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Original Research Article

First report of Megalocytivirus (*Iridoviridae*) in grouper culture in Sabah, Malaysia

Asrazitah Abd Razak[1], Julian Ransangan[1]* and Ahemad Sade[2]

[1]Microbiology and Fish Disease Laboratory, Borneo Marine Research Institute, Universiti Malaysia Sabah, Jalan UMS, 88400, Kota Kinabalu, Sabah, Malaysia
[2]Fisheries Department Sabah, Wisma Pertanian, Jalan Tasek, 88628 Kota Kinabalu, Sabah, Malaysia
**Corresponding author*

A B S T R A C T

Groupers are popular aquaculture species in Sabah, Malaysia. However, its aquaculture production is often limited by disease outbreaks. Although many diseases are known to affect groupers, iridovirus infection is a major concern because it causes high mortality within a short period of time. Recently, a disease resembled to iridovirus occurred and caused heavy losses to grouper aquaculture in Sabah. This has prompted us to conduct a study with the aim to determine if iridovirus present in the culture groupers. In this study, we examined 212 fish specimens, which represented all the major culture grouper species in Malaysia. The examination was carried out using single- and nested-PCR methods and followed by DNA sequencing. Two genes (major capsid protein and ATPase) were targeted for the PCR amplification and DNA sequencing. The finding showed 15.6% (33/212) of the grouper specimens were severely infected by iridovirus. Meanwhile, 17.4% of the specimens exhibited latent infection or asymptomatic carriers. Phylogenetic analysis revealed that the iridovirus in this study was clustered together with the infectious spleen and kidney necrosis virus (ISKNV) under the genus Megalocytivirus (*Iridoviridae*). Generally, poor biosecurity measures in the aquaculture farms seemed to be the major factor responsible for the viral occurrence. Hence, adherence to good aquaculture practices plus strict enforcement of aquaculture biosecurity policy may help control the spread of the virus in the farms.

Keywords

Grouper; Megalo-cytivirus; ISKNV; nested-PCR; Sabah; Malaysia

Introduction

Groupers are popular aquaculture fish species in Sabah, Malaysia. They attain high market demand both in local and international markets and constitute the top menus for restaurants, hotels and resorts, especially during festive seasons.

However, the supply of groupers from aquaculture is often limited due to diseases, which occur throughout the production cycle (Muroga, 2001; Hyatt and Whittington, 2005; Bondad-Reantaso et al., 2005; Harikishnan et al., 2010).

Recently, a disease outbreak occurred in grouper aquaculture in Sabah that has resulted in heavy mortalities over a short period of time. The fish exhibited dark skin coloration and abnormal swimming behavior, suffered from skin lesion, hemorrhage and fin erosion. Assuming based on the clinical signs of affected fish; the outbreak might be due to *Iridoviridae*. *Iridoviridae*, particularly the Infectious Spleen and Kidney Necrosis Virus (ISKNV) under the genus Megalocytivirus, has been widely reported to cause high mortality amongst groupers (Chia et al., 2004; Eaton et al., 2007; Chinchar et al., 2008), and variety of other freshwater and marine fish species (Fauquet et al., 2005; Eaton et al., 2008; Murwantoko et al., 2009). However, presence of *Iridoviridae* in fish is difficult to determine because it can persist for very long time in host cells without manifesting any detectable effects. Under this situation, the host can become the asymptomatic carriers of the virus (Jeong et al., 2006). Hence, the ability to detect the carrier fish could help prevent future disease outbreak from occurring in the aquaculture. However, many of the existing diagnostic methods may not be able to detect when the virus exists in a minute amount. To date, the only method that has the ability to detect small amount of virus presence in tissue sample is the nested-PCR (Chao and Yang, 2002; Wang et al., 2007).

Although it has issues related to carry-over contamination, it can be avoided by strict adherence to good laboratory practices. Motivated by the occurrence of high grouper mortality with clinical signs resembled to iridovirus, this study was conducted with the aim to determine the presence of the virus in culture grouper. To the best of our knowledge, this is the first report related to iridovirus in grouper culture in Sabah, Malaysia.

Materials and Methods

Fish specimens

In this study, 212 specimens of groupers collected from net-cages throughout Sabah (Fig. 1) were analyzed. The specimens comprised of brown-marbled grouper (*Epinephelus fuscoguttatus*), humpback grouper (*Cromileptes altivelis*), giant grouper (*E. lanceolatus*), orange-spotted grouper (*E. coioides*) and hybrid grouper (*E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)). Although majority of the fish specimens suffered from skin and gill lesions, few fish were observed to have enlarged kidney, liver and spleen.

DNA Extraction

Total genomic DNA was extracted from fish tissues (kidney, liver, spleen) using the DTAB-CTAB method described by Philips and Simons (1995). Briefly, about 30 mg of the pooled tissues were homogenized in 600µl DTAB solution followed by incubation at 75ºC for 5 minutes. Then, 700µl of chloroform solution was added into tissue homogenate before centrifuging at 12,000 rpm for 5 minutes. Subsequently, 400µl of the aqueous solution was added with 100µl CTAB and 900µl of autoclaved distilled water. The mixtures were briefly vortex and incubated at 75ºC for 5 minutes. After the incubation, the mixture was centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded while the pellet was re-suspended in 200µl of 1.2M NaCl solution, incubated at 75ºC for 5 minutes and centrifuged at 12,000 rpm for 5 minutes. Next, the clear suspension was transferred to a new tube containing 400µl of 95% ethanol for DNA precipitation by centrifugation at 12,000 rpm for 5 minutes. Then, the DNA pellet was briefly air dried before dissolving in 30µl 1X TE buffer. Finally, the DNA solution was stored in -20ºC until use.

PCR amplification

Specific fragments of the viral major capsid protein (MCP) and ATPase genes were amplified either by single- or nested-PCR. PCR primers used in the amplification are shown in Table 1. The single-PCR amplification was carried out in 50µl reaction (5X GoTaq® Flexi PCR Buffer (Promega), 0.2mM of dNTPs (Promega), 1.7mM of $MgCl_2$ (Promega), 10µM of each primer, 0.3 units of GoTaq® DNA *Polymerase* (Promega) and ~100ng of DNA) at the following conditions: initial denaturation at 95ºC for 3 min followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min and an extra extension at 72ºC was allowed for 5 min. The nested-PCR amplification was carried according to the conditions described for single-PCR except that 2µl of the PCR product generated during the single-PCR amplification was used as the DNA template. Subsequently, 5µl of the PCR products from single- and nested-PCR amplification was then separated on 1.5% of agarose gel, stained with ethidium bromide (5µg/µl) and visualized under UV light (Alpha Innotech Chemi Imager System). Based on the results of PCR amplification, the iridovirus infection was classified into two categories (severe and latent) according to the classification proposed by Jeong et al. (2006).

DNA cloning and sequencing

PCR fragments of major capsid protein (MCP) and ATPase genes were first purified using MEGAquick-spin™ PCR and Agarose Gel DNA Extraction System (iNtRON Biotechbology, Inc.) according to the manufacture's instruction before ligation into pGEM-T-Easy Vector (Promega).

