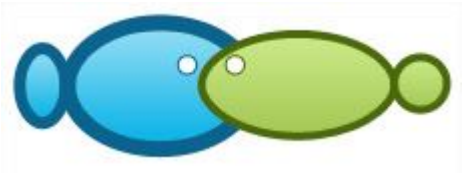

First detection of *Megalocytivirus* in oysters (*Crassostrea iredalei*) from Marudu Bay, Sabah, Malaysia

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Abstract. Oyster cultivation in Malaysia especially in Sabah and Johor has been actively conducted due to its economical values. However, in 2012, occurrences of mortalities due to pathogens in the Marudu Bay farming site had caused large impact on the oysters (*Crassostrea iredalei* Faustino, 1932) production. This study revealed the presence/absence of *Megalocytivirus* in 84 samples of oysters (*C. iredalei*) from Marudu Bay, Sabah and Masai, Johor using Polymerase Chain Reaction (PCR) with primer that selectively amplified Major Capsid Protein (MCP) gene of *Megalocytivirus*. The PCR analysis showed that the occurrence of *Megalocytivirus* in Sabah was 5% and 50% on 2 January 2014 and 8 May 2014 sampling batches, respectively. In Johor, the occurrence was 0% for both samplings on 25 November 2013 and 8 July 2014. Sequencing and phylogenetic analyses of the samples from Sabah (2SL13 and 2SL18) suggested that the strains of the virus shared 99% and 90% similarities respectively with *Megalocytivirus* Sabah and Red Tux Swordtail Iridovirus and belong to the *Megalocytivirus* genus (Genotype 1). Thus, this study demonstrated the first detection of *Megalocytivirus* in a new organism, oyster (*C. iredalei*).
Key Words: *Megalocytivirus*, *Crassostrea iredalei*, Iridoviridae, PCR analysis, Malaysia.

Introduction. Aquaculture is believed to be the fastest growing food-producing sector in the world (FAO 2016a). In 2013, the live weight of the aquaculture production reached 97.2 million tonnes with an estimated value of USD157 billion. The production of farmed food fish such as finfish, crustaceans, molluscs and other aquatic animals increased from 66.5 million tonnes in 2012 to 70.2 million tonnes in 2013 (FAO 2016b). With a growing population and an increasing demand for fish, it has been estimated that the annual demand for fish will increase to 1.93 million tonnes by 2020 (Yusoff 2015). A wide variety of species is cultured, including shellfish, freshwater species and marine finfish in Malaysia. Increasing demand in aquaculture sector makes this industry vital to Malaysian economy. Sabah and Johor represent two most important states for oyster, *Crassostrea iredalei* (Faustino, 1932) cultivation in Malaysia.

Sabah, a state located in East Malaysia, is the largest producer of bivalves such as mussels and oysters, with the culture avenue mainly centres in Marudu Bay. In 2012, the annual production of oysters in Sabah slumped from 917.50 tonnes in 2009 to 649.01 tonnes due to massive mortalities from an unknown cause (DOF 2013). A preliminary study later indicated that the mortalities of the green mussels, *Perna viridis* (Linnaeus, 1758) were caused by co-infections of *Megalocytivirus*, bacteria and parasites (Tan 2013; Bobby 2013; Hong et al 2017). *Megalocytivirus* infections have been previously reported in grouper farms located nearby the oysters and mussels farms (Razak et al 2014) and ornamental fish in Malaysia (Zainathan et al 2017). Phylogenetic analysis revealed that the pathogens (*Megalocytivirus*) were clustered with the Infectious Spleen and Kidney Necrosis Virus (ISKNV) in genus *Megalocytivirus*.

Megalocytivirus is a double-stranded DNA (dsDNA) virus with an approximate genome size of 112.636 bp. They possess icosahedral symmetry capsid and ranges between 140 and 200 nm in diameters (Subramaniam et al 2012). *Megalocytivirus* is characterized based on the presence of enlarged basophilllic cells in the infected organs and differentiated into three major groups namely the ISKNV, Red Sea Bream Iridovirus (RBIV) and Turbot Reddish Body Iridovirus (TRBIV). *Megalocytivirus* infects vital organs such as liver, spleen, muscles, gonads, heart, gills and the gastrointestinal tract (GIT) and kidney. In regards to the classification of the virus, the MCP gene is frequently used as target for phylogenetic analysis because it is highly conserved and diverse within Iridoviridae and within the *Megalocytivirus* genus. MCP has been used to study the viral evolution and phylogenetic relationships between these viruses (Subramaniam et al 2012; Kurita & Nakajima 2012).

