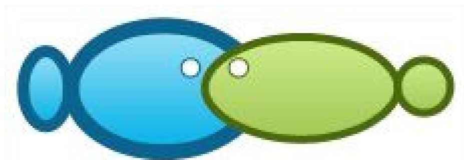

Detection and molecular characterization of *Megalocytivirus* strain ISKNV in freshwater ornamental fish from Southern Malaysia

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Abstract. Malaysia has more than 500 culturists that are involved in the ornamental fish industry for culturing 250 species of 550 varieties including local and exotic species. However, Megalocytiviruses have been associated globally with severe systemic disease and economic loss in farmed food fish and ornamental fish. The aim of this study was to detect the presence or absence of *Megalocytivirus* in a few ornamental species including *Xiphophorus hellerii*, *Xiphophorus maculatus*, *Poecilia sphenops* and *Trichopodus trichopterus* from Southern Malaysia. Out of 195 samples, the PCR analysis demonstrated 26 positive pooled samples (n = 130) for the presence of *Megalocytivirus*. The positive samples did not show any clinical sign of *Megalocytivirus* except pale gills and enlarged liver in *T. trichopterus* and distended body in *X. hellerii*. Sequencing analysis of *Megalocytivirus* major capsid protein (MCP) revealed that the ISKNV strains in this study demonstrated high nucleotide identity to each other and reference ISKNV, 97% to 100%. Based on the phylogenetic tree, the ISKNV strains were closely related to ISKNV and ISKNV strain RSIV-Ku and can be classified into *Megalocytivirus* genotype I.
Key Words: *Megalocytivirus*, ISKNV, genotype I, infection, ornamental fish, Southern Malaysia.

Introduction. The ornamental fish industry is developing in many of the Asian countries due to its high demand in the domestic and international markets. In Malaysia, the ornamental fish culture had started since the 1950s in the states of Johor, Perak and Penang producing fish for exportation purposes to overseas. Currently, in 2015, the production of ornamental fish is recorded to be around 500 million pieces with a valuation of 350 million MYR (Department of Fisheries Malaysia 2016). Frequently exported ornamental fish consists of marine and freshwater species from the families of Cichlidae, Anabantidae, Osteoglossidae, Cyprinidae, Cobitidae, Cyprinodontidae, Poecilidae, Charachidae and Callichthyidae (Department of Fisheries Malaysia 2011). The European Ornamental Fish Import and Exports reported that Malaysia has recorded a valuation of 1,133 million dollars of income through their exportation of ornamental fish to the EU countries (Ornamental Aquatic Trade Association 2015). Although the reports showed a decline in the valuation of ornamental fish in comparison of the previous years, Malaysia is increasing its effort by developing the Economic Transformation Programme and several biosecurity measures to overcome aquaculture issues and to promote regrowth in the ornamental fish industry (Ayson et al 2015).

Among the ornamental fish in Malaysia, *Xiphophorus hellerii*, *Xiphophorus maculatus*, *Poecilia sphenops* and *Trichopodus trichopterus* serve as important species in the trade of ornamental fish. These species are also often associated with the infection of *Megalocytivirus*, which is one of the genera within the family Iridoviridae and can be distinguished by the 120-300 nm diameter of naked or enveloped icosahedral virions (Chinchar et al 2005). Mostly, the occurrence of *Megalocytivirus* infection in ornamental

fish has been reported in the fish imported from Asian countries such as Malaysia and other similar countries to Australia (Rimmer et al 2015; Nolan et al 2015). Until 2013, 97 species of ornamental fish from 111 samples (variety of species) in Australia tested positive for the presence of *Megalocytivirus* (Nolan et al 2015).

However, the extent and intensity of *Megalocytivirus* infection in ornamental fish is still deficient in Malaysia. Previous findings in Malaysia did result in a number of positive samples infected in *X. maculatus* (14/15) from Southern Malaysia, *X. hellerii* (2/15) and negative detection in *T. trichopterus* (0/15) from Northern Malaysia (Subramaniam et al 2014). These samples did not show any sign of distress due to *Megalocytivirus* infection. Ornamental fish infected with *Megalocytivirus* showed signs of stress in their appearances and behaviour which were categorized to be non-specific (Yanong & Waltzek 2013). Asymptomatic signs are a possibility in the ornamental fish cultured in Malaysia, unaware by aqua culturists. Recently, ISKNV was discovered in the tested trash fish used for aquaculture feed in aquaculture farm from East Coast Malaysia (Lajimin et al 2015). The lack of research and knowledge may possess an unknown status regarding the health conditions of these species in Malaysia. A decrease in the trade of ornamental fish has been observed which could be contributed by the presence of *Megalocytivirus* (Department of Fisheries Malaysia 2016).

