BIODIVERSITAS
Volume 19, Number 5, September 2018
Pages: 1730-1736

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d190519

Genotype determination of megalocytivirus from Indonesian Marine Fishes

MURWANTOKO[1,♥], DINI WAHYU KARTIKA SARI[1], CHRISTINA RETNO HANDAYANI[2], RICHARD J. WHITTINGTON[3]

[1]Department of Fisheries, Faculty of Agriculture, Universitas Gadjah Mada. Jl. Flora Bulaksumur, Sleman 55281, Yogyakarta, Indonesia.
Tel.: +62-274-563062, ♥email: murwantoko@ugm.ac.id
[2]Main Center for Brackish Water Aquaculture Development Jepara.Jl. Cik Lanang, Bulu, Jepara 59418, Central Java, Indonesia
[3]Farm Animal and Veterinary Public Health, Faculty of Veterinary Science, University of Sydney. 425 Werombi Road, Camden, NSW 2570 Australia

Manuscript received: 11 December 2017. Revision accepted: 24 August 2018.

Abstract. *Murwantoko, Sari DWK, Handayani CR, Whittington RJ. 2018. Genotype determination of megalocytivirus from Indonesian Marine Fishes. Biodiversitas 19: 1730-1736. Megalocytivirus* is the newest genus within the family of Iridoviridae which can be divided into groups represented by red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body iridovirus (TRBIV), threespine stickleback iridovirus (TSIV). This virus caused serious systemic disease in cultured marine fishes for consumption and ornamental freshwater fishes with significant mortality. The objective of this study was to determine the genotype of megalocytivirus which infected marine fishes from Lampung, Karimun Jawa, Situbondo and Batam based on major capsid protein (MCP), ATPase, DNA polymerase, CY15 and IRB6. The liver, spleen and kidney tissues of humpback grouper (*Cromileptes altivelis*), tiger grouper (*Epinephelus fuscoguttatus*), baramundi (*Lates calcarifer*) were fixed in 10% phosphate-buffered formalin for histological and fixed in 70% ethanol for molecular analysis. Molecular analysis was performed by amplification of MCP, ATPase, DNA polymerase, CY15 and IRB6 genes and followed by sequencing. Genotype was determined by alignment of the sequences with various genotypes of megalocytivirus from Genbank. Histological examination showed that hypertrophy, inclusion body forming bearing cells were found in liver, spleen and kidney tissues. Polymerase chain reaction with MCP primer produced specific DNA bands. Those results confirmed the infection of megalocytivirus on marine cultured fish samples. The analysis from 10 isolates on five genes revealed that two genotypes of megalocytivirus as infectious spleen and kidney necrosis virus (ISKNV) and red sea bream iridovirus (RSIV) genotypes were existed in Indonesia. The ISKNV genotype was confirmed in fish samples from Lampung, Jepara, Bali; while RSIV genotype was found in fishes from Batam, and Situbondo. Interestingly, both ISKNV and RSIV genotypes were confirmed in fish samples from Karimun Jawa. This paper is the first report on the present of ISKNV and RSIV genotypes in Indonesia based on MCP, ATPase, DNA polymerase, CY15 and IRB6 genes.

Keywords: Genotype, ISKNV, marine fish, megalocytivirus, RSIV

INTRODUCTION

Megalocytivirus is the newest genus within the family of Iridoviridae along with the *Iridovirus, Ranavirus* and *Lymphocystivirus* genera (Kurita and Nakajima 2012). Phylogenetic analyses using major capsid protein (MCP) and ATPase genes show that the genus *Megalocytivirus* can be divided into groups represented by red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body iridovirus (TRBIV) (Kurita and Nakajima 2012), threespine stickleback iridovirus (TSIV) (Waltzek et al. 2012). The mortality caused by megalocytivirus infection varies between 30% in juvenile up to 100% in larvae stage (Eaton et al. 2007). This disease have been reported to cause mortality of 80-90% in juvenile grouper during February-August 1993 and February-April 1994 (Danayadol et al. 1996).

The Megalocytivirus was known as agents that caused serious systemic disease in more than 40 species on cultured marine fishes for consumption (Inouye et al. 1992; Chua et al. 1994; Nakajima and Maeno 1998; Gibson-Kueh et al. 2003; OIE 2009) and ornamental freshwater fishes (Anderson et al. 1993; Rodgers et al. 1997; Lu et al. 2005). Diseases caused by *Megalocytivirus* have been reported in Japan, Chinese Taipei, R.R. China, Hong Kong, South Korea, Malaysia, Philippines, Singapore, Thailand (OIE 2009), Australia (Go et al. 2006), Indonesia (Mahardika et al. 2003, 2004, 2009, Murwantoko et al. 2009), North America (Waltzek et al. 2012)

The clinical signs of megalocytivirus-infected fishes were severe anemia, red spots (petechiae) in the gills, swelling of the spleen (Nakajima and Maeno 1998) and kidney (Sudthongkong et al. 2002). Histopathological examination revealed that inclusion body forming bearing cells (IBC) were found in spleen, kidney, hematopoietic tissue and the digestive tract, while necrosis was occurred in kidney tissue (Sudthongkong et al. 2002). However Dong et al. (2017) reported that ISKNV diseases outbreak with none of the infected fish showed the presence of expected hypertrophied cells in histological examination of kidney, liver, spleen and brain tissues. Instead, kidney tissue of the affected fish exhibited hyaline degeneration in the epithelial cells of kidney tubules with notable presence of acidophilic inclusions. Multifocal coagulative

hepatocellular necrosis has been reported on TSIV infected three spine stickleback fishes (Waltzek et al. 2012).