Megalocytivirus are known to infect several fish species such as grouper, red sea bream, rock bream (Jung & Oh 2000; Shi et al 2010; Razak et al 2014). Viruses such as Hepatitis A Virus (HAV) (Sincero et al 2006), Human noroviruses (NoVs) (Lees 2000; Nappier et al 2008), Gill Necrosis Virus (GNV) (Comps et al 1976; Comps 1983), Haemocyte Infection Virus (HIV) and Ostreid Herpes virus 1 (OsHV-1) (Sauvage et al 2009) have been reported in oysters. Recently, *Megalocytivirus* were detected from shellfish in Korea by multiplex nested PCR and the phylogenetic analysis revealed that the *Megalocytivirus* found was from shellfish subtype II (Kim et al 2016).

Mass mortalities of oysters and mussels affected cage cultures in Marudu Bay, Sabah. Since the location of the cultivation area for both mussels and oysters are in the same area and the suspected virus, *Megalocytivirus* can be transmitted via water therefore, it is presumed that the problem could also affect the cultivation and yield of oysters (Tan 2013; Razak et al 2014). The mortalities caused reduction in total production of oysters and many affected farms in Marudu Bay were closed due to the mass mortalities (DOF, Sabah; personal communications). Thus, this study describes the first detection of *Megalocytivirus* in a new organism; slipper cupped oyster, *C. iredalei* by PCR and sequencing analysis.

Material and Method

Sampling. The oysters were randomly collected from oysters' breeding farm at Tanjung Batu in Marudu Bay, Sabah, East Malaysia on January and May 2014 and Johor Strait near Persiaran Senibong, Johor, Southern Malaysia on November 2013 and July 2014. A total of 84 oysters samples were taken for this study. The samples were preserved in Gibco® Roswell Park Memorial Institute (RPMI) 1640 media containing L-glutamine and non-essential amino acids supplemented with 2.5 µg mL^{-1} Amphotericin B (Gibco®), 750 IU mL^{-1} Penicillin (Gibco®) and 750 µg mL^{-1} Streptomycin (Gibco®). The samples were kept in dry ice upon collection to reduce sample damage.

Viral DNA extraction. Viral DNA extraction of gills and GIT was conducted using NucleoSpin® Tissue (Macherey-Nagel) extractions kit according to the protocols provided by the manufacturer. DNA purity was analysed using ScanDrop® (Analytic Jena) prior to PCR amplification. About 25 mg of GIT tissue and gills tissue were cut up into small pieces and placed in a 1.5 mL microcentrifuge tube for sample processing prior to viral DNA extraction. Due to the limited availability of the positive control (infected green mussels DNA from Marudu Bay, Sabah) for this study, synthetic positive control based on the sequence of *Megalocytivirus* Sabah (GenBank accession number JQ253374.1) were used in the current study.

Single step PCR assay. Eight samples of oyster gill and GIT DNA were used in this assay. The single-step PCR assay was carried out using primers that were designed by Dr. Julian Ransangan (UMS), based on the sequence alignment of the MCP gene of *Megalocytivirus* (GenBank accession number JQ253374.1) and intended to generate amplicon of 526 bp (Ransangan 2011 unpublished data; Bobby 2016). The primers were as followed: forward primer MCP Sabah (5'-GCGTTTGATGCGATGGAGACC-3') and reverse

primer MCP Sabah (5'-TGCCTACCGTGTCTCTGCCGT-3'). A total of 25 µL PCR mixture containing: 12.5 µL 2X MyTaq™ Mix (BIOLINE), 9.0 µL RNase-free water, 0.5 µL (10 µM) MCP Sabah F and 0.5 µL (10 µM) MCP Sabah R were added to 2.5 µL extracted DNA. The amplification was conducted with the following programme: 10 minutes at 95ºC, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 66ºC for 30 seconds, extension at 72ºC for 2 minutes and a final extension of 72ºC for 5 minutes. The amplified PCR products from both reactions were analysed by electrophoresis (30 minutes at 90 V) on 1.7% (w/v) agarose gel in TAE buffer and stained with SYBR® Safe-DNA Gel Stain (*Invitrogen*) (substitute for the highly toxic ethidium bromide). Both synthetic positive control and negative control were used in all assays.