Recombinant plasmids containing the gene fragments were transformed into competent *E. coli* strain JM109 (Promega) using the heat-shock method described by Sambrook and Russell (2001). Then, the competent cells were aseptically spread onto Luria-Bertani (LB)/ampicillin /IPTG/X-gal agar plates and incubated at 37ºC overnight. After incubation, white colonies of *E.coli* JM109 were picked and aseptically inoculated into LB tubes containing 100µg/ml ampicillin. Then, the tubes were incubated at 37ºC with shaking at 150 rpm overnight. Subsequently, bacterial cells were harvested by centrifugation at 12,000 rpm for 5 minutes. Plasmids were extracted using alkaline lysis method (Sambrook and Russell, 2001) and purified using DNAspin™ Plasmid DNA Purification Kit (iNtRON Biotechnology, Inc) according to the manufacturer's procedure. DNA insert was verified by *EcoR1* restriction analysis following manufacturer's instruction (New England Biolabs). Then, all plasmids containing correct DNA insert were sequenced at AIT,Singapore Pt Ltd.

Sequence alignment

Multiple alignment of DNA sequences was done against selected sequences of the genes (major capsid protein and ATPase) downloaded from the National Center for Biotechnology Information (NCBI) using the Clustal W method (Thompson, 1994). The list of sequences analyzed in the study is given in Table 2 and Table 3, respectively.

Construction of phylogenetic tree

Phylogenetic trees were constructed by the MegAlign program of the DNASTAR software package that employs the neighbor-joining algorithm of Saitou and Nei (1987). The accuracy of the phylogenetic trees was evaluated using bootsrapping analysis.

Results and Discussion

Virus detection

It was recorded that 33.02% (70/212) of the fish specimens were found positive for iridovirus. From the 70 infected fish specimens, 47.14% (33/70) were amplified for both genes during the single-PCR amplification and classified under severe infection. The rest of infected fish specimens (52.86%) were classified under latent infection since they were only amplified during the nested-PCR amplification (Table 4; Fig. 2). The percentage of fish specimens, according to species, which are severely and latently infected with iridovirus is given in Fig. 3.

Clinical signs of infected fish

Majority of the severely infected grouper specimens exhibited gross external clinical signs of diseases such as fin erosion, dark skin coloration, red spot in gills and skin lesion. The observation recorded were similar to the findings of Chincar et al. (2008); Murwantoko et al. (2009); Yanong and Waltzek (2010). In contrast, the specimens in the latent infection category did not exhibit apparent clinical signs of disease.

DNA Sequence analysis

DNA sequences of major capsid protein and ATPase genes examined in this study have been deposited into GenBank (http://www.ncbi.nih.gov) with the following accession numbers: MCP-JQ253365, JQ253366, JQ253367, JQ253368, JQ253369, JQ253370, JQ253371, JQ253372, JQ253373 and JQ253374; ATPase-KF669901, KF669902, KF669903, KF669904, KF669905, KF669906, KF669907, KF669908, KF669909, KF669910, KF669911, KF669911, KF669912, KF669913 and KF669914. Analysis of major capsid protein gene sequences against other genera within the family of *Iridoviridae* showed that the virus in this study had 96.04% nucleotide similarity to genus Megalocytivirus, 54.25% to Ranavirus, 51.21% to Chloriridovirus, 50.37% to Lymphocytivirus, 48.86% to Iridovirus and 34.4% to out-group (African Swine Fever Virus). Similar finding was also observed using ATPase gene where the DNA sequences had higher nucleotide similarity (97.73%) to Megalocytivirus compared to Ranavirus (63.41%), Chloriridovirus (52.76%), Lymphocytivirus (39.8%), Iridovirus (37.1%) and out-group (37.6%). Phylogenetic trees of the major capsid protein and ATPase genes also showed that the virus was clustered within the clade of Megalocytivirus (Fig. 4 and Fig. 5). Further phylogenetic analysis with the aim to elucidate the viral strain also revealed that the virus was clustered within the ISKNV strain (Fig. 6 and Fig. 7).

It is evident in this study that *Iridoviridae*, particularly the ISKNV within the genus Megalocytivirus could have responsible for the high mortality in grouper culture in Sabah. Megalocytivirus belongs to the fifth genera of *Iridoviridae* (Fauquet et al., 2005; Wang et al., 2007) and members of this genus are known to infect different species of freshwater and marine fishes

(Wang et al., 2007; Kurita and Nakajima, 2012). The results of this study have shown that about 33% of the grouper specimens suffered from Megalocytivirus infection. Out of these positive specimens, significant percentage (17.5%) of the fish specimens was found to be asymptomatic carriers of the virus. Furthermore, all the grouper species examined in this study have shown some degrees of susceptibility to Megalocytivirus including hybrid grouper. This is a worrying situation since these fish may potentially transmit the virus to other culture fish species or even to their counterparts in the wild.

Groupers with severe viral infection were seen lethargic, exhibited skin darkening, showed abnormal swimming behavior, increased respiration, suffered from skin hemorrhage, fin erosion, and red spots in the gills. Infected grouper also exhibited enlargement of kidney, liver and spleen. These clinical signs were similar to the one reported by Chinchar et al. (2008), Murwantoko et al. (2009) and Yanong and Waltzek (2010). Interestingly, some of the externally healthy-looking groupers were also found positive. This condition shows, under certain circumstances, groupers can become asymptomatic carriers of the virus (Choi et al., 2006; Jeong et al., 2006; Wang et al., 2007).

According to Wen et al. (2008) and Yanong and Waltzek (2010), Megalocytivirus requires high water temperature to multiply. The relatively high annual water temperature (28-32^{0}C) of the coastal waters in Sabah (Jiran and Ransangan, 2013) may provide an optimal condition for the virus to remain active all year round. Unlike temperate countries, the virus is only reported to outbreak during summer months (Joon et al., 2003). Majority of groupers are cultured on small scale basis and are generally poorly operated. The farms generally consist of 10-40 cages per farm. Despite small in size (3m x 3m x 3m), cages are heavily stocked. From our observation, the average stocking density of grouper in Sabah was at 450 individuals (25-30cm TL) per cage. Limited space coupled with high stocking density can be stressful to groupers. Such situation can cause fish to become immunologically incompetent and eventually make them susceptible to viral infection. Poorly maintained farms also provide excellent habitats for bio-fouling organisms such as oysters and mussels. When these organisms are heavily growing on the net cage, they can easily cause skin injury to fish especially during net lifting, sorting or harvesting. The injured skin may then develop into wounds, which then provide entry points to fish pathogens, including viruses.

Viruses can also enter fish orally via the consumption of contaminated feeds such as trash fish. Trash fish have been known to carry iridovirus (Mao et al., 1999; Wang et al., 2007). Unfortunately, majority of the aquaculture farms in Sabah depends on trash fish for feeding. It was also evident to our observation that many of the aquaculture farms do not have proper storage facility for trash fish. Rotten trash fish may increase the chances of pathogens contamination. Although pellet feeds are beneficial over trash fish, availability and relatively high cost have often been the issues of their use in aquaculture.

