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# MOLECULAR DETECTION OF T4-like MYOVIRUSES IN HIGH MICROBIAL ABUNDANCE (HMA) MARINE SPONGES, *Aaptos suberitoides* AND *Xestospongia testudinaria* FROM TERENGGANU ISLANDS, MALAYSIA, SOUTH CHINA SEA

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**Abstract:** Sponges and viruses are abundant biotic components of the marine ecosystem, particularly when conducting nutrient recycling. Cyanobacteria are the main source of oxygen supplies and nutrient cycling and provide colouration to the sponge. Meanwhile, the T4-like phages are a group of lytic phages from Myoviridae known to infect cyanobacteria. These symbiotic relationships of sponges-cyanobacteria can be disrupted by the infection of cyanophages that can kill the cyanobacteria. Thus, this study aimed to detect the presence of T4-like myovirus in High Microbial Abundance (HMA) marine sponges such as *Aaptos suberitoides* and *Xestospongia testudinaria* from Bidong Island and Karah Island, Terengganu, Malaysia. A total of 208 replicates, sponges (n = 80), water (n = 64), and sponge mucus (n = 64) were subjected to Polymerase Chain Reaction (PCR) analyses accordingly. A total of four replicates of *A. suberitoides* (n = 4/15) from Karah Island and 26 replicates of water samples (n = 26/32) from Bidong Island (1X and 10X DNA dilution) demonstrated positive results. Sequencing analysis results of the Major Capsid Protein (MCP ) gene demonstrated 97.37% similarities for Bidong water samples and 77.72% similarities for sponge samples *A. suberitoides* for T4-like myovirus gene 23. Remarkably, this is the first detection of T4-like myovirus in both sponge species, *A. suberitoides*, *X. testudinaria*, and seawater in Malaysia.

Keywords: *Aaptos suberitoides*, *Xestospongia testudinaria*, T4-like myovirus, Malaysia, High Microbial Abundance (HMA).

## Introduction

The seas close to Malaysia are one of the biggest areas within the tropical region and they contain an exceptionally high diversity of sponges and corals (May, 2018; Hamza *et al*., 2018). The documentation of marine organisms, particularly in the Indomalayan region, continues to be the foremost task in modern biology (May, 2018). Notably, sponges have been around for a long time, with bound species having a fossil record that dates back more or less 600 million years to the earliest (Precambrian) amount of Earth's history (National Oceanic and Atmospheric Administration, United States Department of Commerce, 2019). Accordingly, the more or less 8,550 living sponge species are classified within the phylum Porifera, which is comprised of four classes: Demospongiae (the most numerous, representing 90% of all living sponges), Hexactinellida (the rare glass sponges), Calcarea (calcareous sponges), and Homoscleromorpha (the rarest and simplest category, solely recently recognised, with approximately 117 species) (National Oceanic and Atmospheric Administration, United States Department of Commerce, 2019).

Furthermore, sponges are ecologically leading members of the benthic fauna in marine and they play a massive part in contributing to nutrient silicon cycling and nitrogen cycling (Fiore *et al*., 2013). Correspondingly, sponges

are widespread in the South China Sea. Some of the sponges collected in Malaysia, the South China Sea are *Aaptos* sp. (Rosmiati *et al*., 2014; Yu *et al*., 2014; Murniasih & Bayu, 2015), *Xestospongia* sp. (Ibrahim *et al*., 2014), *Acanthella* sp. (Xu *et al*., 2012), *Callyspongia* sp. (Chen *et al*., 2014), *Halicondria* sp. (Sun *et al*., 2015), *Theonella* sp., and *Jaspis* sp. (Xu *et al*., 2018). Sponges have been characterised as those with a High Microbial Abundance (HMA) and those with a Low Microbial Abundance (LMA). Accordingly, HMA species harbour dense, diverse microbial communities while LMA species contain fewer and less diverse microorganisms (Ribes *et al*., 2015).

In particular, HMA sponges commonly harbour highly diverse microbial communities that contain bacterial and archaeal types reported in Porifera. These assemblages of microorganisms are known as "sponges microbial signatures" while LMA sponges have lower and variable diversity can be dominated by fewer microorganisms (García-Bonilla *et al*., 2021). Concurrently, two HMA marine sponges (*Xestospongia* sp., *Aaptos* sp.) and an LMA marine sponge (*Haliclona* sp.) are discovered on Bidong Island (Amelia *et al*., 2020). At the same time, three HMA marine sponges (*Xestospongia* sp., *Aaptos* sp., and *Theonella* sp.) and an LMA marine sponge (*Haliclona* sp.) are observed in Karah Island (Jamaludin *et al*., 2023). Thus, *Xestospongia testudinaria* and *Aaptos suberitoides* were selected for this study due to their high abundance on both islands.