Genetic variations do exist within the genus *Megalocytivirus* (Oh et al 2006), and based on the phylogenetic tree generated by Wang et al (2007), two clusters were within the genus *Megalocytivirus*. Cluster I included ISKNV and cluster II included mainly RBIV and orange-spotted grouper iridovirus, OSGIV. However, in another study conducted in Malaysia, the phylogenetic analysis revealed three genotypes within the *Megalocytivirus*, and all of the ISKNV strains that were detected from freshwater ornamental fish from Peninsular Malaysia were placed within genotype 1 (Subramaniam et al 2014). Thus, this study describes the detection and genotype of *Megalocytivirus* in freshwater ornamental fish, namely: *X. hellerii*, *X. maculatus*, *P. sphenops* and *T. trichopterus*.

Material and Method

Sampling. A total of 195 samples of ornamental fish (*X. hellerii*, n = 50, *X. maculatus*, n = 45, *P. sphenops*, n = 50 and *T. trichopterus*, n = 50) were collected from an ornamental fish farm from Southern Malaysia in December 2016. The samples were collected randomly from the farm and transported alive to the Aquatic Animal Health laboratory in Universiti Malaysia Terengganu (UMT). The water quality parameters were recorded for each species. Macroscopic examination was recorded and selected organs such as the gill, stomach, intestine, kidney and spleen were isolated from the whole organ. All the organs were kept in viral transport media (VTM) until DNA extraction. All the isolation processes were conducted under the laminar hood in order to prevent any contaminations to the samples.

PCR analysis. Approximately 25 to 50 mg of the samples including gills, stomach, intestine, kidney and spleen were removed from each fish. Upon removal, the samples were pooled as a pool of five samples. The DNA extraction was conducted using the GF-1 Viral Nucleic Acid extraction kit (Vivantis Technologies) according to the protocols provided by the manufacturer. The nested PCR analysis was carried out using primers that were designed by Rimmer et al (2012) using the nested PCR 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 was conducted followed by forward primer, C1073 (5′ – AATGCCGTGACCTACTTTGC – 3′) and reverse primer C1704 (5′ – GATCTTAACACGCAGCCACA – 3′) in the nested PCR reaction. A total of 25 μL PCR mixture containing: 12.5 μL 2× MyTaq Mix (Bioline), 9.0 μL RNase – free water, 0.5 μL (10 μM) C1105 and 0.5 μL (10μM) C1106 was 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 minutes, annealing at 55°C for 30

seconds, extension at 72°C for 1 minute. A final extension of 72°C for 5 minutes terminated the thermal cycling reaction.

The nested PCR analysis was carried out with a total of 25 µl PCR mixture containing: 12.5 µL 2× MyTaq Mix (Bioline), 9.0 µL l RNase – free water, 0.5 µL (10 µM) C1073 and 0.5 µL (10 µM) C1074 was added to 2.5 µL PCR product. The amplification was conducted with the following program: 10 minutes at 95°C, followed by 35 cycles of denaturation at 95°C for 30 minutes, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute and a final extension of 72°C for 5 minutes. 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 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

Water quality. The water quality parameters such as temperature, dissolved oxygen, salinity, and pH obtained from the tanks holding the species are as followed. All the parameters including temperature, salinity, pH and dissolved oxygen tested were within the required range for ornamental fish except for the high dissolved oxygen for X. *hellerii* culture (Table 1).

Table 1

Water quality parameters taken during sampling for *Xiphophorus hellerii*, *Xiphophorus maculatus*, *Poecilia sphenops* and *Trichopodus trichopterus*

Water quality parameters	*X. maculatus*	*X. hellerii*	*P. sphenops*	*T. trichopterus*
Temperature (°C)	26.8	28.0	25.8	27.3
Dissolved oxygen (mg L^{-1})	7.5	13.8	9.21	7.03
Salinity (ppt)	2.23	1.81	0.14	0.05
pH	7.74	7.57	7.58	8.58

Macroscopic examination. The samples of *X. maculatus* and *P. sphenops* did not show any clinical signs (Figure 1A & 1B). Whereas, *T. trichopterus* demonstrated pale gills (Figure 2A) and enlarged liver (Figure 2C); and *X. hellerii* showed distended body (Figure 2C).

