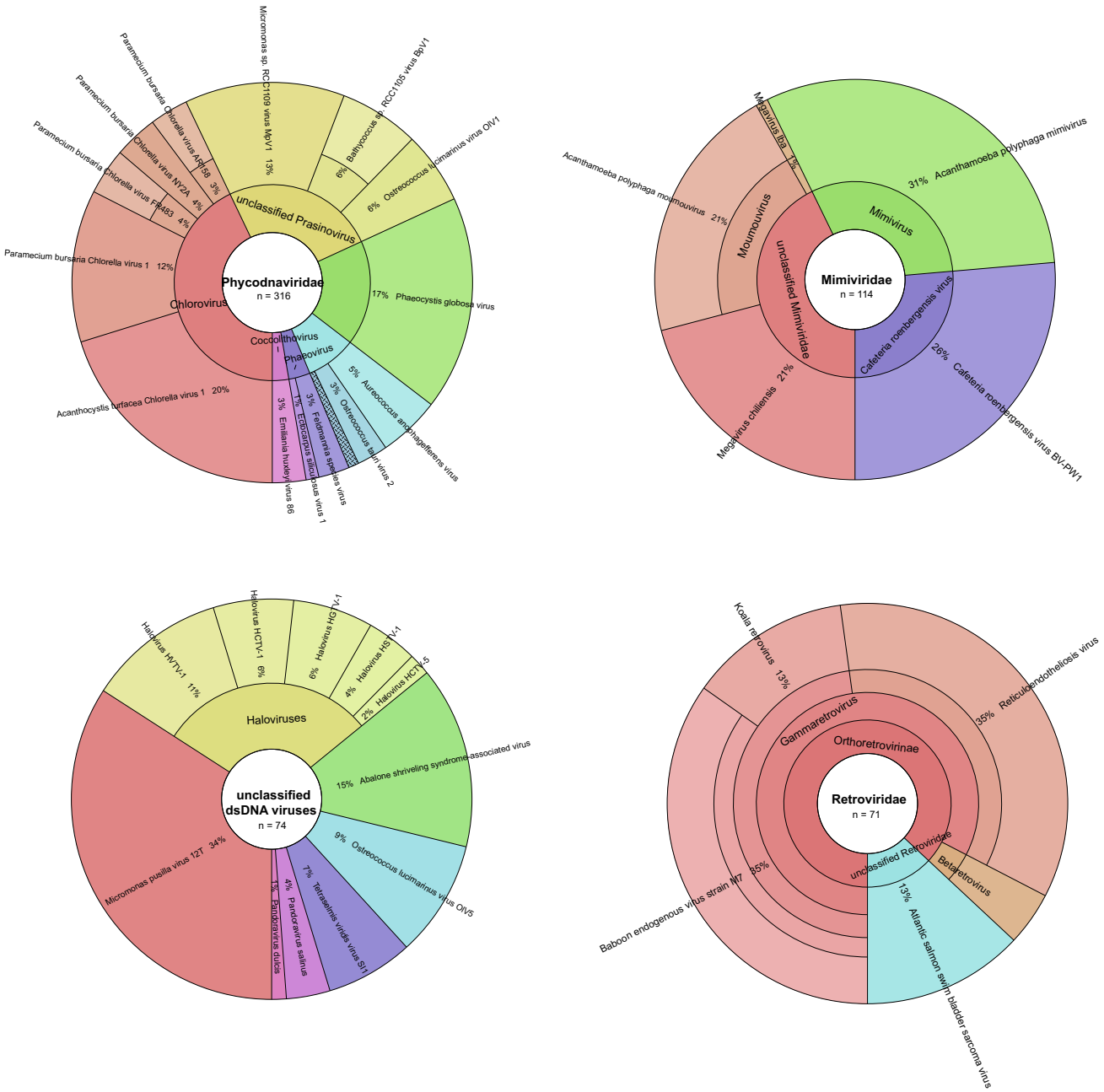

Fig. 5 Taxonomic annotation of predicted genes (DNA viromes) and single reads (RNA viromes), generated from *Symbiodinium* virus clade C1 in culture, computed through MetaVir2 (Roux et al. 2014). Taxonomic composition is computed from comparison with the RefSeq complete viral genomes sequences database from NCBI (released 19 January 2016) using BLASTx (threshold of 50 on the BLAST bitscore) and displayed through Krona graphs

a DNA repair protein with similarity to a gene encoded by Mimivirus; and a NAD-dependent DNA ligase (Table 1). More specifically, this metavirome approach has revealed the presence of at least 48 genes encoded by members of the NCLDV group of viruses: putative family X DNA polymerase; helicases; and transcription factors involved in initiation, elongation, and termination of transcription, as well as putative sugar metabolism enzymes and methyltransferases for post-translational modifications. In particular, this *Symbiodinium* virus shared homology to phycodnavirus-like ribonucleotide reductase large and small subunit genes (Table 1).

