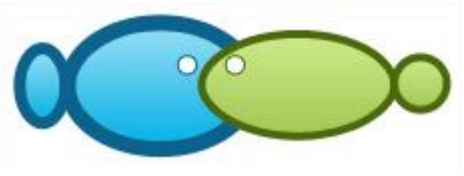

Molecular characterization of infectious spleen and kidney necrosis virus (ISKNV) in Malaysian brown-marbled grouper (*Epinephelus fuscoguttatus*) from West Malaysia

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Abstract. Groupers have become one of the most important aquaculture and traded commodities in the Asia-Pacific region. Groupers, especially species from subfamily Epinephelinae, contribute to 155,000 tonnes of production annually in Asia. One of the major production constraints in grouper culture is the heavy mortality due to viral diseases. With the rapid development of grouper culture, outbreaks of viral diseases occurred frequently in cultured brown-marbled grouper (*Epinephelus fuscoguttatus*) farms. Infectious spleen and kidney necrosis virus (ISKNV) has always been associated with mass mortalities of marine fish including groupers. Thus, this study was conducted to detect the presence or absence of ISKNV in brown-marbled groupers from West Malaysia. A total of 40 brown-marbled groupers were collected from the farm. The PCR analyses were conducted based on major capsid protein genes of *Megalocytivirus*. Out of a total of 40 samples, the PCR analysis demonstrated 2 positive pooled samples (n = 10) for the presence of ISKNV. Sunken eye, skin ulcer, fin rot, darkened body, abnormal body shape and swollen spleen and kidney were observed on positive samples. Sequence analysis of MCP gene showed 95% query cover and 99% similarities respectively with ISKNV, genotype 1.
Key Words: brown-marbled grouper, genotype I, ISKNV, *Megalocytivirus*, West Malaysia.

Introduction. Groupers belong to the subfamily Epinephelinae and are widely distributed in tropical and warm waters worldwide. The subfamily Epinephelinae (Teleostei: Serranidae), is commonly known as groupers and comprises about 162 species of marine fishes in 15 genera (FAO 2021). Many of which are valuable marine-cultured fish species in many countries or regions around the Asia-Pacific region, including mainland China, Indonesia, Malaysia, Philippines, Singapore, Thailand, and Taiwan (Harikrishnan et al 2010, 2011). Due to the increasing demand, groupers are exported in live condition to many countries including Singapore, Japan, Hong Kong, Taiwan, Malaysia, and United States (Che-Zulkifli et al 2020). Grouper industry has been established for the last three decades and it continues to be one of the most value added industries in the Far East and South East Asia especially in Taiwan (Che-Zulkifli et al 2020). The bulk of grouper aquaculture is undertaken in Asia with three countries (China, Taiwan and Indonesia) contributing 95% of reported global production (Glamuzina & Rimmer 2022). Groupers are generally cultured in floating net cages or earthen ponds, but cage culture is more common in Southeast Asia (FAO 2021). Farmed grouper production in representative countries is reported at 174,000 metric tonnes in 2019, and was expected to increase by 6 percent to 185,000 MT in these representative countries by 2020-2021 (Tveteras et al 2019). The demand for grouper is expected to increase to half a megatonne in 2020 (Kuo et al 2020).

In Malaysia, the grouper culture is normally carried out alongside other marine/brackish water finfish such as sea bass (*Lates calcarifer*) and snapper (*Lutjanus*