Many studies have been conducted on megalocytivirus in Indonesia. In 2000, megalocytivirus caused mortality more than 80% in *Epinephelus coioides* in North Sumatera. The megalocytivirus has been detected and caused mortality of up to 100% on green grouper (*E. coioides*) and duskytail grouper (*E. bleekery*) in Bali during acclimation after been caught from marine (Roza et al. 2005). Others studies have been conducted on detection in orange-spotted grouper (*E. coioides*) (Mahardika et al. 2003), coral grouper (*Epinephelus coralica*) (Johnny and Roza 2009), succeptibility of humpback grouper (*Cromileptes altivelis*) (Mahardika et al. 2004), pathogenicity on coral trout grouper (*Plectrophomus leopardus*) (Mahardika et al. 2009), histopatology on experimentaly infected of humpback grouper (*C. altivelis*) (Mahardika and Mastuti 2013). Molecular study on megalocytovirus in Indonesia based on MCP gene showed the megalocytivirus from Jepara (IJP01) and Bali (IGD01) are belonged to ISKNV genotype and among those IJP01 and IGD01 isolates shared 99.8% identity in nucleotide level and 99.4% identity at amino acid level (Murwantoko et al. 2009). In this present study we determined the genotype of megalocytivirus from Batam, Karimun Jawa, Situbondo and Lampung in Indonesia based on MCP, ATPase, DNA polymerase, CY15, IRB6 DNA sequences.

MATERIALS AND METHODS

Fish samples

Marine cultures fishes as humpback grouper (*Cromileptes altivelis*), tiger grouper (*Epinephelus fuscoguttatus*), baramundi (*Lates calcarifer*) showing clinical signs of megalocytivirus infection were collected from Situbondo (East Jawa), Karimun Jawa (Central Jawa), Batam (Riau islands) and Lampung from January to October 2010. The internal organs: liver, spleen, kidney were collected and preserved in normal buffer formalin (NBF) for histological examination and in 70% ethanol for molecular analysis.

Histological examination

Liver, spleen and kidney tissues were fixed in 10% phosphate-buffered formalin for at least 24 h. The decalcifying was done on 10% EDTA in NBF for at least 5 h. The desired organs were dehydrated in a graded alcohol series before routine processing and embedding in paraffin wax. The Sections (5 mm) were stained with haematoxylin and eosin (HE) (Roberts et al. 2012).

Molecular characterization

DNA extraction

DNA was extracted from tissue following Wasko et al. (2003). Ten to 30 mg of tissue was homogenized in 400 µL TNES buffer (10 mM Tris-HCl pH 8; 125 mM NaCl; 10 mM EDTA pH 8; 0,5% SDS; 4M urea). Three µL of RNase (10 mg/mL) was added to the mixture and incubated at 42°C for 1 hour. Following this, 3 µL of proteinase K (10 mg/mL) was added into the mixture and incubated at 42°C for 2-6 hours. The suspension was extracted using same volume of phenol: chloroform: isoamyl alcohol (PCIAA). DNA was precipitated using 1 M NaCl and two volume of cold absolute ethanol and followed by washing with 70% ethanol.

PCR amplification

First PCR amplification for determine status on megalocytivirus infection was conducted under Mastercycler personal Thermal Cycler (Eppendorf) using primers MCP-Irido-F-Bam (ATCAGGATCCATGTCT GCAATCTCAGGTG) and MCP-Irido-R-Eco (CGTCGAA TTCGTCGACAGATGTGAAGTAG) (Murwantoko et al. 2009). Amplifications for sequencing of target genes were performed in Gradient Palm-Cycler (Corbett Research) PCR machine using primers specifically designed for several genes of megalocytivirus (Go et al. 2006) (Table 1).

Table 1. List of primers used to amplify genes of megalocytivirus (Go et al. 2006)

Name	Gene	Sequence	Tm (°C)
MCPI F	MCP	TTCACAGGATAGGGAAGCCTGC	56.7
MCPI R	MCP	TCATCAGCCAGAGCAACCAG	53.2
MCP2 F	MCP	GTCTGCAATCTCAGGTGCAAAC	54.8
MCP2 R	MCP	GATCTTAACACGCAGCCACA	51.8
ATPase 2 F	ATPase	GCCACCGTAATCAGTTTGATCATC	55.7
ATPase 2 R	ATPase	ATGAACCCGCTGCACTATGC	53.8
CY15 F	CY15	TCATCTGCACGTACACCCTG	53.8
CY15 R	CY15	CGCCCACATCCAAATCTATC	48.9
DNA Pol F	DNA polymerase	CAAGGCTGTTGGATTTTGAG	49.7
DNA Pol R	DNA polymerase	AGTCCTGTCCAAGTGCAACC	53.8
IRB6 F	IRB6	AAGTAGTGAGGGCAGAAG	48.0
IRB6 R	IRB6	ATCGTAGTCGTCCATTCC	48.0

Each PCR reaction was performed in a total volume of 50 µL containing final concentration of 25 mM of each dNTP, 2 mM of each primer, PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.6, 2.5 mM $MgCl_2$, 10 mM beta-mercaptoethanol) and 2 units of Taq DNA polymerase. Optimum condition for PCR reaction with hot start (Go et al. 2006) is as follows: one cycle of initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. and a final extension at 72°C for 3 min. PCR products were separated by gel electrophoresis on a 2% agarose gel containing 0.003% ethidium bromide, and compared against molecular size marker number VIII (Roche). Negative controls for the PCR mix and a negative control (water) for DNA template and DNA derived from MCIV was used as a positive control.