Viruses are also reported to spread through transboundary movement of live fish (Subasinge and Bondad-Reantaso, 2008; Walker and Winton, 2010). However, this

Table.1 PCR primers used for the amplification of MCP gene and ATPase genes

Primer	Oligonucleotide sequences (5' – 3')	Position (nt)	GC content (%)	Sequence references
IRVF-1	TYAATGTCTGMRATCTYAGGT	1 – 25	28.57%	KC244182
IRVR-1	GGCTTCCCTATCCTGTRARYW	1393 – 1375	42.86%	HQ105005
IRVF-2	GCTGCGTGTTAAGATCCC	244 – 262	55.56%	AB669096
IRVR-2	CATGACAGGGTGACGTTGG	1189 – 1208	57.89%	HQ317462
MATPaseF1	ATGGAAATCMAAGAGTTGTC	1 – 24	37.50%	AF371960
*	CYTG	699 – 720	52.38%	AF371960
MATPaseR1	TTACRCCACGCCAGCCTTGTA	513 – 532	47.37%	AF371960
*	GGRATGAAGCTCATTGTGC	1136 – 1159	47.83%	AF371960
MATPaseF2	GCTTTAAGGATTACAAGGCTG			
MATPaseR2	GC			

Degenerate bases :**M** (A/C); **R** (A/G); **Y** (C/T); **W** (A/T)
*Adopted from Huang et al. (2011)

Table.2 List of gene sequences used in the multiple alignment analysis for the viral classification

Accession Numbers	Gene	Source of isolate (Common name / Scientific name)	Genus	Year	Country
AB109368	MCP	African lampeye,*Aplocheilichthys normani*	Megalocytivirus	1998	Indonesia
AB109370	MCP	Malabar grouper, *Epinephelus malabricus*	Megalocytivirus	1993	Thailand
AB666320	MCP	Tiger grouper, *E. fuscoguttatus*	Megalocytivirus	2002	Singapore
AY285744	MCP	Dwarf gourami, *Colisa lalia*	Megalocytivirus	2002	Thailand
AY310917	MCP	Sea bass, *Lates calcarifer*	Megalocytivirus	1993	China
AY590687	MCP	Turbot, *Scophthalmus maximus*	Megalocytivirus	2002	China
AY894343	MCP	Orange spotted grouper, *E. coioides*	Megalocytivirus	2004	China
JQ253365	MCP	Giant grouper, *E. lanceolatus*	Megalocytivirus	2011	Malaysia
JQ253366	MCP	Giant grouper, *E. lanceolatus*	Megalocytivirus	2011	Malaysia
JQ253367	MCP	Humpback grouper, *Cromileptes altivelis*	Megalocytivirus	2006	Malaysia
JQ253368	MCP	Humpback grouper, *Cromileptes altivelis*	Megalocytivirus	2006	Malaysia
JQ253369	MCP	Humpback grouper, *Cromileptes altivelis*	Megalocytivirus	2006	Malaysia
JQ253370	MCP	Humpback grouper, *Cromileptes altivelis*	Megalocytivirus	2006	Malaysia
JQ253371	MCP	Humpback grouper, *Cromileptes altivelis*	Megalocytivirus	2006	Malaysia
JQ253372	MCP	Orange spotted grouper, *E. coioides*	Megalocytivirus	2004	Malaysia
JQ253373	MCP	Brown-marbled grouper, *E. fuscoguttatus*	Megalocytivirus	2007	Malaysia
JQ253374	MCP	Humpback grouper, *Cromileptes altivelis*	Megalocytivirus	2007	Malaysia
AAS18087	MCP	Giant grouper, *E. tauvina*	Ranavirus	2004	Singapore
AB474588	MCP	American bullfrog, *Rana catesbeiana*	Ranavirus	2008	Japan
DQ335253	MCP	Chinese soft turtle,*Pelodiscus sinensis*	Ranavirus	2005	China
FR682503	MCP	Large mouthbass,*Micropterus salmoides*	Ranavirus	2010	Germany
JF264361	MCP	Orange spotted grouper, *E. coioides*	Ranavirus	2004	Taiwan
JF264365	MCP	Orange spotted grouper, *E. coioides*	Ranavirus	2004	Taiwan
AY297741	MCP	Olive flounder, *Paralichthys olivaceus*	Lymphocytivirus	2003	Korea
AY303804	MCP	Olive flounder, *Paralichthys olivaceus*	Lymphocytivirus	2003	Korea
AY823414	MCP	Korean rock fish, *Sebastes schlegelii*	Lymphocytivirus	2004	Korea
AY849391	MCP	Olive flounder, *Paralichthys olivaceus*	Lymphocytivirus	2004	Korea
AY849392	MCP	Korean rock fish, *Sebastes schlegelii*	Lymphocytivirus	2004	Korea
EF103188	MCP	Cobia, *Rachycentron canadum*	Lymphocytivirus	2006	China
DQ643392	MCP	Aedes, *Ochlerotatus taeniorhynchus*	Chloriridovirus	2006	USA
NC008187	MCP	Aedes, *Ochlerotatus taeniorhynchus*	Chloriridovirus	2006	USA

AF025774	MCP	Moths, *Wiseana fuliginea*	Iridovirus	1999	New Zealand
AF025775	MCP	Brown beetle, *Costelytra zealandica*	Iridovirus	1999	New Zealand
M32799	MCP	Blackflies, *Simulium* spp	Iridovirus	1990	UK
M33542	MCP	Crane fly, *Tipula poludosa*	Iridovirus	1990	UK
AY578706	MCP	Warthog, *Phacochoerus africanus*	Outgroup*(Asfaviridae)*	2004	Namibia,USA
AB666369	ATPase	Reddrum, *Sciaenops ocellatus*	Megalocytivirus	2000	Malaysia
AB666370	ATPase	Seabass, *Lates calcarifer*	Megalocytivirus	2000	Malaysia
AB666371	ATPase	Orange spotted grouper, *E. coioides*	Megalocytivirus	2000	Philippines
AB666372	ATPase	Orange spotted grouper, *E. coioides*	Megalocytivirus	2004	Hong Kong
AB666373	ATPase	Orange spotted grouper, *E. coioides*	Megalocytivirus	2004	Hong Kong
AY608684	ATPase	Turbot, *Scophthalmus maximus*	Megalocytivirus	2000	China
AB043979	ATPase	African lampeye, *Aplocheilichthys normani*	Megalocytivirus	2000	Indonesia
AB666356	ATPase	Orange spotted grouper, *E. coioides*	Megalocytivirus	2004	Hong Kong
AB666359	ATPase	Yellow fin seabream, *Acanthopagrus latus*	Megalocytivirus	2004	Hong Kong
AB666368	ATPase	Greater amberjack, *Seriola dumerili*	Megalocytivirus	1998	Japan
KF669901	ATPase	Giant grouper, *E. lanceolatus*	Megalocytivirus	2011	Malaysia
KF669902	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Megalocytivirus	2012	Malaysia
KF669903	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Megalocytivirus	2012	Malaysia
KF669904	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Megalocytivirus	2012	Malaysia
KF669905	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Megalocytivirus	2012	Malaysia
KF669906	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Megalocytivirus	2012	Malaysia
KF669907	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Megalocytivirus	2012	Malaysia
KF669908	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Megalocytivirus	2012	Malaysia
KF669909	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Megalocytivirus	2012	Malaysia
KF669910	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Megalocytivirus	2012	Malaysia
KF669911	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Megalocytivirus	2012	Malaysia
KF669912	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Megalocytivirus	2012	Malaysia
KF669913	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Megalocytivirus	2012	Malaysia
KF669914	ATPase	Giant grouper, *E. lanceolatus*	Megalocytivirus	2011	Malaysia
JF264219	ATPase	Giant grouper, *E. lanceolatus*	Ranavirus	2001	Taiwan
JF264220	ATPase	Giant grouper, *E. Lanceolatus*	Ranavirus	2004	Taiwan
JF264226	ATPase	Giant grouper, *E. Lanceolatus*	Ranavirus	2004	Taiwan
AF511654	ATPase	Tiger frog, *Ranatigrina cantor*	Ranavirus	2002	China
M80551	ATPase	Frog virus, *Ranatigrina cantor*	Ranavirus	1993	China
NC005902	ATPase	Olive flounder, *Paralichthys olivaceus*	Lymphocystivirus	2003	China
AY947406	ATPase	Korean rock fish, *Sebaster schlegeli*	Lymphocystivirus	2005	Korea
AY380826	ATPase	Olive flounder, *Paralichthys olivaceus*	Lymphocystivirus	2003	China
AF303741	ATPase	Rice stem borer, *Chilo suppressalis*	Iridovirus	2001	USA
NC008187	ATPase	Aedes, *Ochlerotatus taeniorhynchus*	Chloriridovirus	2006	USA
M88275	ATPase	Warthog, *Phacochoerus africanus*	Outgroup	1993	USA