Nested PCR assay. The nested PCR assay was carried out using the method of Whittington et al (2009) and based on the sequence alignment of the MCP gene of *Megalocytivirus* (GenBank accession number JQ253374.1). Forward primer C1105 (5'-GGGTTCATCGACATCTCCGCG-3') and reverse primer C1106 (5'-AGGTCGCTGCGCATGCCAATC-3') for the primary reaction followed by forward primer C1073 (5'-AATGCCGTGACCTACTTTGC-3') and reverse primer C1074 (5'-GATCTTAACACGCAGCCACA-3') in the nested PCR reaction. A total of 25 µL PCR mixture containing: 12.5 µL 2X MyTaq™ Mix (BIOLINE), 9.0 µL RNase-free water, 0.5 µL (10 µM) C1105 and 0.5 µL (10 µM) C1106 were added to 2.5 µL extracted DNA. For the primary reaction, the amplification was programmed as followed: 10 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minutes. A final extension of 72°C for 5 minutes terminated the thermal cycling reaction. The nested PCR was carried out with a total of 25 µL PCR mixture containing: 12.5 µL 2X MyTaq™ Mix (BIOLINE), 9.0 µL RNase-free water, 0.5 µL (10 µM) C1073 and 0.5 µL (10 µM) C1074 were added to 2.5 µL PCR product. The amplification was conducted with the following programme: 10 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minutes and a final extension of 72°C for 5 minutes. The amplified PCR products from both reactions were analysed by electrophoresis (46 minutes at 70 V) on 1.7% (w/v) agarose gel in TAE buffer and stained with SYBR® Safe-DNA Gel Stain (Invitrogen). Then, the expected bands were excised and purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel) based on the standard protocols. The purified PCR products were sent to 1st Base Laboratory Sdn Bhd for sequencing analysis.

Phylogenetic analysis. The DNA sequencing results were used for the phylogenetic analysis. Nucleotide sequence analyses of the MCP gene from the different *Megalocytivirus* isolates were compared with other known isolates retrieved from the GenBank databases. These isolates include *Megalocytivirus* Sabah (JQ253374), RBIV (AY532613), ISKNV (JX649071), Orange-spotted grouper iridovirus (OSGIV) (AY894243), RSIV (310918), and Red Tux Swordtail Iridovirus (RTSIV) (KF153613). The sequences were used to interrogate the NCBI BLAST database to confirm its likely identity. Then, the multiple alignments were aligned using Clustal X2.0.12 (Larkin et al 2007) with other Megalocytiviruses-related sequences. Finally, the phylogenetic tree was inferred from the MCP gene from all the known Iridoviridae using Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA 6).

Results

Gross observation of the oysters. Fouling organisms attached to the shell surface such as barnacles were observed from all of the samples from Sabah. Black spot and blackish gills were observed on the inner surface of the oysters from Sabah and Johor, respectively (Figure 1).

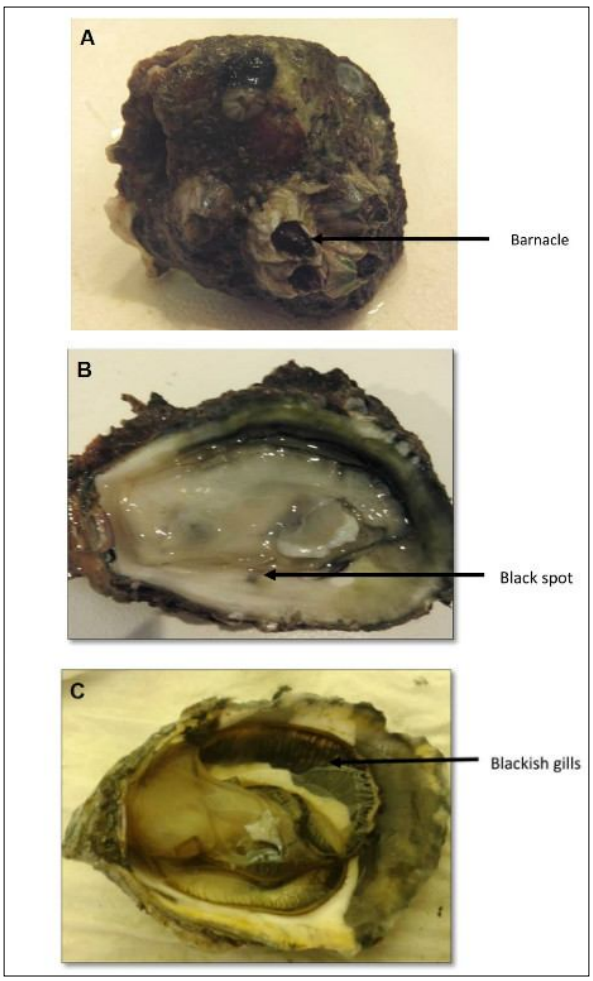

Figure 1. (A). Fouling organisms (barnacles) attached to the oysters outer surface; (B). Black spot observed on the inner surface of the oyster sample from Sabah; (C). Blackish gills observed from the oyster sample from Johor.