PCR products (2.5 µL) were examined on a 2% agarose gel containing 0.003% ethidium bromide. The PCR products were purified using a commercial silica binding column (SV Gel and PCR Clean-Up Kit, Promega Corporation), following the manufacturer's instructions. In case the non specific bands appeared, the desired band was sliced from gel and their DNA was purified using Freeze n Squeeze DNA Gel Extraction Spin Column (Biorad). The PCR product then further purified using shrimp alkaline phosphatase/exonuclease (ExoSap-IT, Amersham Biosciences) with the mixture incubated at 37 °C for 20 min, followed by 80°C for 15 min to denature the enzyme in thermal cycler.

DNA sequencing

Sixteen µL DNA sequencing reaction contained 10 pmol of either the forward or reverse PCR primer, sterile purified water, and 50-200 pg of PCR product. All reactions were performed in a commercial supplier using BigDye Terminator version 3.1 chemistry (Applied Biosystems) and analyzed in a ABI Prism 3100 capillary Genetic Analyser (Applied Biosystems).

Data analysis

Results of DNA sequences were aligned and checked manually to resolve errors. Multiple alignments analysis with addition of Genbank collected sequences was conducted using the MEGA ver. 7.0 (Kumar et al. 2016). Cluster tree diagrams were constructed based on unweighted pair group method using arithmetic average (UPGMA) with 1000 bootstrap replicates for analysis of branch support.

RESULTS AND DISCUSSION

Clinical signs

Humpback grouper (*Cromileptes altivelis*) fishes were collected from Karimun Jawa and Lampung. The fishes were lethargy and stay solely separately from the others in sea cage. Tiger grouper (*Epinephelus fuscoguttatus*) from Batam showed stay in the bottom of tank dark color, lethargy, thin body and cloudy eyes. Lethargy was observed from baramundi (*Lates calcarifer*) collected from Situbondo. Humpback grouper from Karimun Jawa showed the progressive color change of liver to pale, enlargement of spleen, with normal size of kidney. Tiger grouper showed enlargement of spleen and pale color of liver (Figure 1). The fishes from other areas showed pale liver or enlarge spleen.

Histopathological examination

Histology examinations were conducted on liver (Figure 2.A), spleen (Figure 2.B,C), kidney tissues (Figure 2.D). Liver tissue composed of hepatocytes which are often swollen with glycogen. The pancreatic tissues are scattered in liver tissue. In spleen showed diffuse red and white pulps and erythrocytes are distributed. Kidney showed renal tubules surrounded by hematopoietic tissue. The pathological changes observed in several tissue samples such as liver and spleen of humpback grouper (*C. altivelis*) from Lampung, and the spleen and kidney of tiger grouper (*E. fuscoguttatus*) from Batam. hypertrophy, inclusion body forming bearing cells are found from those tissues. There are many pathological changes observed in the tissues, including hypertrophy, inclusion body forming bearing cells in several tissues such as liver, spleen and kidney (Figure 2)

Molecular characterisation

DNA amplification using primers MCP-Irido-F-Bam and MCP-Irido-R-Eco showed that 10 fish samples which originally came from Batam, Lampung, Karimun Jawa and Situbondo were infected by megalocytivirus as indicated by presence a DNA band at 1000 bp (data not shown). Among those samples, five samples were successfully amplified on major capsid protein (MCP) gene using designed primer (Go et al. 2006). Sequencing of those DNA could read the samples from 1266 to 1313 nucleotides and the sequences can be found in supplementary data. The sequences of MCP have been deposited in Genbank with accession number MH764414-MH764418. Cluster analysis together with data in Genbank showed that an isolate from Batam (Btm Fish_35) and three isolates from Karimun Jawa, KJw fish_23, _27 and _28 were belonged to RSIV genotypes. Alignment analysis of those sequences using BLAST showed that this isolate has 100% identity with RSIV (AB461856) and OGIV (AY 894343). Interestingly an isolate from Karimun Jawa, KJw Fish_21 was belonged to ISKNV genotype. Analysis with BLAST showed the KJw_Fish_21 had 100% identity with ISKNV (AF370008), DGIV (AY989901), MCIV (AY936203) and 99% with GSIV (JF264354). Comparing with previous study (Murwantoko et al. 2009), this Karimun Jawa isolated shared 100% nucleotide identity with Bali isolate (IGD01) and 99% nucleotide identity with Jepara isolate (IJP03) (Figure 3).