In general, the sponge symbiotic bacterial community plays a significant role in metabolic functions. This includes nitrogen cycling (Hoffmann *et al*., 2009), phosphorus cycles (Zhang *et al*., 2015), and sulphur cycling (Jensen *et al*., 2017), and a total of 40% of the sponge volume is comprised of microbes and the most dominant inhabitants are cyanobacteria (Konstantinou *et al*., 2018). Furthermore, the symbiosis between marine sponges and cyanobacteria depends on whether the sponge species is an obligate or non-obligate interaction (Steindler *et al*., 2005). In addition, some sponges have mutualistic interactions with cyanobacteria, in which sponge hosts provide habitats for cyanobacteria. In return, cyanobacteria provide sponges with additional nutrition via nitrogen fixation and photosynthesis, chemical defence via the production of bioactive compounds and Ultraviolet (UV) protection (Konstantinou *et al*., 2018).

Cyanophages belonging to the Myoviridae are widespread and ubiquitous in the ocean. Cyanophages evolved more than 3 billion years ago when cyanobacteria diverged from other prokaryotes (Suttle, 2000). The family Myoviridae consists of 30 genera. The discerned features of the Myoviridae family include a central core designed of stacked rings of six subunits and enclosed by a helical contracted sheath that is separated from the top by a neck. Notably, the genus *Cyanomyovirus* is also known as the T4-like cyanophage/myoviruses. T4-like myoviruses possess elongated heads (110 nm), long collar tails (114 nm), short spike base plates, and six long kinked tail fibres (Lavigne *et al*., 2009). A high number of cyanophages co-exist with cyanobacteria (Zborowsky & Lindell, 2019). In marine waters, the most abundant cyanophages are the genetically diverse Myoviridae, which infect *Synechococcus* spp. (Gómez *et al*., 2004).

Meanwhile, *Synechococcus* unicellular marine cyanobacterium infection viruses were first isolated in 1993 (Suttle, 2000). However, the symbiotic relationship between sponges and cyanobacteria can be disrupted by the presence or infection of cyanophages that can be fatal to cyanobacteria. Suttle (2000) estimated that approximately 3% of marine *Synechococcus* sp. is removed by cyanophages daily. At the same time, Jahn *et al*. (2021) reported that bacteriophages, in general, are estimated to lose 20% to 50% of marine bacteria per day. In terms of biotechnological applications, bacteriophages, including Myoviridae have been used as anti-staphylococcal therapeutic cocktails (Kornienko *et al*., 2020), pathogen controls, detection methods such as phage display and pathogen detection, and ability to generate nanostructures (Santos & Azeredo, 2019).

Conversely, according to a review by Jamal *et al*. (2021), the virus families that have been observed in sponges using various detection methods include Myoviridae, Mimiviridae, Siphoviridae, Poxviridae, Phycodnaviridae, and Podoviridae. In particular, Bacteriophages from these families (Myoviridae, Podoviridae, and Siphoviridae) were dominant species observed in sponges such as *Lubomirskia baikalensis* (Butina *et al*., 2015; 2019), *Carteriospongia foliascens* (Pascelli *et al*., 2018), *Stylissa carteri*, *X. testudinaria*, *Amphimedon queenslandica*, *Ianthella basta* (Laffy *et al*., 2018), *Rhopaloeide odorabile*, and *Hymeniacidon perlevis* (Harrington *et al*., 2012). Similarly, these viruses were detected using PCR, Reverse Transcription Polymerase Chain Reaction (RT-PCR), Transmission Electron Microscopy (TEM), Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE), and flow cytometry (Jamal *et al*., 2021).

Nonetheless, there are still gaps in information about sponge-related microbiome composition. This includes the impact of environmental factors on the sponge-related microbiome structure and their dynamics in tropical regions, including Malaysia, compared to other temperature and subtropical areas. Moreover, unlike microbiome analysis, analysis of viral communities associated with marine animals (not specified for sponges) is still in the primary stage. Hence, it is vital to investigate sponge viral composition to understand the relationship between marine sponges and viral composition associated with marine sponges. Notably, viruses are one of the great reservoirs of understudied genes with a potential biotechnological application such as the discovery of vaccination (Martínez *et al*., 2014).

Thereby, the objective of the present study was to detect the presence of T4-like myovirus from two HMA sponge species, *X. testudinaria*, *A. suberitoides*, and seawater samples from two Terengganu islands, Malaysia, based on different PCR assays.