remains a challenge in Sabah because local hatcheries are unable to meet the increasing demand for grouper seeds (Othman, 2008). Moreover, the price of imported fish seed is more attractive than the locally produced seeds. Unfortunately, most of these imported seeds may have not necessarily passed through health screening. This increases the possibility of viral transmission and thus results in disease outbreak. Megalocytivirus can be the greatest threat to the sustainability of grouper aquaculture. Adherence to good aquaculture practices plus strict enforcement of aquaculture biosecurity policy through the Malaysian Aquafarm Certification Scheme may help control further spread of the virus and sustain the grouper aquaculture in Sabah.

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Table.3 List of gene sequences used in the multiple alignment analysis for viral strain determination

Accession Numbers	Gene	Source of isolate (Common name / Scientific name)	Strain	Year	Country
AB109368	MCP	African lampeye, *Aplocheilichthys normani*	ISKNV	1998	Indonesia
AY285745	MCP	African lampeye, *Aplocheilichthys normani*	ISKNV	1998	Indonesia
AB666342	MCP	Flatheat mullet, *Mugil cephalus*	ISKNV	2000	Singapore
AB666348	MCP	Dwarf gourami,*Colisa lalia*	ISKNV	2000	Singapore
AB666337	MCP	Reddrum, *Sciaenops ocellatus*	ISKNV	2000	Malaysia
AB666338	MCP	Barramundi perch, *Lates calcarifer*	ISKNV	2000	Malaysia
JQ253373	MCP	Brown marbled grouper, *E. fuscoguttattus*	ISKNV	2007	Malaysia
AB666340	MCP	Orange spotted grouper, *E. coioides*	ISKNV	2004	Hong Kong
AB666341	MCP	Orange spotted grouper, *E. coioides*	ISKNV	2004	Hong Kong
HM067835	MCP	Marble sleepy goby, *Oxyeleotris marmorata*	ISKNV	2009	China
HQ317461	MCP	Mandarin fish, *Synchiropus splendidus*	ISKNV	2006	China
HQ317462	MCP	Mandarin fish,*Synchiropus splendidus*	ISKNV	2007	China
HQ317460	MCP	Mandarin fish,*Synchiropus splendidus*	ISKNV	2009	China
JF264354	MCP	Giant sea perch, *Lates calcarifer*	ISKNV	2005	Taiwan
JF264345	MCP	Orange spotted grouper, *E. coioides*	ISKNV	2006	Taiwan
JF264349	MCP	Orange spotted grouper, *E. coioides*	ISKNV	2008	Taiwan
JQ253365	MCP	Giant grouper, *E. lanceolatus*	ISKNV	2011	Malaysia
JQ253366	MCP	Giant grouper, *E. lanceolatus*	ISKNV	2011	Malaysia
JQ253367	MCP	Humpback grouper, *Cromileptes altivelis*	ISKNV	2006	Malaysia
JQ253368	MCP	Humpback grouper, *Cromileptes altivelis*	ISKNV	2006	Malaysia
JQ253369	MCP	Humpback grouper, *Cromileptes altivelis*	ISKNV	2006	Malaysia
JQ253370	MCP	Humpback grouper, *Cromileptes altivelis*	ISKNV	2006	Malaysia
JQ253371	MCP	Humpback grouper, *Cromileptes altivelis*	ISKNV	2006	Malaysia
JQ253372	MCP	Orange spotted grouper, *E. coioides*	ISKNV	2004	Malaysia
JQ253373	MCP	Brown-marbled grouper, *E. fuscoguttatus*	ISKNV	2007	Malaysia
JQ253374	MCP	Humpback grouper, *Cromileptes altivelis*	ISKNV	2007	Malaysia
AB080362	MCP	Red sea bream, *Pagrus major*	RSIV	1992	Japan
AB461855	MCP	Japanese amberjack, *Seriola quinqueradiata*	RSIV	2004	Japan
AB461856	MCP	Red sea bream, *Pagrus major*	RSIV	2005	Japan
AB666335	MCP	Red sea bream, *Pagrus major*	RSIV	2001	Japan
AB666326	MCP	Orange spotted grouper, *E. coioides*	RSIV	2004	Hong Kong
AB666321	MCP	Red sea bream, *Pagrus major*	RSIV	2004	Hong Kong
AB666322	MCP	Orange spotted grouper, *E. coioides*	RSIV	2004	Hong Kong
AB666318	MCP	Hybrid sea bass, *Moronesaxatilis x Micropterussalmoides*	RSIV	2004	Hong Kong
AB666319	MCP	Brown marbled grouper, *E. fuscoguttattus*	RSIV	2000	Singapore
AB666320	MCP	Brown marbled grouper, *E. fuscoguttattus*	RSIV	2002	Singapore
AY285746	MCP	Malabar grouper, *E. malabricus*	RSIV	1992	Thailand
AB109370	MCP	Malabar grouper, *E. malabricus*	RSIV	1993	Thailand
AY532608	MCP	Barred knifejaw, *Oplegnathus fasciatus*	RSIV	2001	Korea
AY532613	MCP	Sea bass, *Lateolabrax japonicus*	RSIV	2001	Korea
DQ198145	MCP	Olive flounder, *Paralichthys olivaceus*	RSIV	2005	Korea
HQ263620	MCP	Stone flounder, *Kareius bicoloratus*	RSIV	2010	China
AY532611	MCP	Rock bream, *Oplegnathus fasciatus*	TRBIV	2001	Korea
EU276417	MCP	Olive flounder, *Paralichthys olivaceus*	TRBIV	2007	Korea
HM067603	MCP	Sea perch, *Lateolabrax sp.*	TRBIV	2010	Korea
GQ273492	MCP	Turbot, *Scophthalmus maximus*	TRBIV	2006	China
JF264361	MCP	Orange spotted grouper, *E. coioides*	Outgroup	2004	Taiwan
AB007367	ATPase	Red sea bream, *Pagrus major*	RSIV	1992	Japan
AB666362	ATPase	Pacific bluefin tuna, *Thunnus orientalis*	RSIV	1996	Japan
AB666368	ATPase	Greater amberjack, *Seriola dumerili*	RSIV	1998	Japan
AB666357	ATPase	Giant grouper, *E. lanceolatus*	RSIV	2004	Hong Kong
AB666354	ATPase	Orange spotted grouper, *E. coioides*	RSIV	2004	Hong Kong
AB666350	ATPase	Hybrid sea bass, *Moronesaxatilis* x *Micropterussalmoides*	RSIV	2004	Hong Kong
AB666352	ATPase	Brown marbled grouper, *E. fuscoguttatus*	RSIV	2002	Singapore
AB666351	ATPase	Brown marbled grouper, *E. fuscoguttatus*	RSIV	2000	Singapore
AF462344	ATPase	Giant sea perch, *Lates calcarifer*	RSIV	2001	Taiwan
AF462343	ATPase	Grouper sp, *Epinephelus* spp	RSIV	2001	Taiwan
AY894343	ATPase	Orange spotted grouper, *E. coioides*	RSIV	2004	China
GQ202217	ATPase	Spotted knifejaw, *Oplegnathus punctatus*	RSIV	2007	China
AY779031	ATPase	Large yellow croacker, *Pseudosciaena crocea*	RSIV	2002	China
AB043979	ATPase	African lampeye, *Aplocheilichthys normani*	RSIV	1998	Indonesia
AB666379	ATPase	Dwarf gourami, *Colisa lalia*	ISKNV	2001	Japan