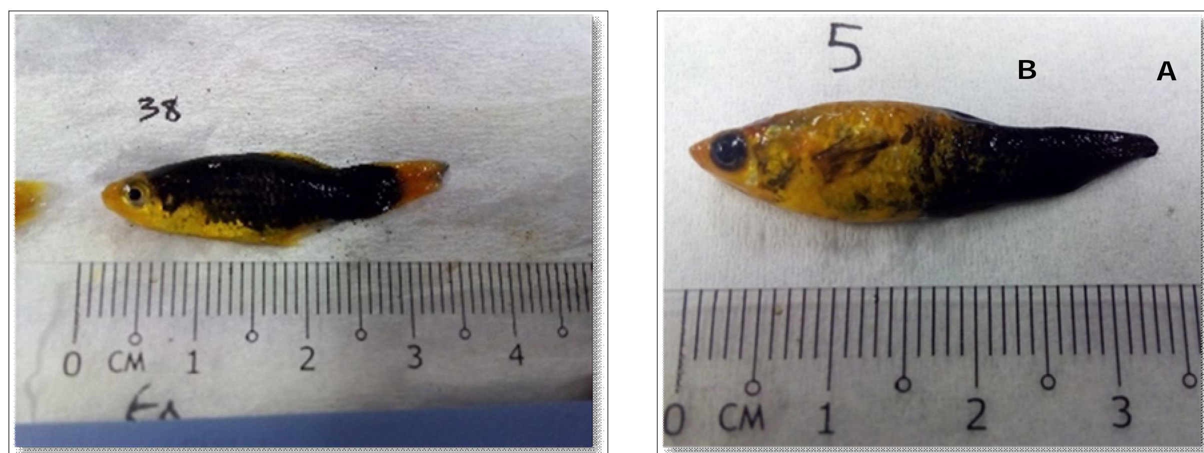

Figure 1. No clinical signs were observed on *X. maculatus* (A) and *P. sphenops* (B).

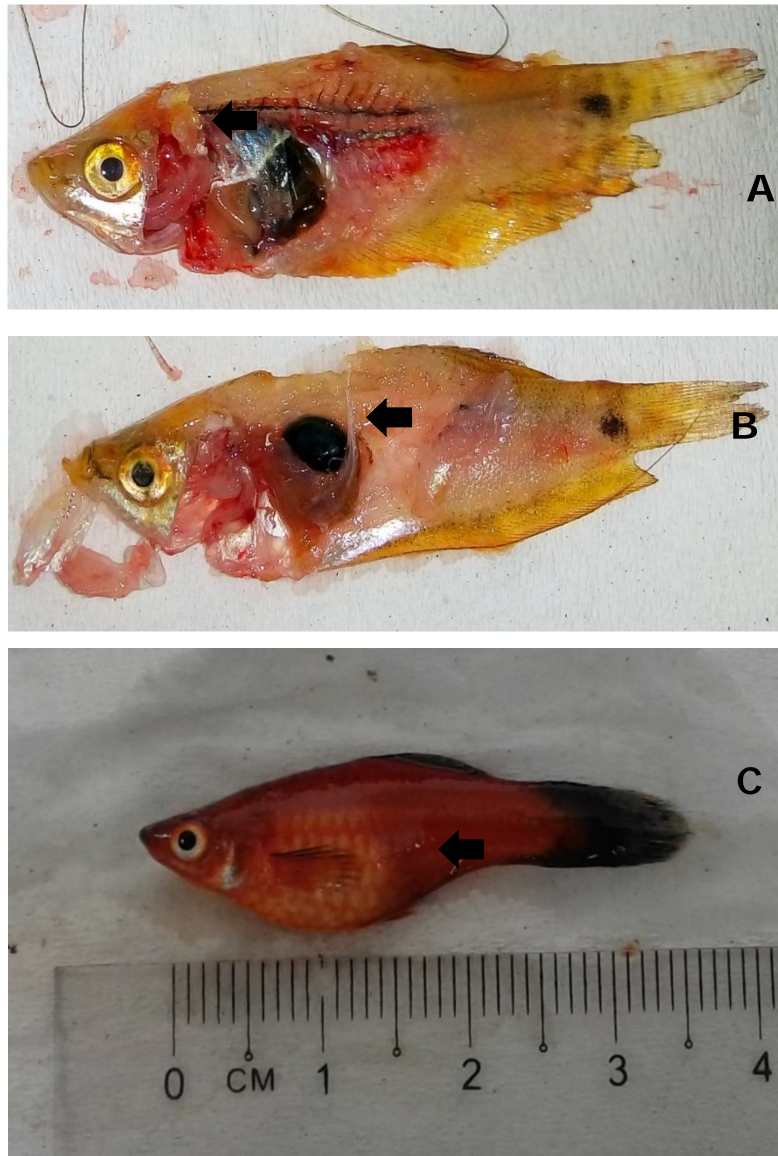

Figure 2. Pale gills (A) and enlarged liver (B) were observed in *T. trichopterus* (arrow), whereas, distended body was observed in *X. hellerii* (C) (arrow).

Primary PCR analysis. The conventional PCR analysis demonstrated positive result for 5 pooled samples (sample 1, 2, 3, 7 and 10) of *T. trichopterus* for the presence of *Megalocytivirus.* Conventional PCR result produced amplicons of the expected size at 430 bp in the reactions (Figure 3A). Whereas, two pooled samples of X. *hellerii* (pooled sample 5 and 6) showed positive results for the presence of *Megalocytivirus* (Figure 3B). Sample 1 from T. *trichopterus*, samples 5 and 6 from X. *hellerii* were sent for sequencing analysis to determine the genotype of *Megalocytivirus*.