## Materials and Methods

### *Sampling Sites and Sample Processing*

The sponge tissues (n = 80), sponge mucus (n = 64), and water samples (n = 64) were collected from Bidong Island (Station 7) and Karah Island (Station 9) (5°37'N, 103°5'E) on 15 September 2020. In total, 20 sponge samples, including *X. testudinaria* and *A. suberitoides* tissues were collected from both sites via scuba diving. Concurrently, the top rim of the sponge (from pinacoderm to mesophyll) was cut at approximately 3 x 3 x 3 $cm^3$. Then, five replicates for each species from each site were packed in individual ziplock bags filled with 1,000 µL Viral Transport Medium (VTM) [Invitrogen, United States of America (USA)]. Subsequently, the sponge samples were cut into smaller pieces, homogenised in VTM, and centrifuged for 15 minutes at 4°C and 14,000 *x g*. The supernatants were harvested and kept at -80°C, the remaining sponge samples were kept in VTM and stored at -80°C. Each sponge sample from both sites was replicated into 40 replicates based on the two PCR assays and two dilutions of samples (5 replicates x 2 methods x 2 dilutions x 2 sites = 40 replicates).

A total of four mucus samples were collected from each sponge species and site. Each mucus sample from both sites was replicated into 64 replicates based on the two PCR assays, two dilutions of samples, and two sponge samples (4 replicates x 2 PCR methods x 2 dilutions x 2 sites x 2 sponge species = 64 replicates). The sponges were then exposed to the air to allow it to secrete the mucus. The sponge mucus samples were collected using sterile cotton swabs and kept in 15 mL microcentrifuge tubes containing 3 mL VTM. Two litres of seawater from the top and bottom of the seawater were collected using the Van Don water sampler from both sites. Similarly, the water samples were replicated: 4 replicates x 2 PCR methods x 2 dilutions x 2 sites x 2 types of water samples = 64 replicates. Note that the samples were collected in replicates in order to increase the

chances of detection for the presence of viruses. The two different dilutions of samples (1X and 10X) were selected to determine the best sample dilution as 1X might consist of high Deoxyribonucleic acid (DNA) concentration, which does not allow the reaction to occur appropriately as it inhibits the reaction (Wu *et al*., 2010). At the same time, triplicate in-situ environmental measurements were conducted. In-situ measurements were conducted using YSI multiparameter for Dissolved Oxygen (DO), pH, temperature, and salinity. Then, the samples were transported back to the laboratory (on ice) at Universiti Malaysia Terengganu (UMT) within two hours. All samples were collected under the research pass MEA 40/200/19/3680 by the Economic Planning Unit, Prime Minister's Department, Malaysia.

### *Water Sample Processing and DNA Extraction*

The water samples were processed immediately after sampling and reaching the laboratory. They were filtered using 0.22 µm filters (47 mm diameter, cellulose acetate membrane filter, BIOFLOW, cat no. MALCA47022). The filter papers were cut into four pieces for upper and bottom water samples using sterile scissors and then placed horizontally into 50 mL centrifuge tubes. The water samples were kept in 200 µL sterile DNA buffer (Huang *et al*., 2015). Prior to extraction, the water samples were diluted in sterilised distilled water as 1X and 10X dilutions.

Hexadecyltrimethylammonium Bromide (CTAB) DNA extractions were executed on seawater samples. The CTAB DNA extraction methods were slightly modified by the National Oceanic and Atmospheric Administration, United States Department of Commerce (2019). Samples of the filter paper were placed in 400 µL of 2X CTAB mixture (Tris, pH 8.0 [0.0121 g/mL], CTAB [0.002 g/mL], EDTA [0.00744 g/mL], NaCl [0.0818 g/mL]). A total of 5 µL of 20 mg/mL of Proteinase K was added and mixed thoroughly with vortex, samples were incubated in a water bath for 3 hours at 64°C. A total of 400 µL of chloroform was added in the same Eppendorf tube, mixed thoroughly, and centrifuged at 12,000 *x g* at 4°C for 20 minutes. The aqueous layer (approximately 400 µL) was transferred to a new sterile Eppendorf tube to precipitate the DNA. A total of 400 µL isopropanol was added and centrifuged at 12,000 *x g* at 4°C for 20 minutes. Consequently, the samples were spun with 1 mL of 70% ethanol and centrifuged at 12,000 *x g* at 4°C for 20 minutes. The pellet was air-dried completely prior to resuspending in 50 µL of TE buffer.

### *DNA Extraction of Sponge and Mucus Samples*

All sponge samples were diluted in sterilised distilled water as 1X and 10X dilutions prior to viral DNA extraction. The DNA extraction was performed using the GF-1 Viral Nucleic Acid extraction kit (Vivantis Technologies Sdn. Bhd.) for the sponge and mucus samples using the protocol provided by the manufacturer. Prior to PCR analysis, the extracted DNA was kept at -20°C. All samples were subjected to different PCR analyses to increase the chances of detection based on the Myoviridae major capsid protein gene (File'e *et al*., 2005; Zhong *et al*., 2014).