johnii, *Lutjanus argentimaculatus*) in floating net cages. Among the groupers, *Epinephelus suillus*, *E. malabaricus*, *E. tauvina*, *E. bleekeri*, *E. lanceolatus*, *E. fuscoguttatus*, *E. areolatus*, *Plectropomus leopardus*, *P. maculatus*, and *Cromileptes altivelis* are the most common species being cultured in net-cages, particularly in the West Coast of Peninsula of Malaysia (Department of Fisheries 2010). The major grow-out sites for grouper in Malaysia are in Sabah, particularly Tuaran and Sandakan, and Sarawak (where wild seed are also captured) in East Malaysia (Pomeroy et al 2002). In Peninsular Malaysia, grouper cultures are mainly in Langkawi (5,440 tons), Pulau Ru (6,400 tonnes), Pulau Tioman (6,400 tonnes) and Sg. Johor (6,400 tonnes) in 2010 (Department of Fisheries 2010). The total Malaysian grouper production in 2020 has reduced to 4000 tonnes (Department of Fisheries 2020). For the fish farmers in Malaysia, collecting wild seed is the major source of local supply of grouper seed but they have been also importing large numbers of hatchery-produced fish fry/juveniles from Taiwan (Che-Zulkifli et al 2020).

Grouper culture can also be severely affected by a series of infectious diseases due to the high-density and intensive farming being undertaken to meet the increasing aquaculture food demand (Harikrishnan et al 2010, 2011). Iridoviridae, particularly the infectious spleen and kidney necrosis virus (ISKNV) has been widely reported to cause high mortality among groupers (Chao et al 2004; Eaton et al 2007; Chinchar et al 2009; Ariff et al 2019), and a variety of other freshwater and marine fish species (Fauquet et al 2005; Eaton et al 2008; Murwantoko et al 2009). Previous detections of ISKNV in Malaysia were observed in ornamental fish from South Malaysia (Zainathan et al 2017, 2019), major group of groupers including *E. fuscoguttatus* (Razak et al 2014), trash fish such as *Sardinella* sp., *Decapterus* sp., *Amblygaster leiogaster* and *Decapterus macarellus* from East Malaysia (Lajimin et al 2015). Thus, this study describes the molecular characterization of ISKNV in Malaysian brown-marbled grouper, *E. fuscoguttatus* from West Malaysia.

Material and Method

Sampling. A total of 40 individuals of brown-marbled grouper were sampled from aquaculture farm in West Malaysia in December 2016. The samples were transported on ice back to the Fish Health laboratory in Universiti Malaysia Terengganu. Selected organs including spleen and kidney were harvested from each sample. The sample processing was conducted according to Zainathan et al (2017). The selected organs from pooled samples were cut into small pieces and kept in viral transport media (VTM) into 1.5 mL micro centrifuge tubes. Upon removal, the samples were pooled as a pool of four samples. The DNA extraction was conducted using the GF–1 Viral Nucleic Acid extraction kit according to the protocols provided by the manufacturer.

PCR analysis. The PCR analysis was conducted using primers that were designed by Rimmer et al (2012) using PCR method of Whittington et al (2009) and based on sequence alignment of the MCP gene of Megalocytivirus (GenBank accession number JQ253374.1) (Nolan et al 2015). The primer pair was as follows: forward primer C1105 (5'GGGTTCATCGACATCTCCGCG-3') and reverse primer C1106 (5'-AGGTCGCTGCGCATGCCAATC-3'). A total of 25 µL PCR mixture containing: 12.5 µL MyTaq 2X Master Mix (BIOLINE), 9.0 µL RNase – free water, 0.5 µL (10 µM) of forward primer (C1105) and 0.5 µL (10µM) of reverse primer (C1106) were added to 2.5 µL extracted DNA. The PCR amplification was 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 minute. A final extension at 72°C for 5 minutes terminated the thermal cycling reaction. The amplified PCR products from both reactions were then analysed by electrophoresis (45 minutes at 70 V) on 1.7% (w/v) agarose gel in tris-acetate-EDTA (TAE) buffer and stained with SYBR Safe – DNA Gel Stain (Invitrogen). Synthetic positive control based on the sequence of *Megalocytivirus* Sabah (GenBank accession number JQ253374.1) was used as positive control in the study. The expected bands were excised and purified using GF – 1 Gel and PCR Clean – up (Macherey – Nagel) based on the standard protocols. The DNA sequencing results were used for the phylogenetic analysis. 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 *Megalocytivirus* – related sequences. Finally, the phylogenetic tree was inferred from the MCP gene from all the known Iridoviridae using Molecular Evolutionary Genetics Analysis (MEGA).