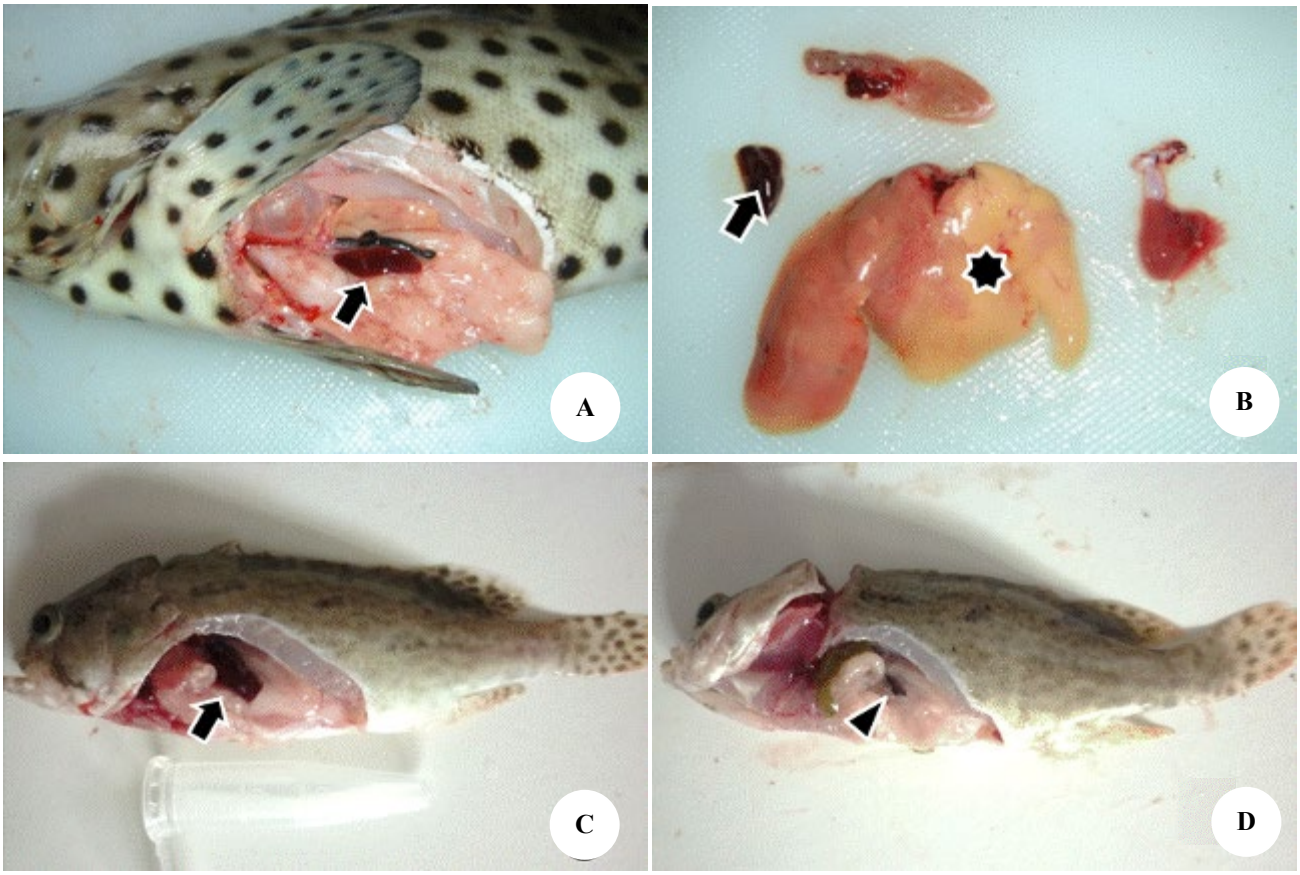

Figure 1. Gross sign of fish samples. The enlargement of spleen (arrow) and progressive color change of liver to pale (*) were found in humpback grouper *C. altivelis* (A and B) and enlargement of spleen of tiger grouper *E. fuscoguttatus* (arrow) (C) and relatively normal size of spleen of *E. fuscoguttatus* (head arrow) (D)

ATPase fragment corresponds to the central region of ATPase from eight isolates have been successfully sequenced with size range from 616 to 805 nucleotides. The sequences of ATPase have been deposited in Genbank with accession number MH764419-MH764426 and the sequences can be found in supplementary data. The sequences of ATPase were used to construct cluster tree under UPGMA, as indicated in Figure 4. Cluster analysis together with data in Genbank showed those isolates were distributed into two genotypes of megalocytivirus; the ISKNV and RSIV. Analysis using BLAST, samples form Lampung, Lpg Fish 3 and Lpg Fish 9 showed 100% identity with ISKNV (AF371960, KP292962), MCIV (AY 936204), DGIV (AY989902). Alignment analysis also showed, a sample form Batam (Btm Fish 35), and five samples from Karimun Jawa (KJw Fish 23,-26,-27,-28 and-29) were belonged to RSIV genotype and showed those sequences had 100% identity with OGIV (AY894343), GSIV (AF462344), RSIV (AP017456), and had 99% identitiy with RSIV (AB007367) (Figure 4). Consistent with the result from MCP sequences, many isolates from Karimun Jawa and isolate from Batam were belonged to RSIV.