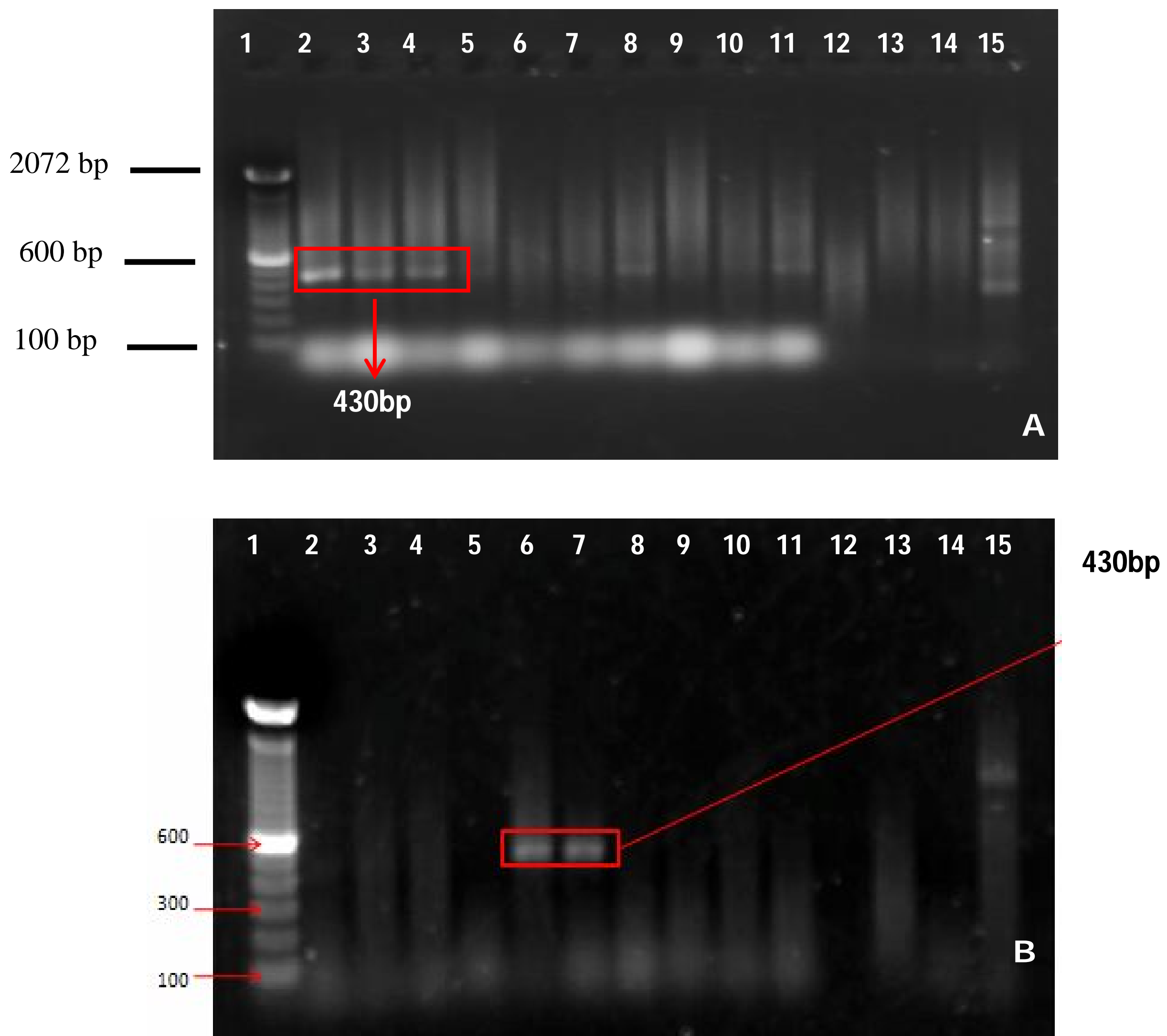

Figure 3. Agarose gel (1.7%) electrophoresis of conventional PCR products for 10 pooled samples of *T. trichopterus* (A) and *X. hellerii* (B). Lane 1: 100bp ladder, Lane 2-11: pooled sample 1 to 10, Lane 12: PCR negative, Lane 13: negative control for extraction, Lane 14: negative control for PCR, and Lane 15: synthetic positive control, *Megalocytivirus*.