PCR optimization of MCP primers by single-step PCR. Upon amplification, a single band was observed at an expected size of 526 bp which was amplified at 66°C for the annealing temperature. From the result (Figure 2), the annealing temperature used in PCR thermo profile was 66°C.

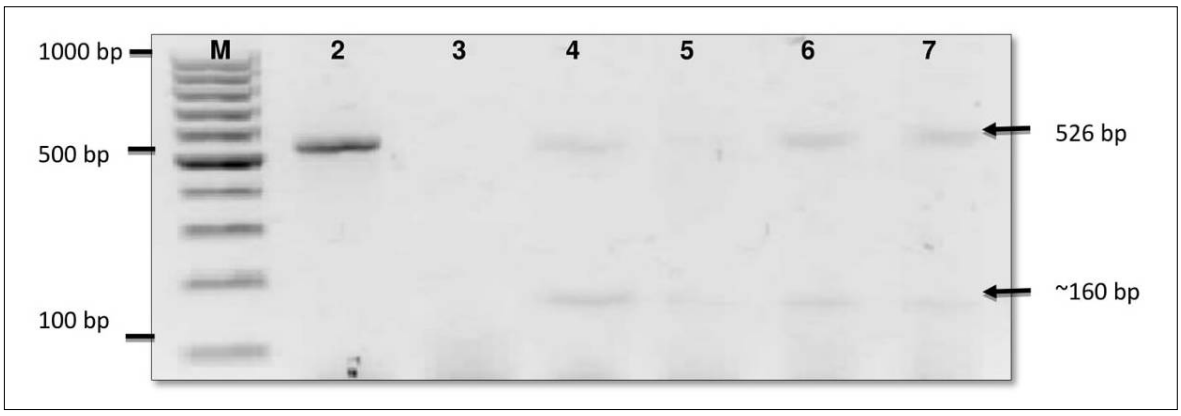

Figure 2. Single-step PCR amplification of positive control (infected green mussels from Sabah) using gradient PCR with various annealing temperature. M: 100 bp DNA ladder as a molecular weight marker, Lane 2: 70°C, Lane 3: 69.2°C, Lane 4: 67.5°C, Lane 5: 66°C and Lane 6: water as a negative control for the reaction.

PCR optimization of synthetic positive control by single-step PCR assay. The synthetic positive control was synthesized by First Base laboratories based on the *Megalocytivirus* Sabah sequence. Optimization of the synthetic control was conducted based on the optimization of MCP Sabah primers with some modifications in the PCR thermo profile. The annealing temperature used was 66ºC. Four dilutions of synthetic control were analysed in duplicates (indicated in Figure 3).

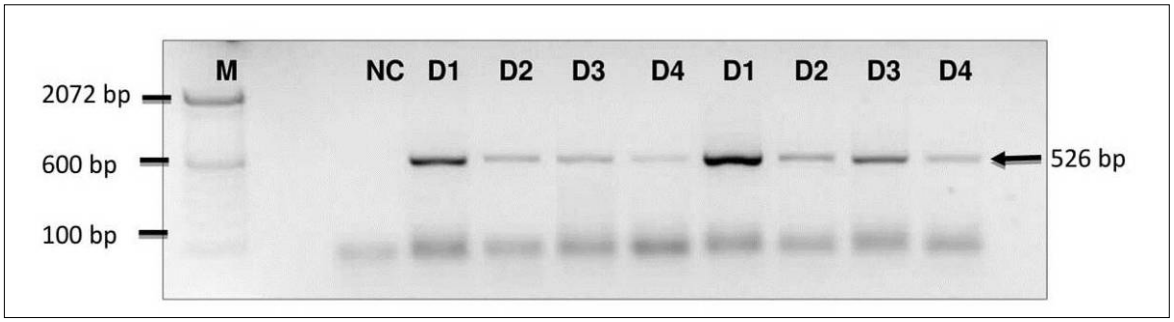

Figure 3. Optimization of synthetic positive control of MCP gene for *Megalocytivirus*. M: 100 bp DNA ladder as a molecular weight marker, NC: Negative control, D1: Dilution 1, D2: Dilution 2, D3: Dilution 3 and D4: Dilution 4. All samples were analyzed in duplicates.

Single-step PCR assay. Five samples out of eight showed positive bands at expected band of 526 bp for the presence of *Megalocytivirus* (Figure 4).