The sequences of DNA polymerase, CY15 and IRB from Karimun Jawa (KJw fish 21, KJw Fish 23), Situbondo (Stb fish 32) and Lampung (Lpg fish 3) have been determined and those sequences can be found in supplementary data. Those sequences of DNA polymerase, CY15 and IRB6 have been deposited in Genbank with accession number MH764410-MH764413 , MH764427-MH764434. Cluster analysis under UPGMA on megalocytivirus isolates based on DNA polymerase, CY15 and IRB6 was presented in Figure 5. Cluster analysis together BLAST analysis showed the KJw fish_28 and Stb fish_32 were belonged to RSIV genotype with 100% identity against RSIV (AP017456), GSIV (KT804738) and OGIV (AY894343). The Lpg fish_3 and KJw fish_21 were belonged to ISKNV and those sequences showed 100% identity with ISKNV (AF371960). Consistent with the above results KJw fish_28 is belonged to RSIV genotype, and KJw fish_21 was belonged to ISKNV genotype (Figure 5).

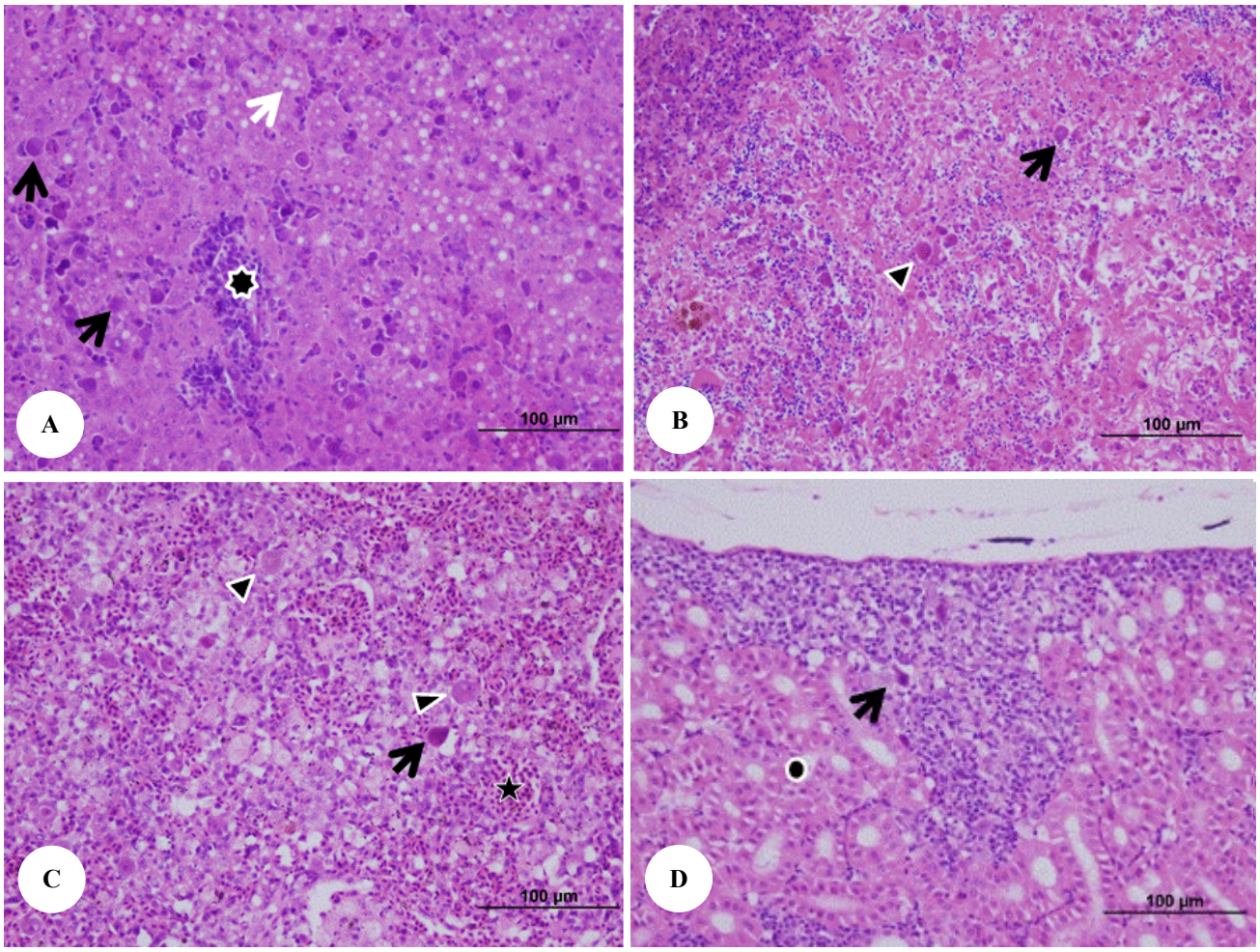

Figure 2. The histology of liver of humpback grouper (*C. altivelis*) from Lampung showed hepatocytes with glycogen (white arrow), pancreatic tissue (*), inclusion body forming bearing cells (IBC) (black arrow) (A).Histology of spleen of humpback grouper from Lampung (B) and of tiger grouper (*E. fuscoguttatus*) from Batam. (C) showed erythrocytes (star), hypertrophied spleenocytes (head arrow) and IBC (black arrow). Histology of kidney of tiger grouper from Batam showed renal tubules (dot) and IBC (black arrow)