Nested PCR analysis. The secondary reaction of conventional PCR analysis of pooled samples of gills, stomach, intestine, kidney and spleen demonstrated positive results for the presence of *Megalocytivirus*. The nested PCR analysis produced amplicons of the expected size (167 bp) for *X. maculatus* (n = 8 pooled samples) (Figure 4A), *X. hellerii* (n = 3 pooled samples), *P. sphenops* (n = 8 pooled samples) (Figure 4B). Sample 2 and 4 from *X. maculatus* and *P. sphenops*, respectively were excised and sent for sequencing analysis to determine the genotype of *Megalocytivirus*. Two samples of *X. hellerii* (sample 8 and 9) positive for the presence of *Megalocytivirus* were chosen for further sequencing analysis. In total, 26 pooled samples (n = 130) of ornamental fish were found to be positive for the presence of *Megalocytivirus* based on both PCR analyses.

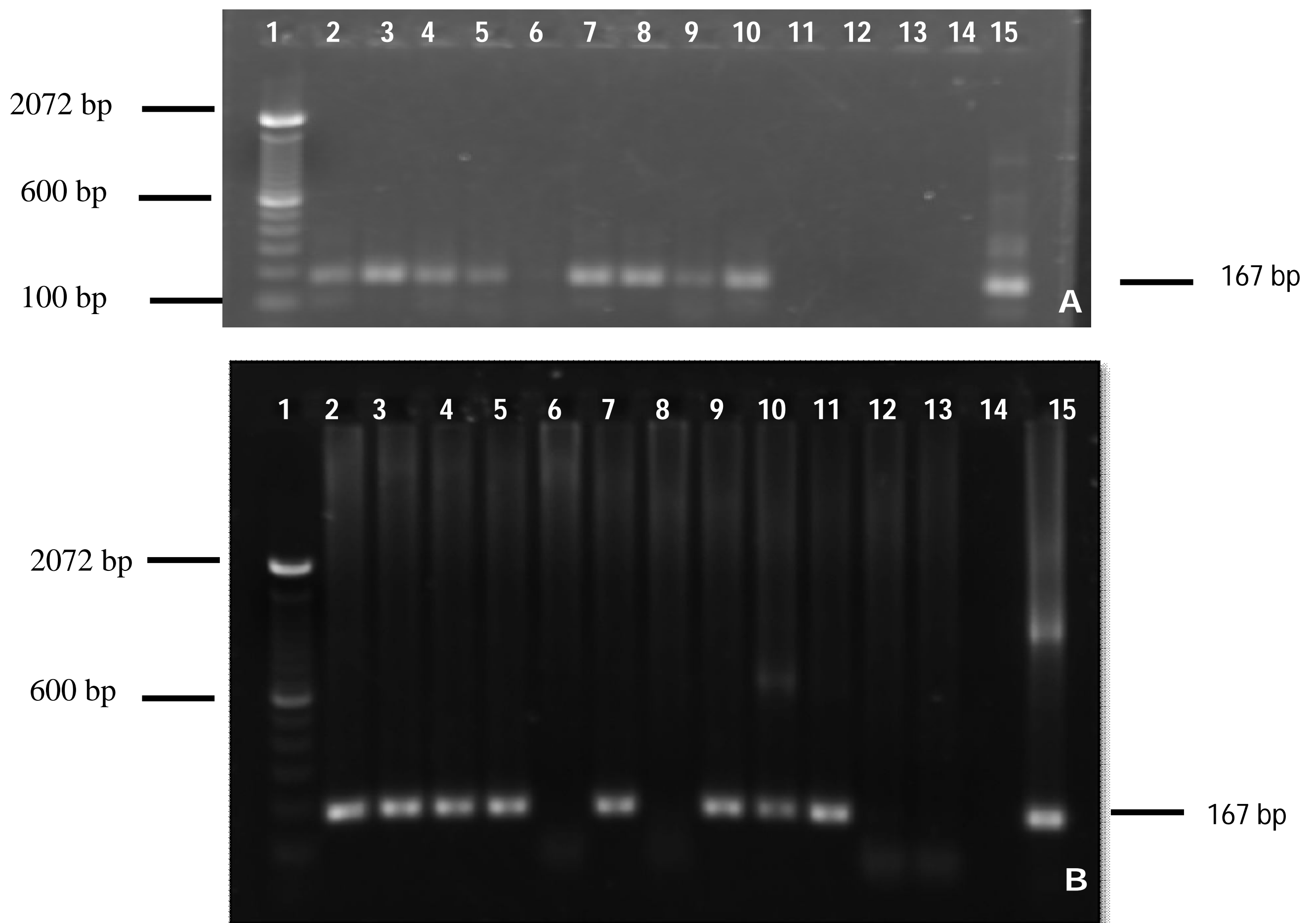

Figure 4. Agarose gel (1.7%) electrophoresis of conventional PCR products for 10 pooled samples of *X. maculatus* (A) and *P. sphenops* (B). Lane 1: 100bp ladder, Lane 2-11: pooled sample 1 to 10, Lane 12: PCR negative, Lane 13: negative control for extraction, Lane 14: negative control for PCR, and Lane 15: synthetic positive control, *Megalocytivirus*.