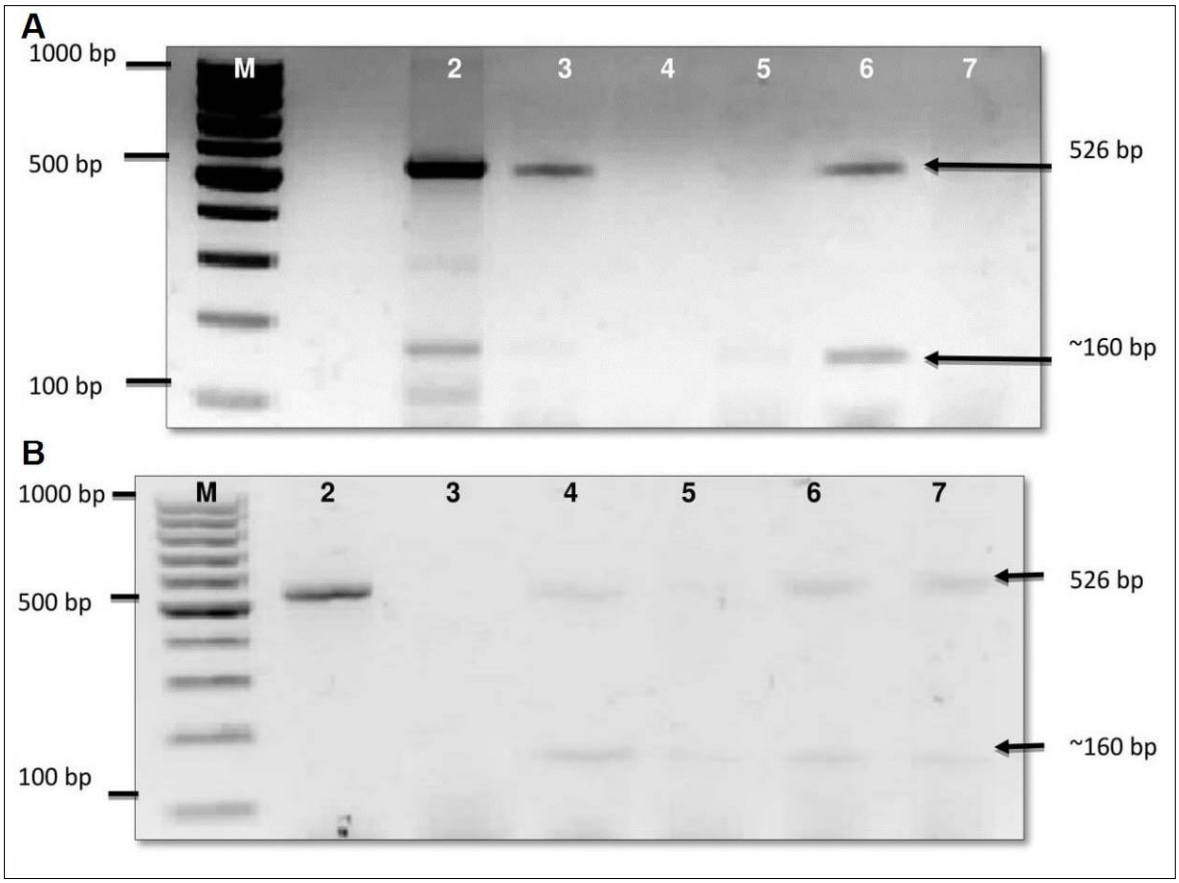

Figure 4(A). Single-step PCR amplification of oysters GIT DNA. M: 100 bp DNA ladder as a molecular weight marker, Lane 2: Positive control, Lane 3: Sample 1, Lane 4: Sample 2, Lane 5: Sample 3, Lane 6: Sample 4 and Lane 7: Water as a negative control for the reaction. (B), Single-step PCR amplification of Oysters Gill DNA. M: 100 bp DNA ladder as a molecular weight marker, Lane 2: Positive control, Lane 3: Water as a negative control for the reaction, Lane 4: Sample 1, Lane 5: Sample 2, Lane 3: Sample 3 and Lane 7: Sample 4.

Nested PCR amplification of DNA from Sabah and Johor. The results showed that the prevalence in Sabah was 5% and 50% on 2 January 2014 and 8 May 2014 sampling batches, respectively. In Johor, the prevalence was 0% for both samplings on 25 November 2013 and 8 July 2014 (not shown). Multiple bands were visualised for all of the samples from Sabah but at different sizes (approximately at 350 bp and 900 bp) in the primary reactions. The nested PCR analysis produced consistent amplicons of the expected size in the secondary reactions (approximately at 144 bp) (Figure 5).