### *PCR Analysis According to MZIAlbis and MZIA6 Primer*

Based on File'e *et al*. (2005), MZIAlbis primer (5'-GATATTTGIGGIGTTCAGCCIATGA-3') and MZIA6 primer (5'-CGCGGTTGATTTCCA GCATGATTTC-3') were used for the detection of Myoviridae MCP gene. The expected size of the primers was 500 bp. A total of 25 µL PCR mixture containing 12.5 µL exTEN 2X PCR Master Mix, 9.0 µL autoclaved double distilled water ($ddH_2O$), 0.5 µL (10 µM) of forward primer, and 0.5 µL (10 µM) reverse primer were added to 2.5 µL extracted DNA. The amplification for File'e *et al*. (2005) method was programmed as follows: Denaturation at 94°C for 90 seconds, 30 cycles of denaturation at 94°C for 45 seconds, annealing at 50°C for 1 minute, extension at 72°C for 45 seconds, and a final extension at 72°C for 5 minutes. The amplified PCR products from both reactions were then

analysed by electrophoresis, which was run for 60 minutes at 100 V on 2.0% (w/v) agarose gel in 1X Tris-acetate-ethylenediaminetetraacetic acid buffer and stained with SYBR® Safe - DNA Gel Stain (Invitrogen, Waltham, Massachusetts, USA).

### *PCR Analysis According to Myoviridae Major Capsid Protein Gene*

According to Zhong *et al*. (2014) method, the amplification was programmed as follows: 95°C for 15 minutes, denaturation at 95°C for 30 seconds (34 cycles), annealing at 50°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 5 minutes. The program for the second stage PCR was 5 minutes of denaturation at 95°C, denaturation at 95°C for 30 seconds (24 cycles), annealing at 50°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 5 minutes. The amplified PCR products from the reactions were then analysed by gel electrophoresis for 45 minutes at 70 V on 2.0% (w/v) agarose gel in 1X Tris-acetate-ethylenediaminetetraacetic acid buffer and stained with SYBR® Safe DNA Gel Stain (Invitrogen, Waltham, Massachusetts, USA).

### *Sequencing and Phylogenetic Analysis*

Two representative positive samples (BB4 and KA5) were sent for sequencing analyses at the Apical Systems Sdn. Bhd., Malaysia. BB4 was the bottom water sample from Bidong Island and KA5 stands for the *A. suberitoides* sample from Karah Island. These samples were selected based on the DNA band intensity of the gel electrophoresis, which will allow for a sufficient DNA concentration for sequencing analysis. Accordingly, the results were analysed and comparisons were made with GenBank sequences using BLAST NCBI (http://blast.ncbi.nlm.nih.gov). Consequently, the sequences were aligned using BioEdit Sequence Alignment Editor software and the generation of phylogenetic trees was made using aligned sequences with MEGA 7.0.26 software. Note that the bootstrap value was 100.

## Results

### *Water Quality Parameters from the Sampling Locations*

The water quality parameters presented a normal range at Bidong Island (Table 1) and Karah Island, according to Hussin (2014) and Zaleha *et al*. (2016) (Table 2).

### *Conventional PCR Assays*

Some water samples were positive for the detection of the Myoviridae major capsid protein gene based on File'e *et al*. (2005) and Zhong *et al*. (2014) (Table 2). Based on File'e *et al*. (2005), none of the samples demonstrated positive results except for 1X top water samples

Table 1: Water quality parameters of high microbial abundance marine sponge at Bidong Island (A) (5°36.790'N, 103°03.504'E) and Karah Island (B) (5°35.955'N, 103°03.820'E)

| Water Parameter (A) | Units | Value (Top) | Value (Middle) | Value (Bottom) |
|---|---|---|---|---|
| Temperature | °C | 30.4 | 30.4 | 30.4 |
| Dissolved Oxygen | mg/L | 1.40 | 2.85 | 5.76 |
| pH | - | 8.47 | 8.48 | 8.39 |
| Salinity | ppt | 31.90 | 31.96 | 31.89 |
| **Water Parameter (B)** | **Units** | **Value (Top)** | **Value (Middle)** | **Value (Bottom)** |
| Temperature | °C | 30.30 | 30.30 | 30.40 |
| Dissolved Oxygen | mg/L | 5.07 | 5.83 | 4.80 |
| pH | - | 8.49 | 8.49 | 8.46 |
| Salinity | ppt | 31.94 | 31.94 | 31.80 |

from Bidong Island (n = 3/8 replicates) (Table 2). Similarly, the primary PCR analysis of Zhong *et al*. (2014) detected T4-like myovirus in 1X (n = 16/16) and 10X water samples from Bidong Island (n = 7/8 replicates). These results also indicated that a higher number of DNA