Sequence and phylogenetic analysis. The sequence of the sample representatives from both primary and nested PCR analysis were determined based on major capsid protein (MCP) region of *Megalocytivirus*. The samples were sent for sequencing and phylogenetic analysis was conducted to verify any similarities and variation of the *Megalocytivirus* strains using the NCBI Blast. Alignment of the nucleotide sequences of the amplified PCR products confirmed that the samples sent were from the same member of *Megalocytivirus* genus ISKNV strain. The strains detected in this study were identical to each other with nucleotide sequence identity that ranged from 97 to 100% (Table 2). Similarly, the nucleotide sequence identity between the ISKNV strains in this study and reference ISKNV was within 97 to 100%. Based on the generated phylogenetic tree (Figure 5), the reference ISKNV and all ISKNV strains that were detected in Southern Malaysia were grouped within genotype 1. The ISKNV strains from this study demonstrated high similarity to ISKNV strain RSIV-Ku (KT781098.1) based on phylogenetic tree.

Table 2

Percentage of nucleotide sequence identity of the major capsid protein gene between strains of *Megalocytivirus* (ISKNV) detected in this study and reference viruses from genus *Megalocytivirus* retrieved from Genbank database

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*
1		99	97	97	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99	99
2			97	100	99	99	99	100	100	100	100	100	100	100	100	100	100	100	100	100
3				98	97	99	97	97	97	97	97	97	97	97	97	97	97	97	97	97
4					98	97	100	100	100	100	100	100	100	100	100	100	100	100	100	100
5						98	99	99	99	99	99	99	99	99	99	99	99	99	99	99
6							99	99	99	99	99	99	99	99	99	99	99	99	99	99
7								100	100	100	100	100	100	100	100	100	100	100	100	100
8									100	99	99	99	99	99	100	99	99	100	100	100
9										99	99	99	99	90	100	99	100	100	100	100
10											99	99	99	99	99	99	99	99	99	99
11												99	99	99	99	99	99	99	99	99
12													99	99	99	99	99	99	99	99
13														99	99	100	99	99	99	99
14															99	99	99	99	99	99
15																99	99	100	100	100
16																	99	99	99	99
17																		99	99	99
18																			100	100
19																				100
20																				

1 = ISKNV (*X. hellerii*/Johor/2016/XH5), 2 = ISKNV (*X. hellerii*/Johor/2016/XH6), 3 = ISKNV (*X. hellerii*/Johor/2016/XH8), 4 = ISKNV (*X. hellerii* /Johor/2016/XH9), 5 = ISKNV (*X. maculatus*/Johor/2016/XM2), 6 = ISKNV (*P. sphenops*/Johor/2016/PS4), 7 = ISKNV (*T. trichopterus*/Johor/2016/TT1), 8 = ISKNV RSIV-Ku (KT781098.1), 9 = ISKNV (KX354220.1), 10 = *Megalocytivirus* Sabah (JQ253371.1), 11 = *Megalocytivirus* Sabah (JQ253374.1), 12 = *Megalocytivirus* Sabah (JQ253369.1), 13 = ISKNV (KY074549.1), 14 = *Megalocytivirus* Sabah (JQ253366.1), 15 = *Megalocytivirus* Sabah OSGIV (JQ253372.1), 16 = *Megalocytivirus* Sabah (JQ253368.1), 17 = ISKNV strain DGIV (AB666344.1), 18 = ISKNV (KX354219.1), 19 = ISKNV (KX354218.1), 20 = ISKNV *Tilapia* (KU254555.1).