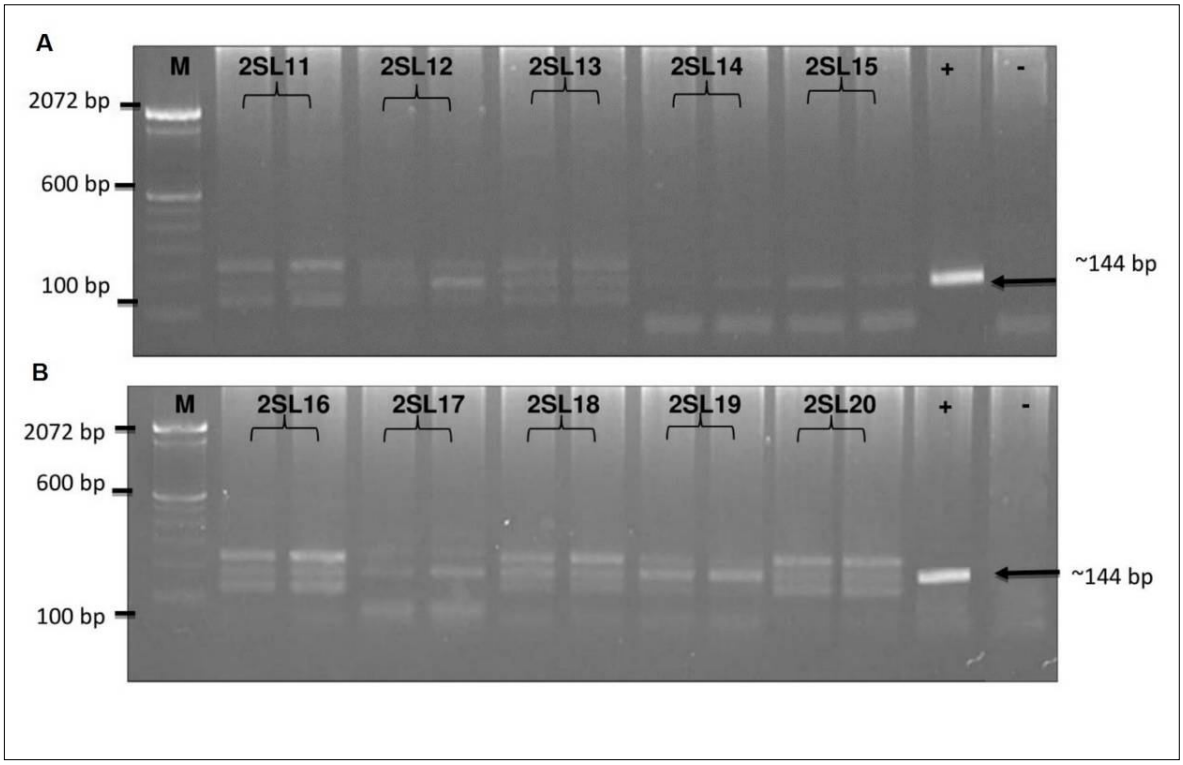

Figure 5(A). Nested PCR results: amplification of gills samples from Sabah according to Rimmer et al (2012) primers. M: 100 bp ladder, Lane 2-11: sample 11 to 15 and Lane 12: positive control. Amplification of the *Megalocytivirus* gene at different band size, ~100 bp to 250 bp. (B), Nested PCR results: amplification of gills oyster samples from Sabah according to Rimmer et al (2012) primers. M: 100 bp ladder, Lane 2-11: sample 16 to 20 and Lane 12: negative control. Amplification of the *Megalocytivirus* gene at different band size, ~100 bp to 250 bp.

Sequence and phylogenetic analysis. Sequences of sample representatives covering 139 bp and 144 bp region of *Megalocytivirus* Sabah, Sabah/RAA1/2012 strain BMGIV48 (GenBank accession no. JQ253374.1) were determined for samples 2SL13 and 2SL18, respectively. The amplified products were sequenced and compared with other known Megalocytiviruses such as *Megalocytivirus* Sabah (JQ253374), ISKNV (JX649071), RTSIV (KF153613), RSIV (AY310918), OSGIV (AY894343) and RBIV (AY532613) to evaluate the genetic variation of the viruses. Multiple alignment of nucleotide sequences of the amplified PCR products confirmed that sample 2SL13 and 2SL18 were from the same lineage with *Megalocytivirus* Sabah with minor variations and belonging to Genotype 1. The alignment showed that 2SL13 and 2SL18 were different for 26 base pairs and 30 base pairs, respectively. Their phylogenetic relationship with other *Megalocytivirus* genotypes isolates were also investigated (Figure 6). Phylogenetic trees of the MCP genes also showed that the virus was clustered within *Megalocytivirus* genus. Based on the species demarcation of *Megalocytivirus*, the strains were considered as new strains of the same virus as *Megalocytivirus* Sabah. The BLAST analysis of the 2SL13 (139 bp) and 2SL13 (144 bp) sequence obtained revealed that it shared 99% and 90% sequence identity with a region of the *Megalocytivirus* Sabah/RAA1/2012 strain BMGIV48 MCP gene (GenBank accession number JQ253374.1) and Red Tux Swordtail Iridovirus (RTSIV) (GenBank accession number KF153613), respectively.