Results. The average total length and body weight of the samples were 5.79 cm and 7.02 g respectively (Table 1). A total of 50-92.5% out of 40 samples showed sunken eye, skin ulcer, fin rot, darkened body, abnormal body shape, swollen spleen and kidney (Figure 1). The PCR analysis of pooled samples demonstrated two bands observed at expected size of 430 bp. This result indicated positive results for the presence of *Megalocytivirus* for pooled samples 6 and 9 (Figure 2). Both pooled samples 6 (sample 21-24) and 9 (sample 33-36) demonstrated almost all of the clinical signs consistent with *Megalocytivirus* infection (Table 1).

Table 1

Length, weight and clinical signs observed on *E. fuscoguttatus* samples during the study

Sample	*Length (cm)*	*Weight (g)*	*Clinical sign (external)*					*Clinical sign (internal)*
			Sunken eye	*Skin ulcer*	*Fin rot and ragged*	*Darkened body*	*Abnormal body shape*	*Swollen spleens and kidneys*
1	7.73	8.5	√		√	√		√
2	7.53	8.6	√		√	√	√	√
3	4.14	7.1	√	√	√	√	√	
4	4.40	7.3	√	√			√	
5	5.07	7.4	√	√	√	√	√	√
6	7.28	8.2	√	√	√	√	√	√
7	8.04	8.2	√	√	√	√	√	√
8	6.36	7.8	√	√	√	√		√
9	9.74	8.4	√	√	√	√		√
10	6.04	7.2	√		√	√		√
11	5.31	7.8	√	√	√	√		√
12	4.97	7.3	√	√	√	√		√
13	5.62	7.1		√	√	√		√
14	5.30	7.6		√	√	√	√	√
15	5.62	7.7	√	√	√	√	√	√
16	5.30	8.1	√	√	√	√		
17	5.26	7.6	√	√	√	√		√
18	5.60	7.0	√	√	√	√	√	√
19	4.71	7.0		√	√	√		√
20	6.75	8.4		√	√	√	√	√
21	6.56	8.4	√	√	√	√	√	√
22	4.84	7.5	√	√	√	√	√	√
23	7.81	8.1	√	√	√	√		√
24	7.89	8.2	√	√	√	√		√
25	9.66	9.5	√	√	√	√		
26	5.12	7.6	√			√		
27	7.54	8.2		√	√	√		√
28	4.53	7.0	√	√		√	√	√
29	6.20	7.5	√	√	√	√		√
30	3.41	6.7	√	√	√	√		√
31	4.06	6.9		√		√	√	√
32	2.89	5.8		√	√	√		√
33	7.26	8.3	√	√	√	√	√	√
34	2.59	6.5	√		√			√
35	5.41	7.8	√		√	√	√	√
36	4.84	7.4	√	√	√	√	√	√
37	5.56	7.4	√	√	√		√	√
38	3.63	6.4	√			√		√
39	7.29	8.3	√	√	√	√	√	√
40	3.75	6.5	√	√	√	√	√	√
Total number			33	33	35	37	20	35
%			82.5	82.5	87.5	92.5	50	87.5

Figure 1. (A) Sunken eye, darkened body and abnormal body shape and (B) fin rot and skin ulcer were observed on brown-marbled grouper (*E. fuscoguttatus)* samples during the sampling (arrow).