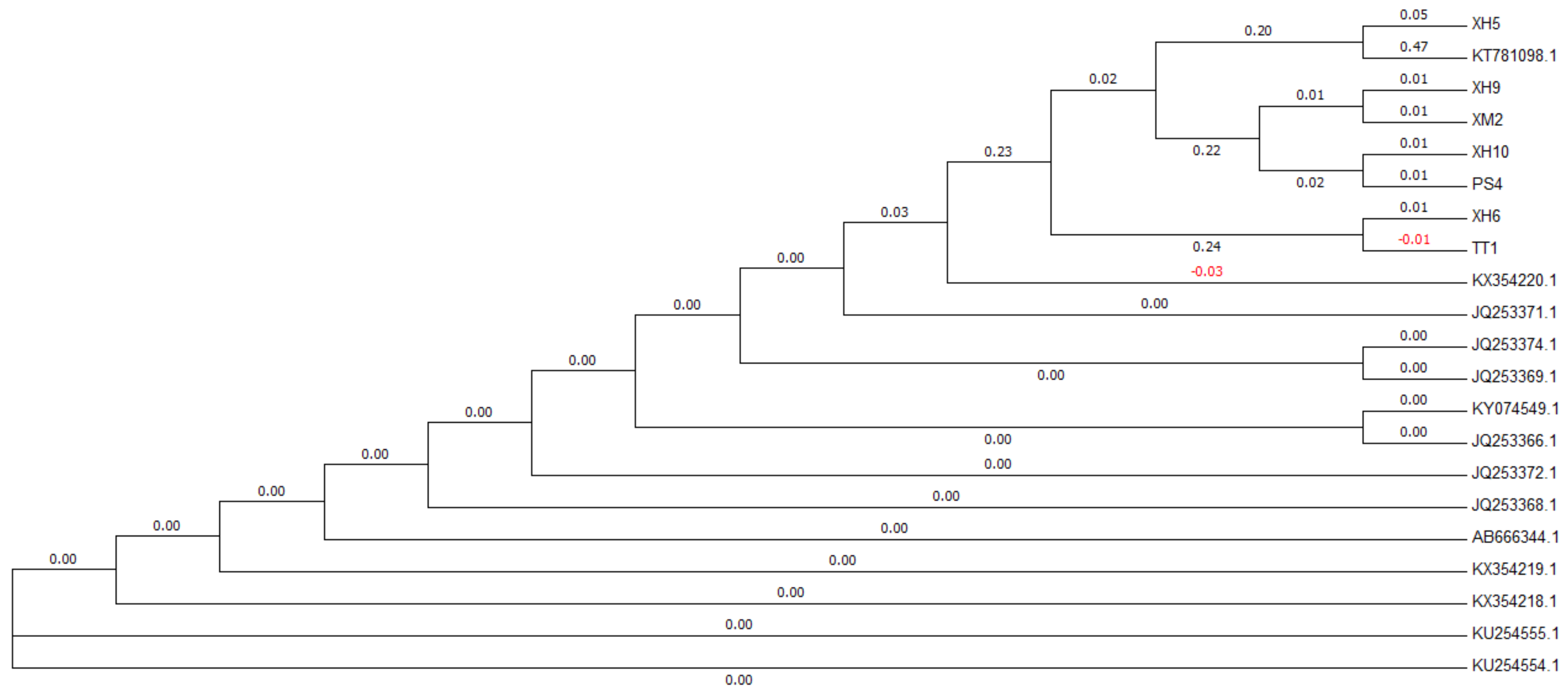

Figure 5. The phylogenetic tree, based on major capsid protein gene sequences of *Megalocytivirus* (ISKNV) detected in ornamental fish from Southern Malaysia and reference viruses from genus *Megalocytivirus*. The scale bar represents distance values. Note: XH = *X. hellerii*, XM = *X. maculatus*, TT = *T. trichogaster*, PS = *P. sphenops*, published Genbank sequences: ISKNV (KX354220.1), *Megalocytivirus* Sabah (JQ253371.1), *Megalocytivirus* Sabah (JQ253374.1), *Megalocytivirus* Sabah (JQ253369.1), ISKNV (KY074549.1), *Megalocytivirus* Sabah (JQ253366.1), *Megalocytivirus* Sabah OSGIV (JQ253372.1), *Megalocytivirus* Sabah (JQ253368.1), ISKNV strain DGIV (AB666344.1), ISKNV (KX354219.1), ISKNV (KX354218.1) and ISKNV *Tilapia* (KU254555.1).

Discussion. This study describes the detection of *Megalocytivirus* ISKNV strain in freshwater ornamental species from Southern Malaysia; *X. hellerii, X. maculatus, P. sphenops* and *T. trichopterus*. In this study, *X. maculatus* and *P. sphenops* sampled appeared to be healthy and no gross signs were observed in the internal organs of the fish. Similarly, *X. maculatus* tested for the prevalence of *Megalocytivirus* in Australia were carried out on healthy platy samples that did not show any gross signs (Mohr et al 2015). In a study in Germany, only hypertrophic cells or the presence of inclusion bodies were observed under histopathological examinations in *X. maculatus* while examinations for gross clinical signs did not show any skin lesions or parasites attached to their bodies, internally and externally (Jung–Schroers et al 2016). Furthermore, a report by Jeong et al (2008) stated that 7 out of 10 ornamental fish tested positive for *Megalocytivirus* did not display any clinical signs. Whereas, in another study, positive PCR samples in five fish species, namely common platy, pearl gourami, zebrafish, swordtail and ram cichlid did not exhibit any clinical signs of ISKNV disease (Subramaniam et al 2014). The pattern of ISKNV infection on *X. maculatus* suspected for *Megalocytivirus* in all cases shows that affected freshwater ornamental fish including *X. maculatus* and *P. sphenops* could be in a long-term carrier state (Jeong et al 2008; Paperna et al 2001; Subramaniam et al 2014).