Retroviral sequence similarities dominate RNA virome of cultured *Symbiodinium*

The best BLASTx taxonomic hits to RNA viruses were to members of the *Retroviridae* (Table 2; Fig. 5). Putative

genes included an envelope gene, reverse transcriptase, integrase, and a polyprotein gene (Table 2). A number of reads with sequence homology to phycodnaviruses, including a short read to the OtV-2 MCP gene, were present in the RNA virome.

Discussion

The initial aim of this study was to employ flow cytometry to test the hypothesis that stress, such as elevated temperature or UV shock, induces latent viruses to actively infect cultured type C1 *Symbiodinium* cells. However, during stress experiments, no discernible differences were observed between control and treated cultures, which led to further analysis using TEM and molecular approaches. Three different putative viruses were present in the cultures, regardless of stress treatment. Future studies of viral infection in *Symbiodinium* may include a comparison of the screening techniques involved.

A dsDNA virus related to the NCLDV family infects *Symbiodinium* C1 cultures

Flow cytometric analysis of four monoclonal type C1 *Symbiodinium* cultures revealed a virus population consisting of VLPs with similar granularity to OtV-1 viral particles, but with larger genomes (≥200 kb). These populations were persistent across all cultures, with no differences between control and experimentally UV- or heat-shocked treatments. TEM imaging, virome analysis, and previous transcriptome sequencing (Levin et al. 2016) all provided additional support for a large dsDNA virus related to the NCLDV family of viruses infecting the type C1 *Symbiodinium* in culture. These findings are consistent with previous reports of VLPs resembling members of the NCLDVs in the surface mucus layer of scleractinian coral species (Davy and Patten 2007) and in association with cultured *Symbiodinium* cells (Lawrence et al. 2014). In the latter study, VLPs were detected using TEM in different *Symbiodinium* clades (i.e. A, B, C, and E), indicating viruses are likely a common feature of *Symbiodinium* cultures. Transcriptomic evidence of a dsDNA virus putatively infecting *Symbiodinium* in *Montastraea cavernosa* coral tissue (Correa et al. 2012) and the same *Symbiodinium* type C1 cultures examined here (Levin et al. 2016) have also been reported, as well as additional '-omic' evidence of dsDNA viruses targeting *Symbiodinum* (Wood-Charlson et al. 2015).

The NCLDVs likely form a monophyletic group of virus families whose members infect a range of eukaryotes (Yutin et al. 2009). NCLDVs can replicate either solely in the cytoplasm or have stages of replication in both the nucleus and cytoplasm (Iyer et al. 2001, 2006). Most described NCLDVs encode genes required for cellular functions, e.g. transcription, translation, and replication (Yutin et al. 2009), and, atypically for viruses, they can undertake their own replication and transcription without a strong reliance on the host cell machinery to conduct protein synthesis (Yutin and Koonin 2012). A number of these core NCLDV genes matched predicted gene sequences in the *Symbiodinium* type C1 DNA virome, providing strong support for *Symbiodinium* infection by an NCLDV. As with other viruses of the NCLDVs, the dsDNA virus targeting *Symbiodinium* appears to have a complex life strategy, encoding protein synthesis genes enabling independence from the host.

Observed sequence similarities to giant viruses (e.g. *Mimiviridae*) within the *Symbiodinium* C1 virome likely reflect current database limitations as a number of 0.2 μm filtration steps were incorporated in the laboratory methods

Table 2 Top BLASTx results for four RNA retrovirus consensus reads sequenced from *Symbiodinium* type C1 culture strain against both NCBI non-redundant (nr) database and viral RefSeq database

Retrovirus consensus read #	Putative function	Top BLASTx hit result in NCBI nr	*E* value	Query cover (%)	Identity (%)	Closest BLASTx hit result in NCBI Viralrefseq	*E* value	Query cover (%)	Identity (%)
1	Endonuclease	*Ovis aries* NYNRIN-like protein: XP_006772113.1	9e−17	86	53	Baboon endogenous virus: YP_009109695.1	4e−14	77	53
2	Envelope protein	*Nomascus gabriellae* envelope polyprotein: ABB52639.1	4e−45	98	80	Atlantic salmon swim bladder sarcoma virus: YP_443923.1	2e−24	99	49
3	RVgp2 protease/ polymerase	*Ovis aries* NYNRIN-like protein: XP_012013024.1	3e−28	80	59	Reticuloendotheliosis virus: YP_223871	3e−15	75	44
4	Reverse transcriptase	*Ochotona princeps* Polyprotein: XP_012786164.1	9e−36	92	64	Koala retrovirus: YP_008169843.1	2e−30	80	60

that should have precluded sampling of particles >200 nm. However, these results indicate that either common genes are shared between the dsDNA viruses associated with *Symbiodinium* C1 and algal/giant viruses in the databases, or ~200-nm-diameter viruses with similarity to *Mimiviridae*, such as Lausannevirus (Thomas et al. 2011), may infect *Symbiodinium* C1. Viromes generated from the coral species *Pocillopora damicornis* and the sponge species *Rhopaloeides odorabile,* using the same filtration method as described here, have also been reported to encode predicted genes with BLAST matches to *Mimiviridae* (Laffy et al. 2016).