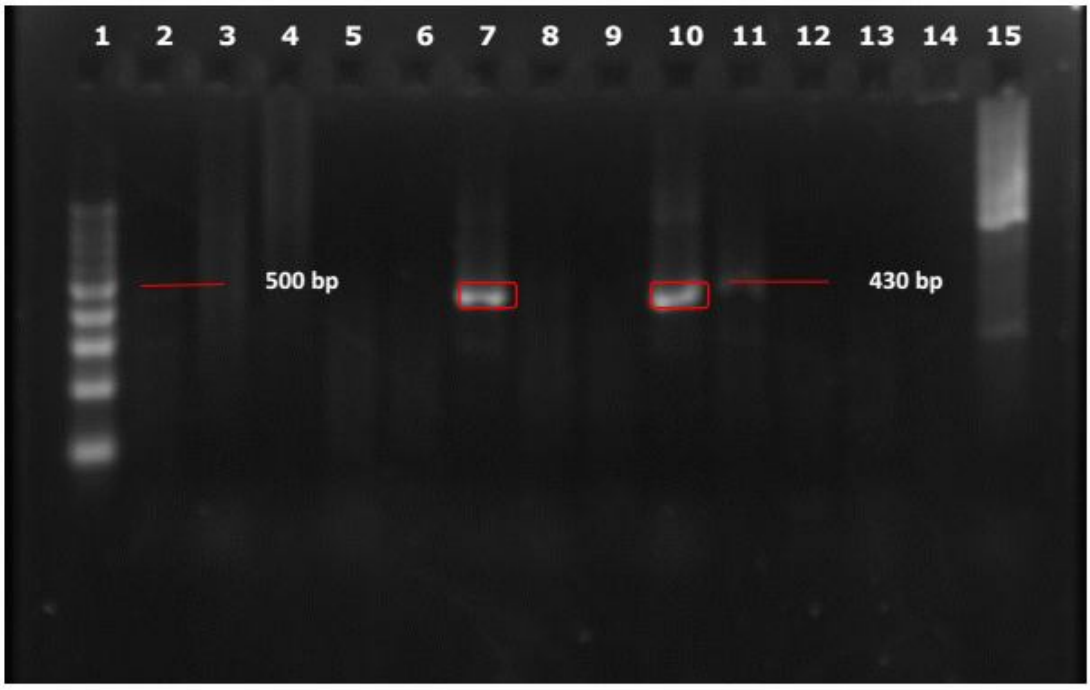

Figure 2. Agarose gel (1.7%) electrophoresis of conventional PCR products for 10 pooled samples of *E. fuscoguttatus* from West Malaysia. Lane 1 (from left) 100 bp DNA ladder, Lane 2-11: pooled samples 1-10, Lane 12: negative control for extraction, Lane 13: negative control for PCR, Lane 15: synthetic positive control, *Megalocytivirus*.

Sequencing and phylogenetic analysis. The sequence of the sample representative from the PCR analysis from 430 bp region of *Megalocytivirus* Banting, was determined for sample 6 (S6). Alignment of the nucleotide sequences of the amplified PCR products confirmed that the sample sent, S6, was from the same member of *Megalocytivirus* genus ISKNV strain and genotype I (Figure 3). DNA sequencing confirmed that S6 showed query coverage at 95% and 99% identity similarities to MCP nucleotide sequences of *Megalocytivirus*. Sample 6 demonstrated 99% similarities to nucleotide sequences of ISKNV (ISKNV KX354220.1; ISKNV KX354219.1; ISKNV KX354218.1; ISKNV KY074549.1; ISKNV KU254555.1 and ISKNV KU254554.1), ISKNV strain RSIV-Ku (GenBank Accession KT781098.1), *Megalocytivirus* Sabah (*Megalocytivirus* Sabah strain BMGIV JQ253374.1; *Megalocytivirus* Sabah strain OSGIV JQ253372.1 and *Megalocytivirus* Sabah strain HGIV JQ253371.1) (Figure 3).