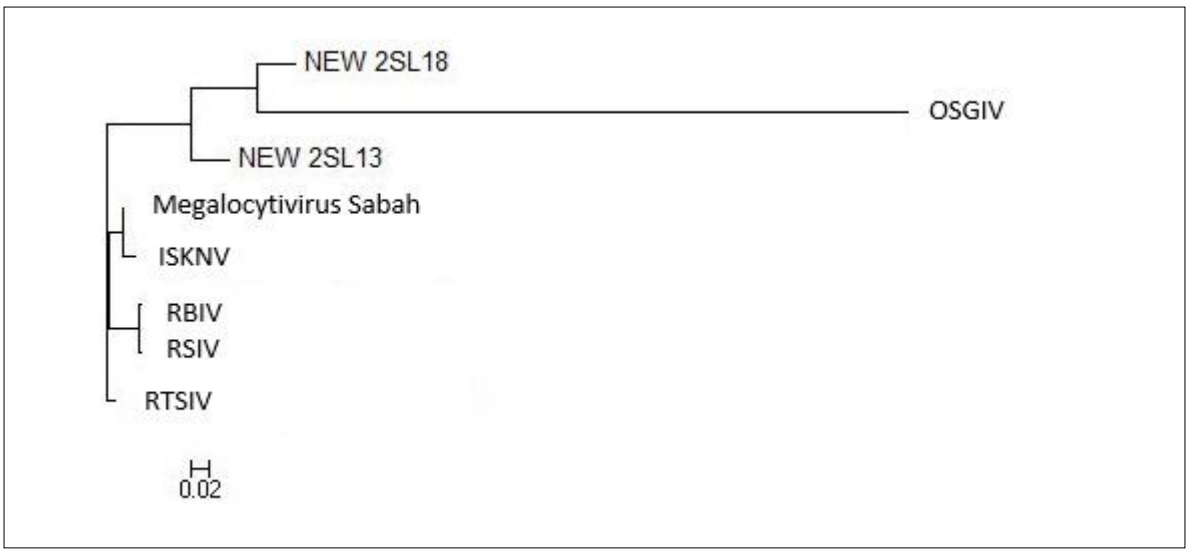

Figure 6. Phylogenetic neighbour-joining tree deduced from analysis nucleotide sequences of MCP gene of genus *Megalocytivirus*. The scale bar represents distance values. Note: These isolates included *Megalocytivirus* Sabah (JQ253374), RBIV (AY532613), ISKNV (JX649071), OSGIV (AY894343), RSIV (AY310918), and RTSIV (KF153613).

Discussion. From the gross external observation of the oysters, fouling organism such as barnacles (unidentified species) were found attached to the shell surface. Barnacles are well known as the common colonists that normally present on the shell surfaces and not a threat to the oysters health (FAO 2016c). However, the presence of the marine bio-fouling organisms especially barnacles will lead to significant operating costs and reduce the aesthetic quality of the oysters (Arakawa 1980). The presence of unwanted organisms on cultured oysters can reduce the oyster growth rate and quality (infected by pathogens). The internal surface observation revealed the presence of the black spot and blackish gills. Black spot and blackish gills indicate that the oysters were unhealthy due to certain factors (FAO 2016c). Normally, sick oysters display wide open shells due to the weaken adductor muscle. In certain cases, appearance of one or more yellow spots on gills and labial palps can be observed from infected oysters by Irido-like virus infections such as GNV. Yellow or green pustules have been observed on the adductor muscle and mantle of diseased oysters (Renault & Novoa 2004). The malfunction of mantle and gills can cause the oysters to be susceptible to opportunists and parasitic infections (FAO 2016c).

In the preliminary results, DNA of GIT and gills were screened with MCP Sabah primers. Mass mortalities of bivalves during sample collection period restricted adequate sample size. Collectively, 70% of the oyster's samples were positive for *Megalocytivirus*. The *Megalocytivirus* DNA detected from GIT of the infected oysters because the bioaccumulation occurred specifically in the digestive tissues (Maalouf et al 2011). A study by Sincero et al (2006) also reported that Hepatitis A virus (HAV) was detected in the oyster's GIT tissue. Viral DNA can be detected from the oyster's gills due to their feeding activities. As filter feeders, oysters pump water over their gills (Thiagarajan et al 2006). Therefore, any small particles including bacteria, virus and parasite will pass through the gills first before it is passed to the alimentary tract. *Megalocytivirus* was detected in oysters GIT and gills because it is commonly found in multiple organs including spleen, kidney, eye, pancreas, liver, heart, brain, intestine and gills (Gibson-Kueh et al 2003). Thus, the nature of oyster as a filter feeder increases virus accumulation in the tissues and this may act as the viral transmitter (Jin et al 2014).