Table 2: Conventional PCR results for the detection of T4-like myovirus in water samples, sponge mucus samples, and sponges: *A*. *suberitoides* and *X*. *testudinaria* based on different PCR methods

| Location | Method | Samples | Species/Part | DNA Devilution | PCR Result | Total Positives for Replicates |
|---|---|---|---|---|---|---|
| Karah Island | File'e *et al*. (2005) | Sponge | *Aaptos* sp. | 1X and 10X | Negative | - |
| | | | *Xestospongia* sp. | 1X and 10X | Negative | - |
| | | Mucus | *Aaptos* sp. | 1X and 10X | Negative | - |
| | | | *Xestospongia* sp. | 1X and 10X | Negative | - |
| | | Water | Top | 1X and 10X | Negative | - |
| | | | Bottom | 1X and 10X | Negative | - |
| Bidong Island | File'e *et al*. (2005) | Sponge | *Aaptos* sp. | 1X and 10X | Negative | - |
| | | | *Xestospongia* sp. | 1X and 10X | Negative | - |
| | | Mucus | *Aaptos* sp. | 1X and 10X | Negative | - |
| | | | *Xestospongia* sp. | 1X and 10X | Negative | - |
| | | **Water** | **Top** | **1X** | **Positive** | **2/4** |
| | | | Top | 10X | Negative | - |
| | | | **Bottom** | **1X** | **Positive** | **1/4** |
| | | | Bottom | 10X | Negative | - |
| Karah Island | Zhong *et al*. (2014)[1] | Sponge | ***Aaptos* sp.** | **1X** | **Positive** | **1/5** |
| | | | *Aaptos* sp. | 10X | Negative | - |
| | | | *Xestospongia* sp. | 1X | Negative | - |
| | | | *Xestospongia* sp. | 10X | Negative | - |
| | | Mucus | *Aaptos* sp. | 1X and 10X | Negative | - |
| | | | *Xestospongia* sp. | 1X and 10X | Negative | - |
| | | Water | Top | 1X and 10X | Negative | - |
| | | | Bottom | 1X and 10X | Negative | - |
| Bidong Island | Zhong *et al*. (2014)[1] | Sponge | *Aaptos* sp. | 1X and 10X | Negative | - |
| | | | *Xestospongia* sp. | 1X and 10X | Negative | - |
| | | Mucus | *Aaptos* sp. | 1X and 10X | Negative | - |
| | | | *Xestospongia* sp. | 1X and 10X | Negative | - |
| | | **Water** | **Top** | **1X** | **Positive** | **4/4** |
| | | | Top | 10X | Negative | - |
| | | | **Bottom** | **1X** | **Positive** | **4/4** |
| | | | Bottom | 10X | Negative | - |

| | | | | | | |
|---|---|---|---|---|---|---|
| Karah Island | Zhong *et al*. (2014)[2] | **Sponge** | ***Aaptos* sp.** | **1X** | **Positive** | **2/5** |
| | | | ***Aaptos* sp.** | **10X** | **Positive** | **1/5** |
| | | | *Xestospongia* sp. | 1X | Negative | - |
| | | | *Xestospongia* sp. | 10X | Negative | - |
| | | Mucus | *Aaptos* sp. | 1X and 10X | Negative | - |
| | | | *Xestospongia* sp. | 1X and 10X | Negative | - |
| | | Water | Top | 1X and 10X | Negative | - |
| | | | Bottom | 1X and 10X | Negative | - |
| Bidong Island | Zhong *et al*. (2014)[2] | Sponge | *Aaptos* sp. | 1X and 10X | Negative | - |
| | | | *Xestospongia* sp. | 1X and 10X | Negative | - |
| | | Mucus | *Aaptos* sp. | 1X and 10X | Negative | - |
| | | | *Xestospongia* sp. | 1X and 10X | Negative | - |
| | | **Water** | **Top** | **1X** | **Positive** | **4/4** |
| | | | **Top** | **10X** | **Positive** | **3/4** |
| | | | **Bottom** | **1X** | **Positive** | **4/4** |
| | | | **Bottom** | **10X** | **Positive** | **4/4** |

Bolded samples are positive samples detected by the different PCR methods. Total positives for replicates indicate the total number of positives based on the replicates for each type of sample. Zhong *et al*. (2014)[1] represents the first stage PCR and Zhong *et al*. (2014)[2] is the second stage PCR results.

replicates were positive for T4-like myovirus in Bidong Island and 1X dilution of the water samples (Table 2).

Based on the sponge species samples, *A. suberitoides* demonstrated a higher number of positive bands than *X. testudinaria* based on the Zhong *et al*. (2014) (n = 4/15 replicates) from Karah Island. These results suggested that more positive samples were observed in Bidong Island (n = 26) than in Karah Island (n = 4), regardless of sample type and PCR assays. Sponge sample dilution (1X) demonstrated more positive results than 10X sample dilution. In contrast, the mucus samples from both sampling sites and sponge species presented negative results based on all PCR methods. In terms of different PCR methods, nested PCR of Zhong *et al*. (2014) demonstrated a higher number of samples (water and sponge samples) than File'e *et al*. (2005) for the presence of T4-like myovirus.