Whereas, pale gills and enlarged liver were observed in positive samples of *T. trichopterus* and distended body in *X. hellerii*. The clinical signs observed in this study are similar to other studies reported in *Megalocytivirus* (Yanong & Waltzek 2010; Subramaniam et al 2014). Distended stomach or body cavity is one of the common clinical sign for *Megalocytivirus* (Yanong & Waltzek 2010). He et al (1998) reported 100% mortality in farmed mandarin fish, *Siniperca chuatsi* and the affected fish exhibited clinical signs such as anorexia, abnormal swimming, faded body pigmentation and pale gills (Subramaniam et al 2014). During necropsy, *Megalocytovirus*-infected fish are often observed to cause damage to many internal organs (tissue death, necrosis) especially the spleen (which is often enlarged), kidney, and liver (Yanong & Waltzek 2010).

A total of 26 pooled samples (n = 130) samples of *X. hellerii*, *X. maculatus*, *P. sphenops* and *T. trichopterus* sampled in December 2016 demonstrated positive result for the presence of *Megalocytivirus*. The result from this study is consistent with several other papers which indicated positive infection of *Megalocytivirus* in these freshwater ornamental fish (Subramaniam et al 2014; Nolan et al 2015; Rimmer et al 2015; Mohr et al 2015; Jung-Schroers et al 2016). The study on *Megalocytivirus* within ornamental fish in Malaysia by Subramaniam et al (2014) had 14 out of 15 *X. maculatus* and *X. hellerii* (2/15) samples tested positive for *Megalocytivirus*. However, the *T. trichopterus* in this study demonstrated positive results for the presence of *Megalocytivirus*, which was absent in the study by Subramaniam et al (2014). Previous studies have shown that 100% of *P. sphenops* samples demonstrated positive result in the presence of *Megalocytivirus* (Jeong et al 2008). The presence of *Megalocytivirus* was confirmed by PCR in 3 species of freshwater ornamental livebearer fish which included *X. maculatus*, *Poecilia latipinna* and *X. hellerii* (Nolan et al 2015). *Megalocytivirus* has been reported to infect the susceptible gourami species including three-spot gourami (*T. trichopterus*), pearl gourami (*Trichopodus leerii*), silver gourami (*Trichopodus microlepis*), dwarf gourami (*Trichogaster lalius*) and also thick-lipped gourami (*Colisa labiosa*) (Paperna et al 2001; Gibson-Kueh et al 2003; Go & Whittington 2006; Jeong et al 2008).

All the positive samples were from the same member of *Megalocytivirus* under ISKNV strain and shared high nucleotide identity, 97 to 100% and fit under genotype 1. These findings are similar to the study by Subramaniam et al (2014). Sequence analysis of MCP gene showed that all the strains detected in the study were closely related to the reference ISKNV and shared 99.8 to 100% of nucleotide sequence identity (Subramanian et al 2014). In addition, MCP sequences of *Megalocytivirus* in ornamental fish samples from 2002-2010 had almost complete identity to each other (99.9-100%) and to ISKNV providing further evidence that these Megalocytiviruses were genetically distinct (Go & Jeffrey 2015; Go et al 2016). The sequencing results showed that the samples were closely related with infectious spleen and kidney necrosis virus (ISKNV) strain RSIV-Ku, complete genome (GenBank accession number KT781098.1) and belonged to Genotype 1 according to phylogenetic tree. Based on the phylogenetic tree generated by Wang et al

(2007), there were two clusters within the genus *Megalocytivirus*. Cluster I included ISKNV and cluster II included mainly RBIV and orange-spotted grouper iridovirus, OSGIV. However, in another study conducted in Malaysia, the phylogenetic analysis revealed three genotypes within genus *Megalocytivirus*, and all the ISKNV strains that were detected from freshwater ornamental fish from Peninsular Malaysia were placed within genotype 1 along with reference ISKNV, DGIV, MCIV and ALIV (Subramaniam et al 2014). Genotype 2 mainly included RSIV detected in red sea bream from Japan, RBIV detected in striped beakfish from China and GSDIV detected in brown-spotted grouper from Thailand (Subramaniam et al 2014). Genotype 3 includes TRBIV and FLIV that were detected in flatfish from China and Korea, respectively.

Conclusions. In conclusion, a total of 130 samples of freshwater ornamental fish were positive for the presence of *Megalocytivirus* and confirmed as ISKNV strain, genotype 1. All the positive samples showed high nucleotide identity with reference ISKNV strain and ISKNV strain RSIV-Ku. The spread of *Megalocytivirus* must be minimized due to high mortality of infected fishes and lack of technology in development of rapid, sensitive and specialized viral diagnostic tests for early detection. A new health certification is required by the Malaysian government for affected fish species for maintaining Malaysia's wild fish and farmed fish populations as for being free from the Infectious spleen kidney necrosis virus disease. Thus, it is recommended that further studies should be carried out so that the farmers can prevent and manage disease outbreaks.

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