PCR-based detection with MCP Sabah primer by Rimmer et al (2012) in the current study revealed that the occurrence of *Megalocytivirus* in Sabah (50% occurrence) is higher compared to Johor (0% occurrence). In Sabah, the cultivation area of the diseased mussels and fish was located close to the oyster's farm. Since *Megalocytivirus* can be transmitted via water and the shellfish has the ability to co-accumulate several pathogens over long period, the infection can be transmitted to other cultures nearby

including the oyster farms (Go et al 2006; Kim et al 2016). In this context, it was demonstrated that bivalve molluscs inhabiting areas nearby fish farms are the potential sources of viral transmission (Jin et al 2014; Kim et al 2016). The ability of *Megalocytivirus* to survive in favourable seawater conditions increased the chances of the viral transmission (Albert & Ransangan 2013; Jin et al 2014).

However, the presence of *Megalocytivirus* was not detected in the current study by single-step PCR using MCP Sabah primer. Therefore, nested PCR method of Whittington et al (2009) was conducted using primers designed by Rimmer et al (2012). These primers are general primers and capable of detecting all Megalocytiviruses (Whittington et al 2009; Nolan et al 2015). The presence of *Megalocytivirus* in this study was detected in the secondary reaction of the nested PCR analysis due to the low concentration of the virus present or latent infection of *Megalocytivirus* (Razak et al 2014; Kim et al 2016). Nested PCR is highly sensitive and has the ability to detect small amount of virus presence in tissue samples (Chao et al 2002; Wang et al 2007; Razak et al 2014; Kim et al 2016). Previously, Iridovirus infection in hybrid grouper, giant seaperch and largemouth bass were confirmed using nested PCR analyses (Chao et al 2002).

In this study, sequencing and phylogenetic analysis of the representative samples showed that the sequences shared 99% and 90% respectively sequence identity with a region of the Megalocytivirus Sabah/RAA1/2012 strain BMGIV48 major capsid protein gene (GenBank accession number JQ253374.1). Similarly, sequence analysis of representative PCR products of fish samples in Australia showed 100% identity to a previously characterized *Megalocytivirus* (Sabah/RAA1/2012 strain BMGIV48) (Nolan et al 2015). Based on the species demarcation of genus *Megalocytivirus*, the Megalocytiviruses show > 94% of sequence identity based on their ATPase and MCP gene. In addition, sequence analysis and serological studies of all *Megalocytivirus* isolated to date were from the strains of the same viral species (Chinchar et al 2005). Therefore, sequencing and phylogenetic analysis suggested that 2SL13 and 2SL18 were strains of the same viral species belonging to the *Megalocytivirus* genus in the Iridoviridae family.

This is the first detection of *Megalocytivirus* in oysters (*C. iredalei*). Interestingly, oysters are not known to be infected or act as a host for *Megalocytivirus*. However, it could act as the transient reservoir for *Megalocytivirus* or other viruses (Lees 2000; Potasman et al 2002; Nishida et al 2003; Renault & Novoa 2004; Kim et al 2016). As a host, a shellfish has the ability to accumulate viruses over long period and the viruses are able to multiply within the cells of the organisms and cause disease. Oysters infected by viruses usually manifest certain characteristics such as lesions, and mantle perforation. Histological studies of few oysters sample from Sabah were grouped into lesion as necrosis, hyperplasia and tearing/thinning of structure of the gill intestinal epithelium and digestive diverticula were observed respectively (Hong et al 2017). This evidence suggests that the oysters might be infected by the *Megalocytivirus*. However, further confirmation by the isolation of the virus from diseased host and infecting it to a susceptible host via Koch Postulates must be conducted in order to determine whether oyster is the host for *Megalocytivirus*.

Conclusions. In conclusion, sequencing and phylogenetic analysis suggested that 2SL13 and 2SL18 were strains of the same viral species belonging to the *Megalocytivirus* genus (Genotype 1) in the Iridoviridae family. Therefore, further study is required to confirm oysters as the host for the *Megalocytivirus* that caused the mortalities and morbidity in oyster's farm in Marudu Bay, Sabah.

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