AB666372	ATPase	Orange spotted grouper, *E. coioides*	ISKNV	2004	Hong Kong
AB666373	ATPase	Orange spotted grouper, *E. coioides*	ISKNV	2004	Hong Kong
AB666380	ATPase	Dwarf gourami, *Colisa lalia*	ISKNV	2000	Singapore
AB666375	ATPase	Flathead mullet, *Mugil cephalus*	ISKNV	2000	Singapore
AB666376	ATPase	Dwarf gourami, *Colisa lalia*	ISKNV	2000	Singapore
AY319288	ATPase	Dwarf gourami, *Colisa lalia*	ISKNV	2000	Malaysia
AB666369	ATPase	Reddrum, *Sciaenops ocellatus*	ISKNV	2000	Malaysia
AB666370	ATPase	Sea bass, *Lates calcarifer*	ISKNV	2000	Malaysia
KF669901	ATPase	Giant grouper, *E. lanceolatus*	ISKNV	2011	Malaysia
KF669902	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	ISKNV	2012	Malaysia
KF669903	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	ISKNV	2012	Malaysia
KF669904	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	ISKNV	2012	Malaysia
KF669905	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	ISKNV	2012	Malaysia
KF669906	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	ISKNV	2012	Malaysia
KF669907	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	ISKNV	2012	Malaysia
KF669908	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	ISKNV	2012	Malaysia
KF669909	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	ISKNV	2012	Malaysia
KF669910	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	ISKNV	2012	Malaysia
KF669911	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	ISKNV	2012	Malaysia
KF669912	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	ISKNV	2012	Malaysia
KF669913	ATPase	Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	ISKNV	2012	Malaysia
KF669914	ATPase	Giant grouper, *E. lanceolatus*	TRBIV	2011	Malaysia
AY608684	ATPase	Turbot, *Scophthalmus maximus*	Outgroup	2002	China
M80551	ATPase	Frog virus, *Ranatigrina cantor*		1993	China

Table.4 List of fish species, number and origin of fish specimens analyzed in this study

Common name / scientific name	Origin	Number of fish specimens	Positive	Single-PCR	Nested-PCR
Brown-marbled grouper (*E. fuscoguttatus*)	Tawau	20	6/20	2	4
	Tuaran	15	5/15	1	4
	Kota Kinabalu	63	13/63	2	11
	Kuala Penyu	4	-	-	-
Humpback grouper (*Cromileptes altivelis*)	Tawau	26	6/26	6	-
	Tuaran	25	7/25	5	2
Giant grouper, (*E. lanceolatus*)	Kota Kinabalu	5	5/5	-	5
	Tawau	3	2/3	2	-
	Kuala Penyu	6	-		
Orange spotted grouper (*E. coioides*)	Tawau	3	1/3	1	-
Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♂)	Kuala Penyu	23	20/23	11	9
	Tuaran	8	5/8	3	2
	LahadDatu	8	-	-	-
Total numbers of specimens		**212**	**70**	**33**	**37**

Fig.1 Map shows the locations of farms (star marks) from which the fish specimens were collected (source: google map).

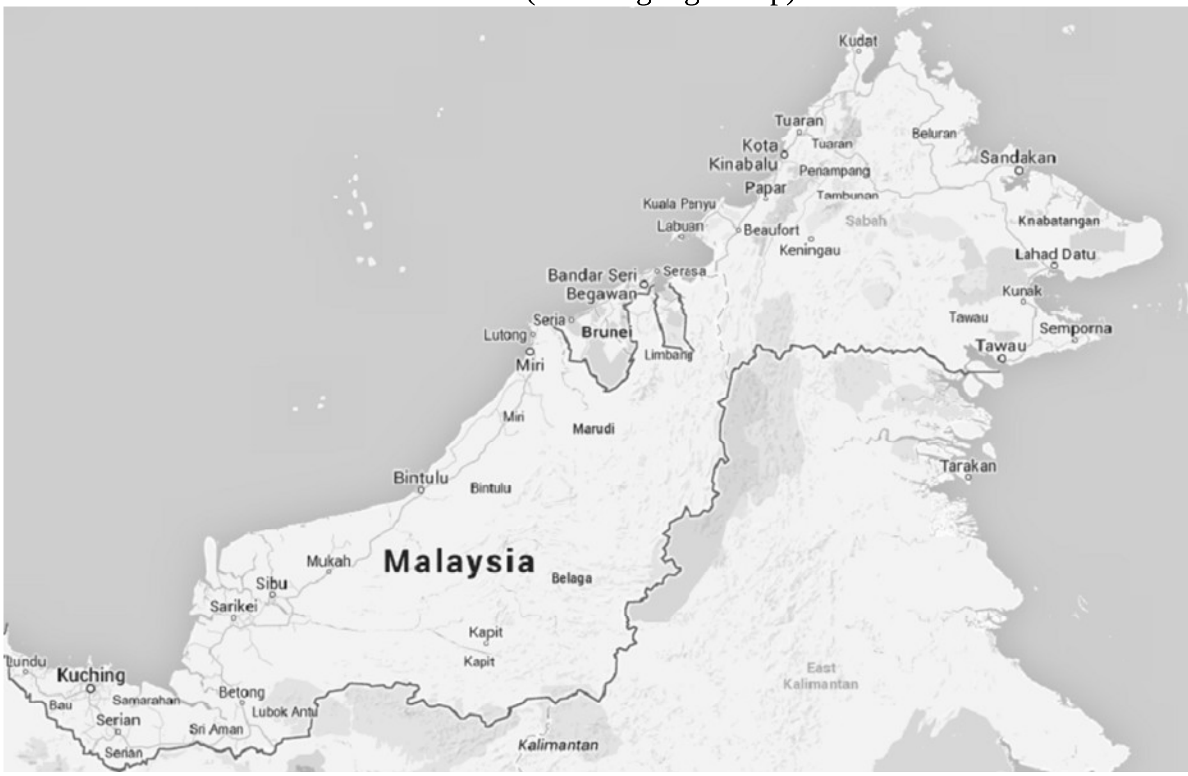


Fig.2 PCR amplification of the major capsid protein gene from Megalocytivirus (*Iridoviridae*). Lane M: 1kb DNA ladder (Promega); lane a: negative control (nuclease free distilled water); lanes 1-3: samples amplified using single-PCR (severe infection); lanes 4-6: samples amplified using nested-PCR (latent infection).