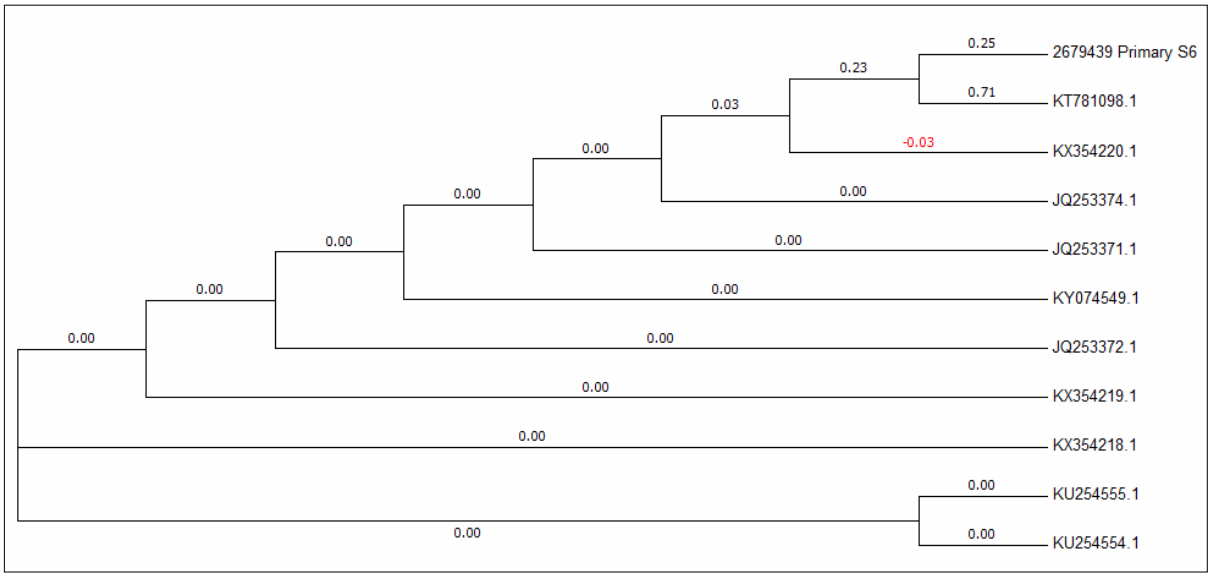

Figure 3. The phylogenetic tree, based on major capsid protein gene sequences of *Megalocytivirus* (ISKNV) detected in *E. fuscoguttatus* from West Malaysia and reference viruses from genus *Megalocytivirus*. The scale bar represents distance values. Note: 2679439 Primary S6 = pooled sample 6, published Genbank sequences: other strains of ISKNV (KX354220.1; KX354219.1; KX354218.1; KY074549.1; KU254555.1 and KU254554.1), RSIV-Ku (KT781098.1) and *Megalocytivirus* Sabah (strain BMGIV JQ253374.1; strain OSGIV JQ253372.1 strain HGIV JQ253371.1).

Discussion. This study describes the detection of *Megalocytivirus* strain ISKNV in brown-marbled grouper, *E. fuscoguttatus* sampled from West Malaysia. The PCR analyses were conducted according to the method by Nolan et al (2015). Out of 40 samples, the PCR analysis demonstrated 2 positive pooled samples (n = 10) for the presence of ISKNV. Sunken eye, skin ulcer, fin rot, darkened body, abnormal body shape and swollen spleen and kidney were observed on the positive samples. Sequence analysis of MCP gene showed 95% query cover and 99% similarities respectively with ISKNV. Moreover, the phylogenetic analysis of MCP gene revealed that viruses from genus *Megalocytivirus* are the closest to the ISKNV complete genome and can be classified into *Megalocytivirus* genotype I.

The positive samples from the study showed clinical signs such as sunken eye, skin ulcer, fin rot, darkened body, abnormal body shape and swollen spleen and kidney. The observed symptoms in the positive samples were similar to previous studies reported in ISKNV infections (Chao et al 2004; Eaton et al 2007; Chinchar et al 2009; Murwantoko et al 2009; Razak et al 2014). Marine ornamental fish affected by ISKNV infection demonstrated similar clinical signs including lethargy and darkened body (Sriwanayos et al 2013). According to Yanong & Waltzek (2010), the fish infected with *Megalocytivirus* develop clinical signs of disease that are non-specific, meaning that they are similar to clinical signs observed with many other diseases which were similar with infected grouper