### *Sequencing and Phylogenetic Analyses*

The phylogenetic analyses demonstrated that all revealed gene fragments were closely related to viruses of the order Caudovirales in the family Myoviridae (Table 3). Correspondingly, the water samples from Bidong Island (BB4) (Figure 1) and sponge sample (*A. suberitoides*) from

Table 3: The nucleotide identity and percentage similarities query cover the sample representative sent for sequencing analysis based on the Myoviridae major capsid gene from Bidong Island and Karah Island

| Sequences | Identity (%) | Query Cover | Accession Number | Virus | Family |
|---|---|---|---|---|---|
| BB4 | 84 | 18% | DQ105885.1 | Uncultured Myoviridae clone | Myoviridae |
| KA5 | 87 | 71% | KY685686.1 | Uncultured Myoviridae clone | Myoviridae |

BB4: Bottom water samples from Bidong Island. KA5: *Aaptos suberitoides* from Karah Island. Data was presented in percentages of similarities for 800 bp. Sequence variations were observed in the samples.

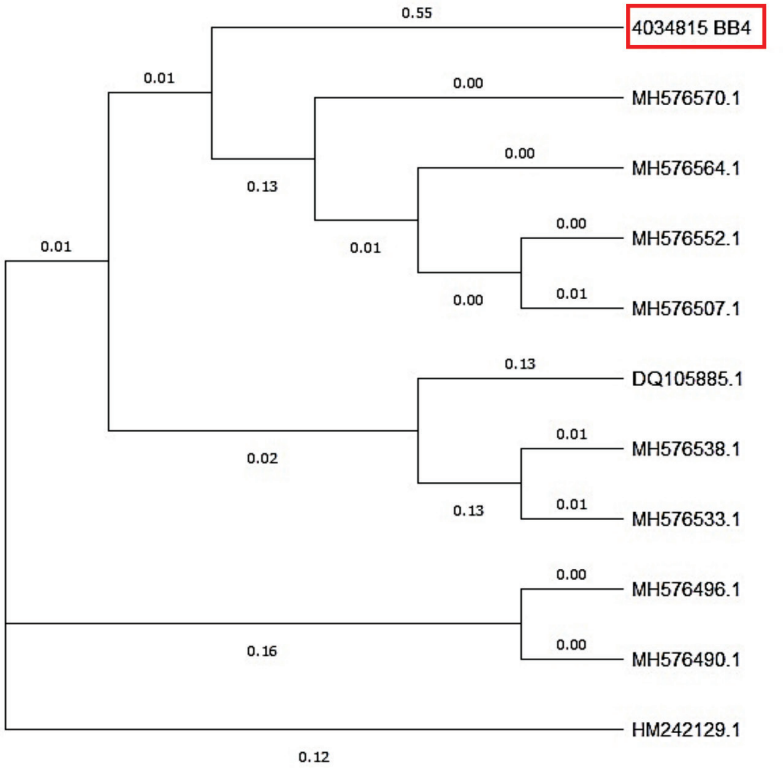


Figure 1: Phylogenetic neighbouring-joining tree for **BB4 (water sample)** for 500 bp within T4-like myoviruses major capsid protein (MZIAlbis and MZIA6 primer) of the nucleotide sequences in this study compared to GenBank nucleotide sequences. A blast search of the nucleotide sequences from this study revealed high sequence similarities to DQ105885.1 (Uncultured Myoviridae clone 3758), MH576496.1 (T4 virus environmental sample clone), MH576490.1 (T4 virus environmental sample), HM242129.1 (Uncultured Myoviridae clone s7-37), MH576570.1 (T4 virus environmental sample clone), MH576564.1 (T4 virus environmental sample), MH576552.1 (T4 virus environmental sample), MH576538.1 (T4 virus environmental sample clone), MH576533.1 (T4 virus environmental sample), and MH576507.1 (T4 virus environmental sample clone). In particular, sample BB4 yielded the highest sequence identity to accession number DQ105885.1, with 84% of the data retrieved from the blast search of the nucleotide sequences, with a bootstrap value of 1,000

Karah Island (KA5) (Figure 2) demonstrated high similarities (84% and 87%) to Myoviridae. However, they demonstrated low query cover in the BB4 water sample (18%), most probably due to the nature of the environmental sample. The alignment of nucleotide sequences of nested reaction amplified PCR products has been verified and the samples for BB4 and KA5 were from the same members of the T4-like myovirus gene 23.