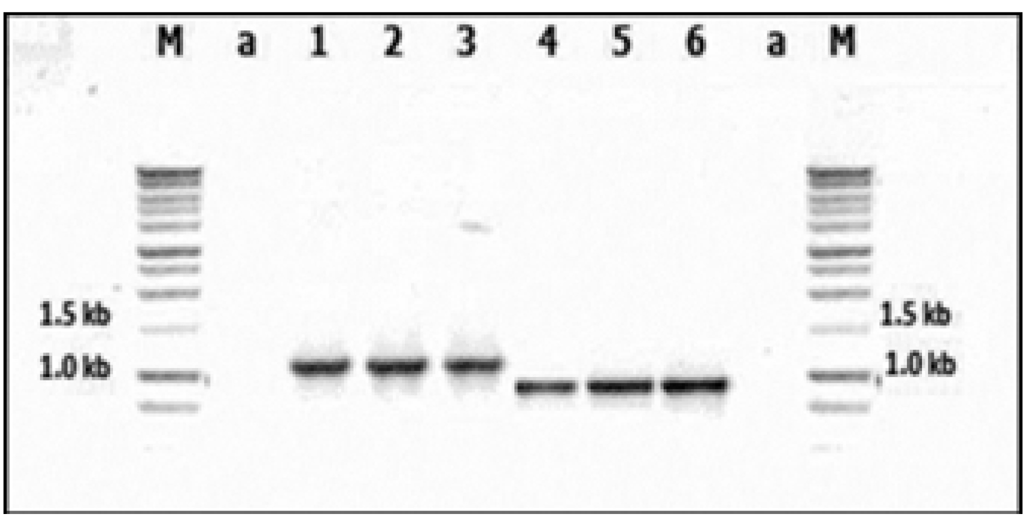


Fig.3 Percentage of groupers detected positive for Megalocytivirus; A: Brown- marbled grouper (*E. fuscoguttatus*); B: Humpback grouper (*Cromileptes altivelis*); C: Giant grouper (*E. lanceolatus*); D: Orange-spotted grouper (*E. coioides*) and E: Hybrid grouper, *E. fuscoguttatus* (♀) x *E. lanceolatus* (♀).

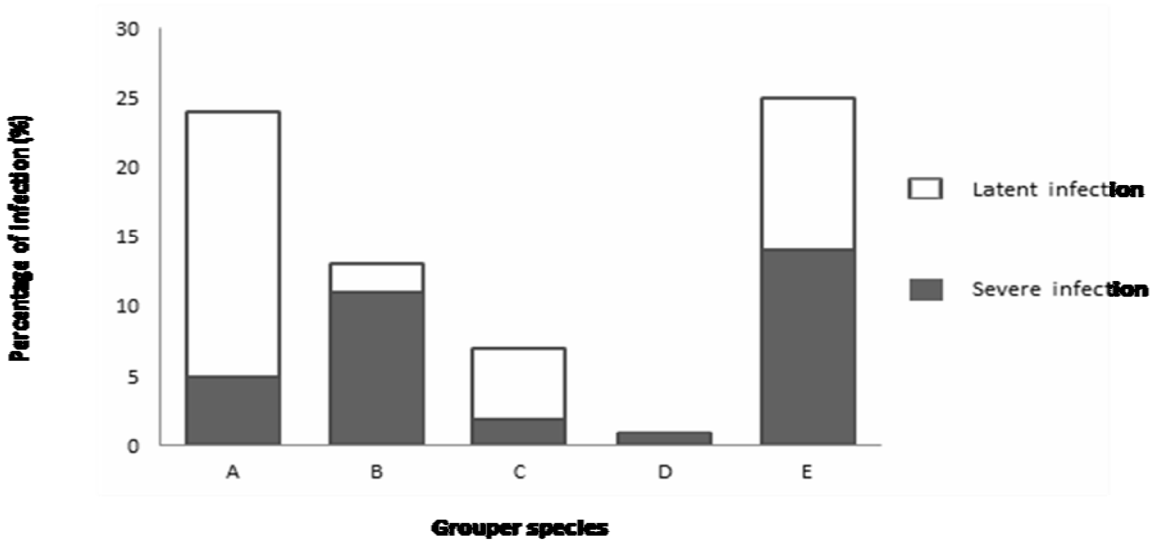


Fig.4 Phylogenetic tree deduced from the variable region (nt22-1101, AB109370) of the major capsid protein gene from all the known *Iridoviridae* genera. Numbers at the tree nodes indicate bootstrap values of 1000 replicates. The distance between sequences is represented by the length of each pair of branches. *Asfaviridae* was included as an outgroup virus

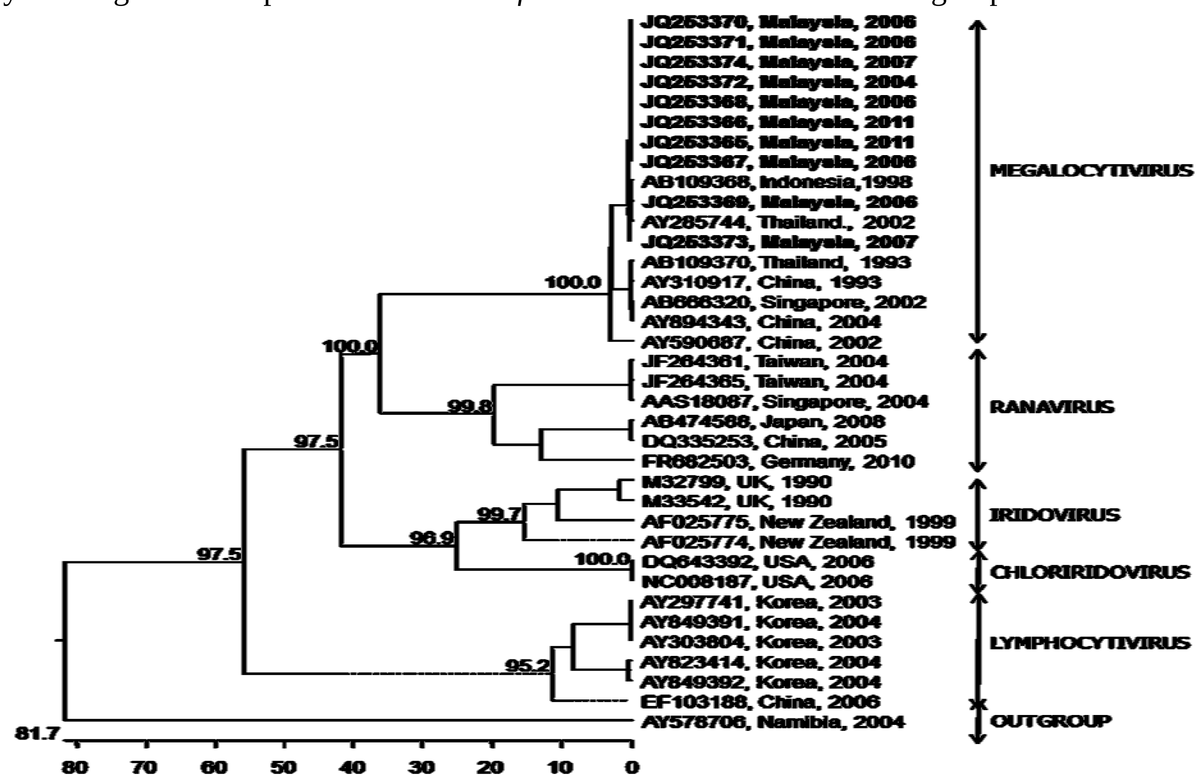


Fig.5 Phylogenetic tree deduced from variable region (nt146-231, AB666373) of ATPase gene from all the known *Iridoviridae* genera. Numbers at the tree nodes indicate bootstrap values of 1000 replicates. The distance between sequences is represented by the length of each pair of branches. *Asfaviridae* was included as an outgroup virus