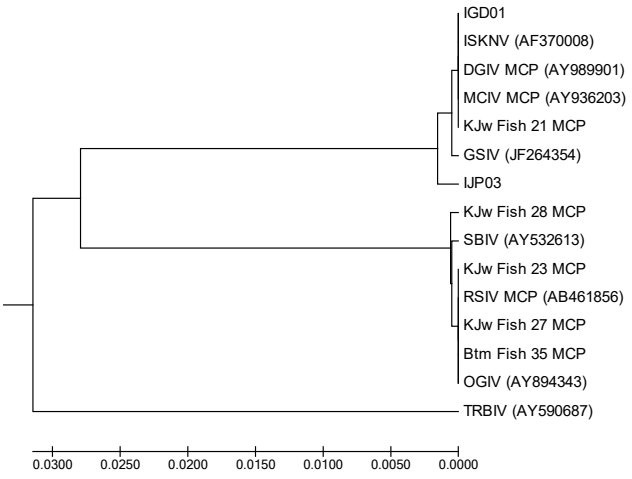

Figure 3. UPGMA dendrogram of the megalocytivirus isolates based on major capsid protein gene sequences

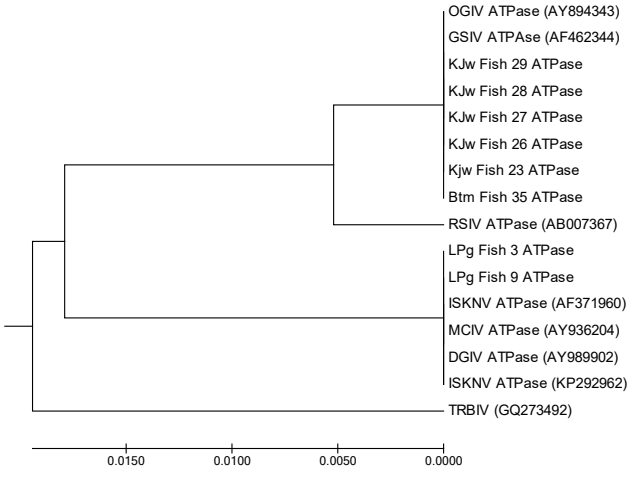

Figure 4. UPGMA dendrogram of the megalocytivirus isolates based on ATPase gene sequences

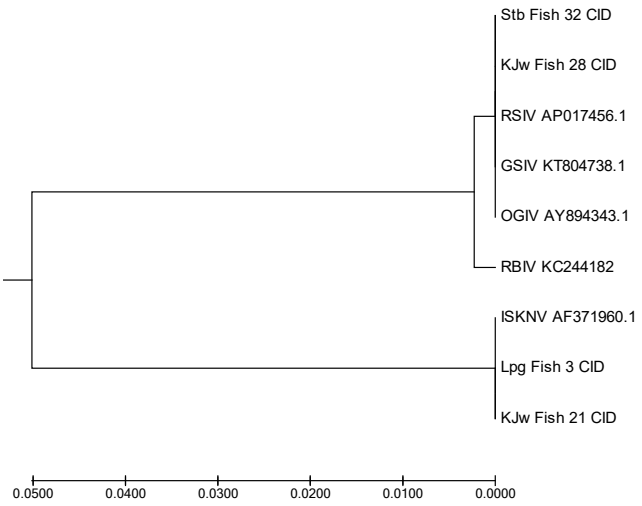

Figure 5. UPGMA dendrogram of the megalocytivirus isolates based on DNA polymerase, CY15, and IRB6 gene sequences

Discussion

The clinical signs of megalocytivirus-infected fishes are lethargic, swim helplessly, and show severe anemia, petechiae of the gills, and enlargement of the spleen (Nakajima and Maeno, 1998) and kidney (Sudthongkong et al. 2002). Our study showed that fish samples were lethargy. enlargement of spleen, pale color of liver (Figure 1). Histological examination from some samples clearly showed the presence of hypertrophy, inclusion body forming bearing cells in several tissues such as liver, spleen and kidney (Figure 2). Those result on megalocytivirus was similar with reported by Sudthongkong et al. (2002) reported the enlarged cells have been termed inclusion body-bearing cells (IBC). This IBC appearance is pathognomonic for megalocytivirus (Kurita and Nakajima 2012). Some samples on this study showed clinical signs, but the IBC was not find in the tissues. Due to the positive PCR amplification using primers MCP-Irido-F-Bam and MCP-Irido-R-Eco, we concluded that those fish samples were infected by megalocytivirus. Subramaniam et al. (2014) have reported many positive cases of ISKNV on ornamental fishes which did not show any clinical signs. Jeong et al. (2006, 2008) also found megalocytivirus in marine fish species that were externally healthy, a condition that could be called persistent or asymptomatic infection. Jeong et al. (2006) proved that he DNA concentration of the megalocytivirus in asymptomatically infected tissues was approximately 10^{-5} times of that of moribund fish infected clinically.

The previous study of Indonesian megalocytivirus using MCP sequences have identified IGD01 (from Jepara) and IJP03 (from Bali) were belonged into ISKNV (Murwantoko et al. 2009). In this study we discovered ISKNV genotype from another area in Indonesia i.e. Karimun Jawa and Lampung (Figure 3). All isolates of ISKNV genotype in this study showed 100% identity with ISKNV (AF370008). This Indonesian ISKNV isolates seems has homogenous genetic compare to the Malaysia, as Zainathan et al. (2017) reported that ISKNV isolates from Southern Malaysia showed 97-100% nucleotide identity with reference ISKNV.