## Discussion

The water quality parameters obtained in this study for both sites indicated normal readings. A practical salinity of seawater is 35 ppt, a standardised temperature is usually 25°C, the pH is from 7.5 to 8.4, and the DO level is 1 to 6 mg/L, according to Fondriest Environmental Learning Centre. The seawater temperature at both Bidong and Karah Islands is higher than that stated by Fondriest Environmental Learning

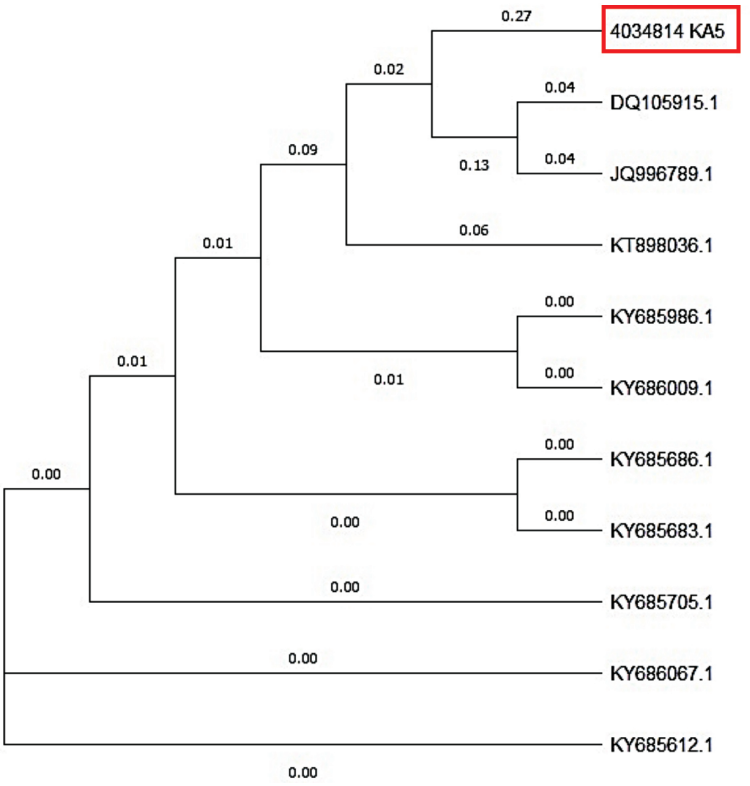


Figure 2: Phylogenetic neighbouring-joining tree for **KA5** (sponge, *A. suberitoides*) for 150 bp within T4-like myoviruses major capsid protein of the nucleotide sequences in this study, compared to Genbank nucleotide sequences. A blast search of the nucleotide sequences from this study revealed high similarities sequences to KY685686.1 (Myoviridae environmental samples clone), KY685683.1 (Myoviridae environmental samples), KY686067.1 (Myoviridae environmental samples clones), KY685612.1 (Myoviridae environmental samples), KY685705.1 (Myoviridae environmental samples clone), KY685986.1 (Myoviridae environmental samples clone), KY686009.1 (Myoviridae environmental samples clone), KT898036.1 (T4 virus environmental sample clone), DQ105915.1 (Uncultured Myoviridae clone), and JQ996789.1 (Uncultured Myoviridae clone). Specifically, sample KA5 demonstrated the highest sequence identity to accession number KY685686.1 with 87% from blast search of nucleotide sequences with a bootstrap value of 1,000

Centre while the pH, DO, and salinity levels are in the normal range. Whereas according to Hussin (2014), the average of water parameters recorded at Bidong Island was the salinity of 29.53 ppt. the temperature at 29.93°C, pH at 8.15, and DO at 3.84 mg/L. This suggests that the value of the water parameters was in a normal range, similar to Zaleha *et al*. (2016). Furthermore, similar water parameters were obtained from a study conducted at Karah Island and Bidong Island to detect the presence of Phycodnavirus from similar species of sponges and seawater (Ambalavanan, 2023). Ambalavanan (2023) also demonstrated that temperature (28°C to 29°C) is the only significant factor that influences the presence of Phycodnavirus. As the temperature increased, a higher number of Phycodnaviruses were reported in *A. suberitoides* from Karah Island. Temperature could also be an environmental factor that influences the presence of Myovirus, as a similar temperature range was observed in the current study.

In this study, the top and bottom water samples from Bidong Island were positive for the presence of T4-like Myovirus and water

sample BB4 presented high similarities (84%) to the Myoviridae family. Cyanomyoviruses are more abundant in the ocean and can infect a broad range of hosts such as *Synechococcus* and *Prochlorococcus* (Huang *et al*., 2015). The presence of cyanomyoviruses can also be due to the high abundance of *Prochlorococcus* and *Synechococcus* as primary producers in the ocean. Moreover, cyanomyoviruses can influence the abundance, diversity, and productivity of these cyanobacteria in the ocean (Huang *et al*., 2015). Similarly, cyanomyoviruses have been observed in seawater in many studies (Sandaa & Larsen, 2006; Sandaa *et al*., 2008; Liu *et al*., 2017; Ambalavannan *et al*., 2021; Jamal *et al*., 2021; Liu *et al*., 2021). According to File'e *et al*. (2005), T4-like myoviruses have been observed in water samples obtained from tropical regions such as the northeastern Gulf of Mexico, northeastern Pacific fjords and bays, and temperate regions such as the Arctic Ocean.