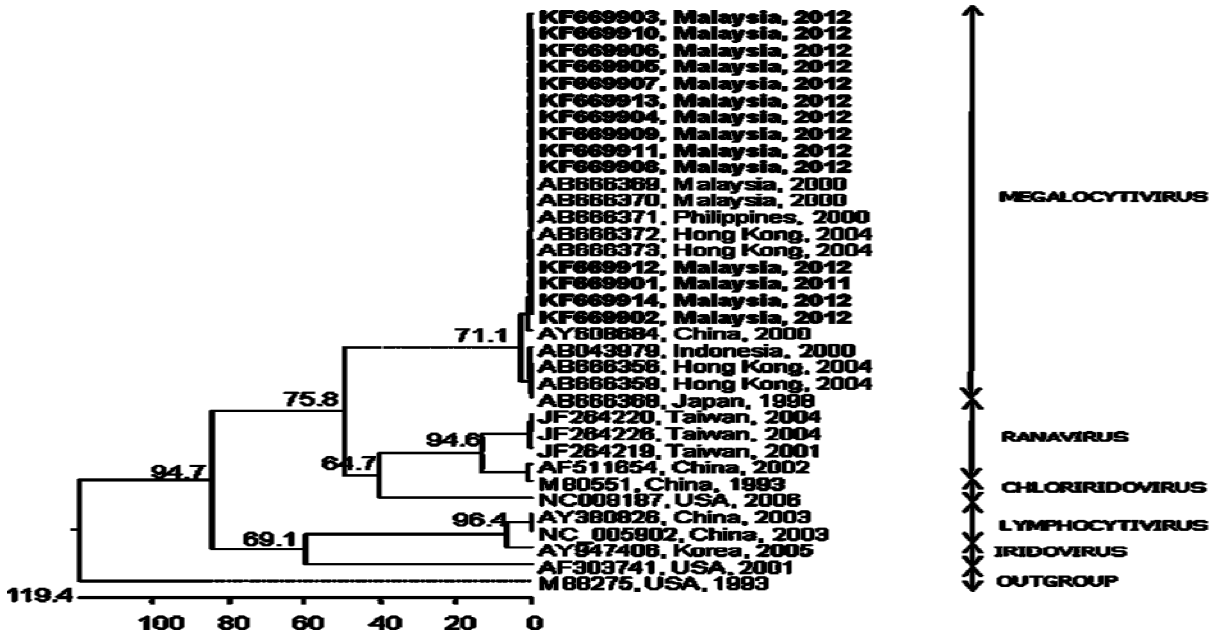


Fig.6 Phylogenetic tree deduced from variable region (nt540-951, AB109370) of the major capsid protein gene from all the knwon strains of Megalocytivirus. Numbers at the tree nodes indicate bootstrap values of 1000 replicates. The distance between sequences is represented by the length of each pair of branches. Ranavirus was included as an outgroup virus.

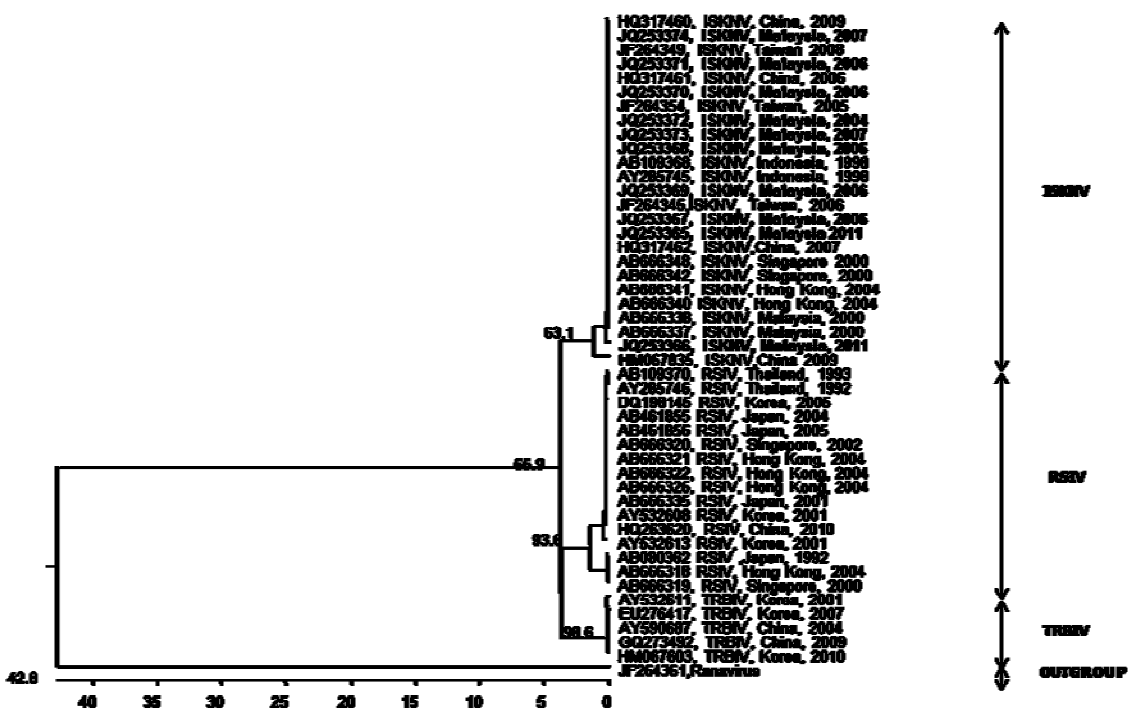
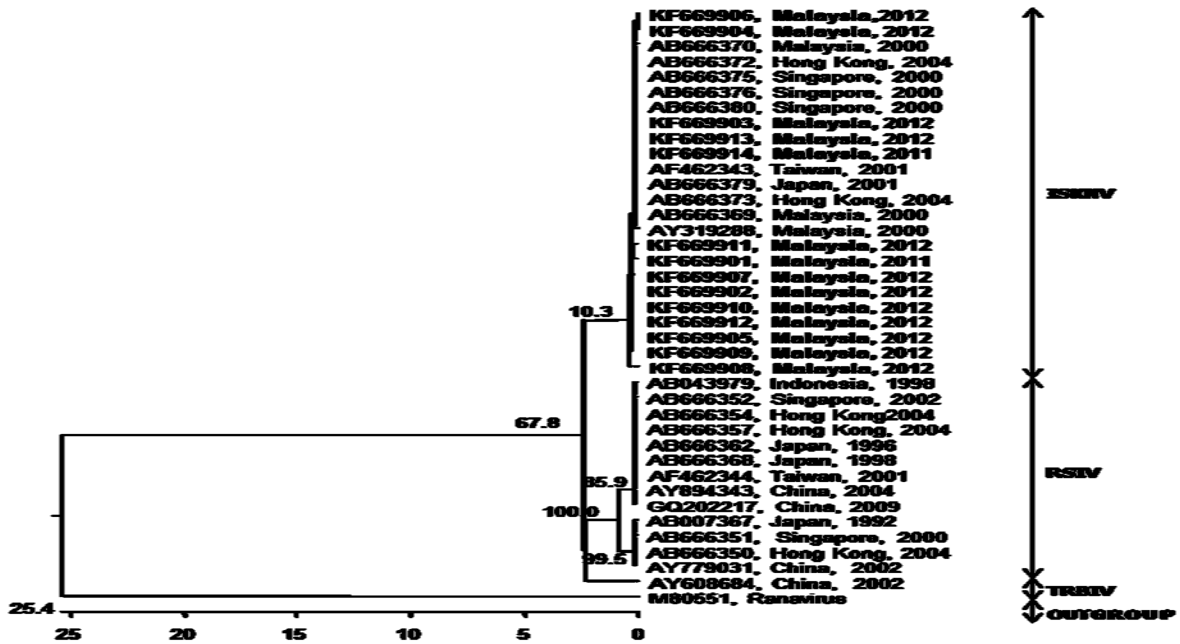