Host range of ISKNV is relatively broad but freshwater and brackish water fish species are predominantly affected species (Kurita and Nakajima 2012). ISKNV diseases outbreak in freshwater fishes have been reported on ornamental fish in Germany (Jung-Schroers et al. 2016), Australia (Mohr et al. 2015; Rimmer et al. 2017), Malaysia (Subramaniam et al. 2014; Zainathan et al. 2017), and in cultured Nile tilapia (*Oreochromis niloticus*) in the US Midwest (Subramaniam et al. 2016). The results of our study showed that ISKNV could be detected from marine fish, humpback grouper (*Cromileptes altivelis*), and tiger grouper (*Epinephelus fuscoguttatus*). Dong et al. (2017) have further reported the occurrence of ISKNV diseases outbreak in farmed baramundi (*L. calcarifier)* in Vietnam.

Based on MCP sequenced, we confirmed a sample form Batam (Btm Fish 35), and five samples from Karimun Jawa (KJw Fish 23,-26,-27,-28 and-29) were belonged to RSIV genotype (Figure 3). Alignment analysis on ATPase sequences showed the KJw fish_28 and a sample from Situbondo (Stb Fish_32) were belonged to RSIV genotype (Figure 4). Several genes-other than those of ATPase and MCP-have been used to genetic analysis of megalocytivirus (Go et al. 2006). We employed a combination of DNA polymerase, CY15 and IRB genes to confirm our results using MCP and ATPase gene. The results showed samples from Situbondo (Stb Fish_32) and from Karimun Jawa (KJw Fish-28) were belonged to RSIV genotype (Figure 5). From those results we confidently confirmed that RSIV genotypes already presence in Indonesia and have been detected on fish from Batam, Karimun Jawa and Situbondo. To our knowledge, this is the first report on the presence of RSIV genotype in Indonesia.

The RSIV-type viruses can be further divided into two sub-clusters: genotype I (RSIV Ehime-1), and genotype II (majority of RSIV, grouper sleeping disease virus (GSDIV), orange spotted *grouper* iridovirus (OGIV), RBIV) (Kurita and Nakajima 2012). The sequences of MCP, ATPase, DNA polymerase, CY15 and IRB6 from RSIV Indonesian isolates showed 100% identity with orange spotted grouper iridovirus (OGIV). Those results showed that Indonesian RSIV were belonged to genotype II RSIV.

Here we confirmed the presence of RSIV and ISKNV genotypes in Indonesia. The distribution map of megalocytivirus in Indonesia are as follows: ISKNV genotype presents in Lampung, Karimun Jawa, Jepara, Bali; and RSIV genotype presents in Batam, Situbondo and Karimun Jawa. Interestingly Karimun Jawa has two genotypes of megalocytivirus, ISKNV and RSIV.

ACKNOWLEDGEMENTS

This research is funded by Research Grant from Directorate General of Higher Education, Ministry of National Education, Republic of Indonesia No.

425/SP2H/PP/DP2M/VI/2010. The authors would like to give high appreciation to the farmers, who permitted to conduct research and many technical supports. We also thank to two anonymous reviewers for giving critical suggestion.

REFFERENCES

Anderson, IG, Prior HC, Rodwell BJ, Harris GO 1993. Iridovirus-like virions in imported Dwarf Gourami (*Colisa lalia*) with systemic amoebiasis. Austr Vet J 70: 66-67

Chao CB, Yang SC, Tsai HY, Chen CY, Lin CS, Huang HT 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.

Chua FHC, Ng ML, Ng, KL, Loo JJ, Wee JY. 1994. Investigation of outbreak of a novel disease, "*Sleepy Grouper Disease*", affecting the Brown-Spotted Grouper, *Epinephelus tauvina* Forskal. J Fish Dis 17: 417-427.

Danayadol Y, Direkbusarakom S, Boonyaratpalin S, Miyazaki T, Miyata M. 1996. An outbreak of iridovirus-like infection in Brown-spotted Grouper (*Epinephelus malabaricus*) cultured in Thailand. Aquat Anim Health Res Inst Newslett 5: 6

Eaton HE, Metcalf J, Penny E, Tcherepanov V, Upton C, Brunetti CR. 2007. Comparative genomic analysis of the Family Iridoviridae: Re-annotating and defining the core set of iridovirus genes. Virol J 4: 11.

Do JW, Cha SJ, Kim JS, An EJ, Park MS, Kim JW, Kim YC, Park MA, Park JW. 2005. Sequence variation in the gene encoding the major capsid protein of koren fish iridoviruses. Arch Virol 150: 351-359

Dong HT, Jitrakorn S, Kayansamtuaj P, Pirarat N, Rodkum C, Rattanajpong T, Senapin S, Saksmerprome. 2017. Infectious spleen and kidney necrosis disease (ISKND) outbreaks in farmed barramundi (*Lates calcarifer*) in Vietnam. Fish Shellfish Immunol 68: 65-73.

Gibson-Kueh, S, Netto P, Ngoh-Lim GH, Chang SF, Ho LL, Qin QW, Chua FHC, Ng ML, Ferguson HW. 2003. The pathology of systemic iridoviral disease in fish. J Comp Pathol 129: 111-119.

Go J, Lancaster M, Deece K, Dhungyel, O, Whittington R. 2006. The molecular epidemiology of iridovirus in Murray Cod (*Maccullochella peelii peelii*) and Dwarf Gouramy (*Colisa lalia*) from distant biogeographical regions suggests a link between trade in ornamental fish and emerging iridoviral diseases. Mol Cell Probes 20 (3-4): 212-222.