Positive bands for T4-like myovirus were detected in *A. suberitoides* marine sponges from Karah Island in this study. Similarly, phylogenetic analysis demonstrated that the KA5 positive sample was classified as belonging to the order Caudovirales in the family Myoviridae. T-4-like myoviruses have never been detected in *A. suberitoides* previously. This is the first detection of the major capsid protein of Myovirus. However, viruses from Myoviridae have been detected from other sponge species, including *X. muta* (Gomez *et al*., 2004) and *X. testudinaria*, using metagenomic analyses previously (Laffy *et al*., 2018). According to the review by Ambalavanan *et al*. (2021), Pascelli *et al*. (2018) detected Myoviruses using TEM in *Xestospongia* sp. and *X. testudinaria*. Similar work also detected VLPs from Myoviridae from other sponges such as *Echinochalina isaaci*, *S. carteri*, and *Amphimedon ochracea*. According to Potapov *et al*. (2019), the *Rezinkovia* sp. and *Swartschewskia papyracea* sponge samples were positive for T4-like myovirus. Previous studies have proven that sequences of the Myoviridae cyanophages have been observed in the Baikal sponge *L. baikalensis* for the first time according to the g20 gene as the marker isolated from *Synechococcus* spp (Butina *et al*., 2015). The positive presence of Myovirus-related DNA in *A. suberitoides* was reported compared to *X. testudinaria* in this study, which might be due to the morphology of *Aaptos* sp. *Aaptos* sp. consists of a choanosome with tiny choanocyte chambers. Thus, it traps more microbial communities. It can filter the seawater, efficiently retaining viruses and other pathogens (Hoffmann *et al*., 2008), and accessible to viral infection (Thomas *et al*., 2010; Nguyen *et al*., 2021).

The presence of T4-like myovirus in the mucus samples of *X. testudinaria* and *A. suberitoides* was negative, contrary to previous studies. This could be due to the different methods employed in this study, as previous studies conducted TEM to detect T4-like myovirus in marine sponges. Based on a previous study, Viral-Like Particles (VLPs) typical of the Myoviridae were observed in *E. isaaci*, *S. carteri*, and *A. ochracea* sponges (Lavigne *et al*., 2009). Eight morphotypes of Filamentous Virus-Like Particles (FVLPs) were observed in the mesohyl matrix, sponge mucus, within sponge cells, and associated with sponge-associated microorganisms (Lavigne *et al*., 2009). These morphotypes varied greatly in size (100 nm to 1,300 nm length, 12 nm to 60 nm width) and shape. Rod-shaped FVLPs were detected in the mucus of *S. carteri*.

In this study, most of the 1X sample dilution of *A. suberitoides* and seawater were positive for the presence of Myovirus, compared to 10X sample dilution. Note that the dilution sample (10X) might be overly diluted and under the detection limit of a conventional PCR assay. On the contrary, a study conducted in marine sponges revealed that *Xestospongia* sp. and *Aaptos* sp. samples demonstrated a high number of positives for the presence of phycodnaviruses based on 10X dilution from Karah Island (Fletcher, 2019; unpublished data). Similarly, Ambalavanan (2023, unpublished) data demonstrated that 10X sample dilution of *Xestospongia* sp., *Aaptos* sp., and seawater yielded more positive samples

for the presence of Phycodnavirus than 1X. Meanwhile, Zhong *et al*. (2014) used a nested PCR assay, demonstrating a higher number of positive samples based on major capsid protein than File'e *et al*. (2005). Accordingly, nested PCR assays are conducted to increase the accuracy and specificity of PCR assays (Laffy *et al*., 2018). Zhong *et al*. (2014) used the same primers for primary and secondary reactions, increasing the sensitivity and reaction cycles needed to detect the virus.

## Conclusions

This study demonstrated the detection of T4-like myovirus in *A*. *suberitoides*, *X*. *testudinaria*, and marine seawater from Bidong and Karah Islands, Terengganu, Malaysia. In this study, the detection of T4-like myovirus was conducted using conventional PCR assay according to File'e *et al*. (2005) and nested PCR method according to Zhong *et al*. (2014). Note that the water quality parameters were normal for both islands. The presence and absence of T4-like myovirus was detected by PCR-based assays. A total of four sponge samples (*A*. *suberitoides*) and 26 replicates of water samples (1X and 10X DNA dilution) were positive for the presence of major capsid protein of T4-like myovirus. Correspondingly, the phylogenetic analyses demonstrated that the positive sponge samples (*A*. *suberitoides*) and positive water samples were similar to T4-like myovirus gene 23. This is the first detection of T4-like myovirus in *A*. *suberitoides* and seawater in Malaysia.

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## Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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