that include lethargy, loss of appetite, darkening, abnormal swimming (including spinning) or position in the water, increased respiration, distended body cavity (coelomic distension), ulceration, hemorrhages (including pinpoint hemorrhages on the skin and gills), pale gills/anemia, fin erosion, white feces, and heavy mortalities. Infected grouper also exhibited enlargement of kidney, liver and spleen which were similar to the one reported by Chinchar et al (2009), Murwantoko et al (2009) and He et al (2002). The spleen is known to be a major target organ of *Megalocytivirus*-infection, and this organ is often used for a tissue filtrate of virus infected fish to determine viral pathogenicity and fish susceptibility to disease (Chao et al 2004; Mahardika et al 2004; Go & Whittington 2006).

General one-step PCR amplification for *Megalocytivirus* indicated specific band at 430 bp for pooled samples 6 and 9 using pair of primers designed by Rimmer et al (2012). Out of 40 samples, the PCR analysis demonstrated 2 positive pooled samples (n = 10) for the presence of *Megalocytivirus*. Similarly to Razak et al (2014), it was shown that about 33% of the grouper specimens suffered from *Megalocytivirus* infection including *E. fuscoguttatus* and 17.5% of the fish specimens was found to be asymptomatic carriers of the virus. Iridoviridae, particularly the ISKNV has been widely reported to cause high mortality among groupers (Chao et al 2004; Eaton et al 2007; Chinchar et al 2009), and a variety of other freshwater and marine fish species (Fauquet et al 2005; Eaton et al 2008; Murwantoko et al 2009). From a total of 210 ornamental fish samples representing 14 species, ISKNV was detected in 36 samples representing 5 fish species in Peninsular Malaysia (Subramaniam et al 2014). According to Wang et al (2007), based on the sampling of > 1600 marine fishes representing 6 orders, 25 families, and 86 species collected in the South China Sea, 13 cultured fish species (141 fish) and 39 wild fish species (102 fish) were confirmed hosts of ISKNV-like viruses including brown-marbled grouper. The average percentage of infection of ISKNV-like viruses was 14.6% and acute infection of ISKNV-like virus was observed in five cultured species (*E. coioides*, *S. ocellatus*, *P. crocea*, *E. awoara*, and *E. malabaricus*), but latent infection occurred in other species.

Alignment of nucleotide sequences of the amplified PCR products confirmed that both pooled samples 6 and 9 were from the same members of ISKNV as sequence analysis of MCP gene showed 95% query cover and 99% similarities respectively with (ISKNV complete genome and can be classified into *Megalocytivirus* genotype I. The grouper samples had 96.04% nucleotide similarity to genus *Megalocytivirus* and were clustered within the ISKNV (Razak et al 2014). From the finding by Wang et al (2007), the analysis of the MCP, VEGF, Capping, and TNFR genes amplified from 40 of the fish species, they showed 100% nucleotide identity with ISKNV. A total of 40 species of marine fish including the groupers (*E. coioides*, *S. ocellatus*, *P. crocea*, *E. awoara*, and *E. malabaricus*) and other species have been reported as hosts for ISKNV. A recent study conducted in juvenile *Epinephelus* spp. farmed in Aceh, Indonesia showed co-infections of ISKNV with NNV and parasitic infections in several grouper species including brown-marbled groupers (Putra et al 2020).

Conclusions. A total of 40 samples of brown marbled grouper, *E. fuscoguttatus* that were collected from aquaculture farm from West Malaysia has been screened for ISKNV; where the samples (n = 40) were screened for ISKNV strain using one-step PCR. In this study, out of 40 samples, the PCR analysis demonstrated 2 positive pooled samples (n = 10) for the presence of ISKNV. In conclusion, *Megalocytivirus* strain ISKNV from genotype I were present in the samples. Thus, it is recommended that further studies should be carried out so that the farmers can prevent and manage disease outbreaks. The general primer of *Megalocytivirus* is recommended to use because it is able to detect ISKNV strain.

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Conflict of interest. The authors declare that there is no conflict of interest.

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