Fig.7 Phylogenetic tree deduced from variable region (nt122-521, AB666373) of ATPase gene from all the known strains of Megalocytivirus. Numbers at the tree nodes indicate bootstrap values of 1000 replicates. The distance between sequences is represented by the length of each pair of branches. Ranavirus was included as an outgroup virus



References

Bondad-Reantaso, M. G., Subasinghe, R.P., Arthur, J.R., Ogawa, K., Chinabut, S., Adlard, R., Tan, Z., Shariff, M., 2005. Disease and health management in Asian aquaculture. Vet. Parasitol. 132, 249-272.

Chao,C.B., Yang, S.C.,Tsai, H.Y., Chen, C.Y., Lin, C.S., Huang, H.T., 2002. A nested PCR for the detection of grouper iridovirus in taiwan (TGIV) in cultured hybrid grouper, giant seaperch and largemouth bass. J. Aquat. Anim. Health. 14, 104-113.

Chia,C.B., Chun, Y.C., Yueh, Y.L., Chan, S.L., Hung, T.H., 2004. Histological, ultrastructural, and *in situ* hybridization study on enlarged cells in grouper *Epinephelus* hybrids infected by grouper iridovirus in Taiwan (TGIV). Dis. Aquat. Organ. 58, 127-142.

Chincar,V.G., Hyatt, A., Miyazaki,T., Williams, T., 2008. Family *Iridoviridae*: poor viral relations no longer. Microbiol. Immunol. 328, 123-170.

Choi, S.K., Kwon, S.R., Nam,Y.K., Kim,S.K., Kim,K.H., 2006. Organ distribution of red sea bream iridovirus (RSIV) DNA in asymptomatic yearling and fingerling rock bream (*Oplegnathus fasciatus*) and effects of water temperature on transition of RSIV into acute phase. Aquaculture. 256, 23-26.

Eaton, H.E., Metcalf, J., Penny, E., Tcherepanov, V., Upton, C., Brunetti, C.R. 2007.Comparative genomic analysis of the family Iridoviridae: re-annotating and defining the core set of iridovirus genes. Virol. J. 4, 11.

Eaton, H.E., Metcalf, J., Brunetti,C.R., 2008. Expression of frog virus 3 genes is impaired in mammalian cell lines. Virol. J. 5, 83.

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A., 2005. *Virus Taxonomy. Eight Report of the International Commitee on Taxonomy of Viruses.* Elsevier Academic Presss, USA, pp 145-161.

Harikrisnan, R., Chellam, B., Moon, S.H., 2010. Molecular studies, disease status and prophylactic measures in grouper aquaculture: Economic importance, diseases and immunology. Aquaculture.10, 1-14.

Hyatt, A.D., Whittington, R.J., 2005. Ranaviruses of fish, amphibians and reptiles: diversity and the requirement for revised taxonomy. In P. Walker, R. Lester and M.G. Bondad-Reantaso (eds.). Disease in Asian Aquaculture V.pp 155-170. Fish Health Section, Asian Fisheries Society, Manila.

Jeong, J.B., Jun, L.Y., Park, K.H., Kim, K.H., Chung, J.K., Komisar, J.L., Jeong, H.D., 2006. Asymptomatic iridovirus infection in various marine fishes detected by a 2-step PCR method. Aquaculture. 255, 30-38.

Jiran,V.A., Ransangan,J., 2013. Effect of water temperature on susceptibility of culture marine fish species to vibriosis. Int. J. Res. Pure Appl. Microbiol. 3(3), 48-52.

Joon, B.J., Lyu, J.J., Min, H.Y., Myoung, S.K., Jack, L.K., Hyun, D.J., 2003. Characterization of the DNA nucleotide sequences in the genome of red sea bream iridoviruses isolated in Korea. Aquaculture. 220, 119-133.

Kurita, J., Nakajima, K., 2012. Megalocytiviruses. Viruses. 4**,** 521- 538.

Mao, J., Green, D.E., Fellers, G., Chinchar., V.G., 1999. Molecular characterization of iridoviruses isolated from sympatric amphibians and fish. Virus Res. 63, 45-52.

Muroga, K., 2001. Viral and bacterial diseases of marine fish and shellfish in Japanese hatcheries. Aquaculture. 202, 23-44.

Murwantoko., Handayani, C.R., Pratiwe,R., 2009. Cloning and sequence analysis of capsid protein gene of iridovirus Indonesian isolates. Indonesian J. Biotechnol. 14, 1117-1123.

Othman, M.F., 2008. The FAO/NACA Regional Workshop on the future of mariculture: a regional approach for responsible development in the Asia-Pacific region. FAO Proceedings. No.11. pp. 207-224.

Philips, A.J., Simon, C., 1995. Simple, efficient and nondestructive DNA extraction protocol for arthropods. Ann. Entomol. Soc. Am. 88, 281-283.

Saitou, N., Nei, M., 1987. The neighbour-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406-425.

Sambrook, J., Russell, D.W. 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Subasinghe, R.P., Bondad-Reantaso, M.G., 2008. The FAO/NACA Asia regional technical guidelines on health management for the responsible movement of live aquatic animals: lessons learned from their development and implementation. Rev. Sci. Tech. OIE. 27, 55-63.

Thompson, J.D., Higgins, D.G., Gibson, T., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence wighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

Walker, P.J., Winton, J.R. 2010. Emerging viral diseases of fish and shrimp. Vet. Res. 41, 51-75.

Wang,Y.Q., Lu, L., Weng, S.P., Huang, J.N., Chan,S.M., He, J.G., 2007. Molecular epidemiology and phylogenetic analysis of a marine fish infectious spleen and kidney necrosis virus like (ISKNV-like) virus. Arch. Virol. 152, 763-773.

Wen, C.M., Lee, C.W., Wang, C.S., Cheng, Y.H., Huang, H.Y., 2008. Development of two cell lines from *Epinephelus coioides* brain tissue for characterization of betanodavirus and megalocytivirus infectivity and propagation. Aquaculture. 278, 14-21.

Yanong, R.E., Waltzek, T., 2010. Megalocytivirus infections in fish, with emphasis on ornamental species. The Institute of Food and Agricultural Sciences (IFAS). University of Florida IFAS Extension FA182. Available: http://edis.ifas.ufl.edu/fa182 (October 2013).