Retrovirus-like viruses infect *Symbiodinium* C1 cultures

RNA viruses, most of which infect eukaryotes, have been largely overlooked in studies of marine viruses (Steward et al. 2013), although some studies have begun to use metagenomics and PCR-based approaches to examine this important assemblage within the marine virioplankton (Lang et al. 2009; Culley et al. 2014). Importantly, recent research has revealed that 60–99% of RNA viruses in metagenomic data sets are not homologous to known viruses (Mokili et al. 2012). Reports of characterized RNA viruses that infect dinoflagellates have been limited to just two host species: *Gymnodinium mikimotoi* (Onji et al. 2003) and the free-living *Heterocapsa circularisquama,* which is infected by a positive-sense ssRNA (HcRNAV, dinornavirus) virus with a 30-nm-diameter capsid (Tomaru et al. 2004). Interestingly, recent metatranscriptome and virome analysis of the corals *Montastraea cavernosa* (Correa et al. 2012) and *Acropora tenuis* (Weynberg et al. 2014) also found evidence of a ssRNA virus with sequence homology to the MCP of the *H. circularisquama* RNA virus (HcRNAV). Whilst sequences with homology to HcRNAV (dinornavirus) were not detected in the RNA virome derived from type C1 *Symbiodinium* monoclonal cultures, this is likely a result of extremely low levels of these viruses present in these particular cultures, as was recently shown (Levin et al. 2016).

Filamentous VLPs were highly prevalent, as evidenced in TEM images, and consistent with reports of filamentous VLPs in other *Symbiodinium* cultures (Lohr et al. 2007; Lawrence et al. 2014). For instance, Lohr and colleagues (2007) reported the presence of VLPs in cultured *Symbiodinium* cells following UV stress that were morphologically similar to members of a family of ssRNA plant viruses, the *Closteroviridae*. It was speculated that these filamentous VLPs indicated a latent infection in the algal cells with UV exposure triggering a switch from latency to a lytic replication event. The filamentous flexible viral capsids of RNA viruses facilitate encapsidation of predominantly ssRNA genomes and are typical of lytic viruses that infect a diverse range of eukaryotic hosts (King et al. 2011). Interestingly, unlike previous reports showing membrane damage in chloroplasts following temperature and light stress (Downs et al. 2013; Lawrence et al. 2014), the TEM data here provide evidence that VLPs are associated with damage within nuclei, prior to any observable adverse effects on other organelles. Given the close association of the observed filamentous VLPs with the *Symbiodinium* nucleus (Fig. 4b–d), we propose that these viruses initiate replication in the host nucleus and may switch from a lysogenic to lytic cycle, although this requires further experimental confirmation.

The RNA virome from type C1 *Symbiodinium* contained sequences with homology to an envelope protein encoded by members of the *Retroviridae* with closest homology to an Atlantic salmon swim bladder sarcoma virus envelope protein (ASSBSV) (Table 2), as well as sequences with homology to a conserved Ebola/HIV-1-like HR1-HR2 superfamily domain. This heptad repeat region is known to encode an envelope glycoprotein that results in infectious retroviruses distinct from retrotransposable elements (Kim et al. 2004). *Retroviridae*-like sequences have previously been observed in the proteome of a *Symbiodinium*-enriched fraction from a colony of the coral, *Stylophora pistillata* (Weston et al. 2012), the coral holobiont *Acropora digitifera* (Dunlap et al. 2013) and other scleractinian species (Correa et al. 2012, 2016), indicating retroviruses may be common viruses of corals.

Of the few reports of heat stress and viral production in algae, most have been in studies of algal endosymbionts, including temperature induction of viruses in *Anemonia viridis* (Wilson et al. 2001). For example, heat shock treatments of the corals *Pavona danai* and *Acropora formosa* resulted in observed increases in VLPs associated with *Symbiodinium* in hospite, and increases in VLPs were also reported in fresh isolates of *Symbiodinium* from *P. danai* and *Stylophora pistillata* after thermal stress (Wilson et al. 2005a; Davy et al. 2006; Correa et al. 2016). In combination with these previous reports, our results point to a role for certain groups of viruses in the coral bleaching response, which requires validation through further experimental and field-based research.

Symbiodinium culture use in coral-related experiments

The findings reported here relate to *Symbiodinium* in culture not in hospite. In the light of the number of *Symbiodinium* strains held in culture and used for experimental research, including the infection of coral larvae, it is timely to investigate whether viruses are present in fresh isolates of *Symbiodinium*, as well as symbiont algal strains held in

culture over longer periods and how these viruses influence host health. Here we provide novel insights into the viruses infecting cultured *Symbiodinium* and present a cautionary tale that these viruses may influence organismal health with implications for experimental reef research utilizing *Symbiodinium* cultures.

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