Inouye K, Yamano K, Maeno Y, Nakajima K, Matsuoka M, Wada Y, Sorimachi M. 1992. Iridovirus infection of cultured Red Sea Bream, *Pagrus major*. Fish Pathol 27: 19-27.

Johnny F, Roza D. 2009. Iridovirus infection case on seed of Coral grouper fish, *Epinephelus corallicola* in hatchery. J Fish Sci 11: 8-12.

Jung-Schroers V, Adamek M, Wohlsein P, Wolter J, Wedekind H, Steinhagen D. 2016. First outbreak of an infection with infectious spleen and kidney necrosis virus (ISKNV) in ornamental fish in Germany. Dis Aquat Organ 119: 239-244.

Kurita J, Nakajima K. 2012. Meglocytivirus. Viruses 4: 521-538

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Ev 33: 1870-1874.

Lu L, Zhou SY, Chen C, Weng SP, Chan SM, He JG. 2005. Complete genome sequence analysis of an iridovirus isolated from the orange-spotted grouper, *Epinephelus coioides*. Virology 339: 81-100

Mahardika K, Mastuti I. 2013. Histopathological study: The formation of enlarged cells in organ of grouper fish following experimental infection of Meglocytivirus. Konferensi Akuakultur Indonesia 2017: 132-138. [Indonesian]

Mahardika K, Koesharyani K, Prijono A, Yuasa K. 2003 Iridovirus infection of orange-spotted grouper (*Epinephelus coioides*). Jurnal Penelitian Perikanan Indonesia 9: 11-15.

Mahardika K, Zafran, Yamamoto A, Miyazaki T. 2004. Susceptibility of juvenile humpback grouper *Cromileptes altivelis* to grouper sleepy disease *iridovirus* (GSDIV). Dis Aquat Organ 59: 1-9.

Mahardika K, Muzaki A, Suwirya K. 2009. Pathogenecity of Grouper Sleepy Disease Iridovirus (GSDIV: Megalocytivirus, Family Iridoviridae) to Coral Trout Grouper *Plectrophomus leopardus.* Indonesian Aquacult J 4: 121-130

Mohr PG, Moody NJG, Williams LM, Hoad J, Cummins DM, Davies KR, Crane MSJ. 2015. Molecular confirmation of infectious spleen and kidney necrosis virus (ISKNV) in farmed and imported ornamental fish in Australia. Dis Aquat Org 116: 103-110.

Murwantoko, Handayani CH.and Pratiwi R, 2009. Cloning and sequence analysis of capsid protein gene of iridovirus Indonesian isolates. Indon J Biotech 14: 117-123.

Nakajima K., Maeno Y. 1998. Pathogenicity of Red Sea Bream and Other Fish Iridoviruses to Red Sea Bream. Fish Pathol 33: 143-144.

OIE. 2009. Red sea bream iridoviral disease, Manual of Diagnostic Tests for Aquatic Animals. OIE, Rome.

Rimmer AE, Whittington RJ, Tweedie A,, Becker JA 2017. Susceptibility of a number of Australian freshwater fishes to dwarf gourami iridovirus (Infectious spleen and kidney necrosis virus). J Fish Dis 40 (3): 293-310.

Roza D, Johny F, Zafran. 2005. Virus Infectious Diseases on Marine Fish Culture and Their Efforts. BBRPBL Gondol, Bali. [Indonesian]

Roberts RJ, Smail DA, Munro ES. 2012. Laboratory methods. In: Roberts RJ (ed.). Fish Pathology. 4th ed. Wiley-Blacwell, UK.

Rodgers HD, Kobs M, Macartney A, Frerichs GN. 1997. Systemic Iridovirus Infection in Freshwater Angelfish, *Pterophyllum scalare* (Lichtenstein). J Fish Dis 20: 69-72.

Shinmoto H, Taniguchi K, Ikawa T, Kawai K, Oshima S. 2009. Phenotypic diversity of infectious red sea bream iridovirus isolates from cultured fish in Japan. Appl Environ Microbiol 75: 3535-3541

Subramaniam K, Shariff M, Omar AR, Hair-Bejo M, Ong BL. 2014. Detection and molecular characterization of infectious spleen and kidney necrosis virus from major ornamental fish breeding states in Peninsular Malaysia. J Fish Dis 37: 609-618.

Subramaniam K, Gotesman M, Smith CE, Steckler NK, Kelley KL, Groff JM, Waltzek TB. 2016. Megalocytivirus infection in cultured Nile tilapia *Oreochromis niloticus*. Dis Aquat Organ 119: 253-258.

Sudthongkong C, Miyata M, Miyazaki T. 2002. Viral DNA sequences of genes encoding the ATPase and the major capsid protein of tropical iridovirus isolates which are pathogenic to fishes in Japan, South China Sea and Southeast Asian countries. Arch Virol 147: 2089-2109.

Wasko AP, Martins C, Oliveira C, Foresti F. 2003. Non-destructive genetic sampling in fish. an improved method for DNA extraction from fish fin and scales. Hereditas 138: 161-165.

Zainathan SC, Ahmad AA, Johan CAC, Halim NIA, Ariff N. 2017. Detection and molecular characterization of *Megalocytivirus* strain ISKNV in freshwater ornamental fish from Southern Malaysia. AACL Bioflux 10: 1098-1109.