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MOLECULAR SCREENING OF INFECTIOUS SPLEEN AND KIDNEY NECROSIS VIRUS IN FOUR SPECIES OF MALAYSIAN FARMED ORNAMENTAL FISH

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ABSTRACT

Malaysia is the 8th largest world producer of freshwater ornamental fish. Ornamental fish are commonly associated with Megalocytiviruses infection, which has led to severe diseases and economic loss. The purpose of this study was to detect the presence or absence of Infectious Spleen and Kidney Necrosis Virus (ISKNV) in four ornamental fish species namely *Xiphophorus maculatus, Poecilia reticulata, Trichogaster leeri* and *Apistogramma ramirezi* from Malaysia. A total of 175 samples were analysed using PCR analysis for detection of ISKNV. The PCR analysis demonstrated 22 positive pooled samples (n = 110) for the presence of *ISKNV*. No clinical signs were observed in positive samples except darkened body in *X. maculatus*. 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 (96% to 100%). Based on the phylogenetic tree, the ISKNV strains were closely related to reference ISKNV and can be classified into *Megalocytivirus* genotype I.

Key words: *Megalocytivirus*, ISKNV, genotype I, ornamental fish, Malaysia

INTRODUCTION

The ornamental fish industry has been recorded as the fastest growing industry in the agricultural sector particularly in many of the Asian countries due to its high demand in the domestic and international markets (Department of Fisheries, Malaysia 2016). Malaysia was enlisted as the fifth largest producer of export valued at US$22.62 million in 2014 (Dey, 2016). Recent reports from the Department of Fisheries, Malaysia presented that Malaysia is the 8th largest world producer of ornamental fish. In 2015, Malaysia produced 383 million pieces of ornamental fish and 371 million bunches of aquatic plants. It was reported that more than 70% of ornamental species produced are exported (Othman *et al.,* 2017). Currently, there are 620 ornamental fish farm/exporters in Malaysia (Department of Fisheries, Malaysia, 2017). The United States appears to be the single largest importer and the European Union is the largest market for ornamental fish (Ng, 2016). The major groups of farm-bred ornamental fish are gold fish, barbs, tetras, swordtails, mollies, gourami, guppies, cichlids, angelfish, fighting fish and platyfish.

Four species of ornamental fish, *Xiphophorus maculatus (X. maculatus)*, *Poecilia reticulata (P. reticulata)*, *Trichogaster leeri (T. leeri)* and *Apistogramma ramirezi (A. ramirezi)* were identified as important species in Malaysia in the trade of ornamental fish (DOF, 2017). The occurrence of *Megalocytivirus* infection has been reported mostly in the fish imported from Singapore, Malaysia and Sri Lanka at export locations (Nolan *et al.,* 2015). *Megalocytivirus* infection in Malaysian ornamental fish has been reported by Subramaniam *et al.* (2014) and Zainathan *et al.* (2017), including the detection of *Megalocytivirus* in ornamental fish exported to

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overseas at border quarantine facilities. Freshwater ornamental species from Malaysia such as *Xiphophorus hellerii, Xiphophorus maculatus, Poecilia sphenops* and *Trichopodus trichopterus* demonstrated positive results for the presence of *Megalocytivirus* with non-specific clinical signs such as pale gills, enlarged liver and distended body (Zainathan *et al.,* 2017). Subramaniam *et al.* (2014) also proved that the ISKNV-infected fish from four different families from major ornamental fish breeding states in Peninsular Malaysia were asymptomatic and appeared clinically healthy.

Genetic variations have been demonstrated within the genus *Megalocytivirus* (Oh *et al.,* 2006). Wang *et al.* (2007) demonstrated two genotypes within *Megalocytivirus* including: cluster I: ISKNV and cluster II: RBIV and OSGIV. However, previous phylogenetic analyses discovered three genotypes within the *Megalocytivirus*, including the ISKNV strains that were detected from Peninsular Malaysia, placed within genotype 1 (Subramaniam *et al.,* 2014). Sequencing analysis of *Megalocytivirus* major capsid protein (MCP) demonstrated that the ISKNV strains in different species of ornamental fish in Malaysia demonstrated high nucleotide identity to each other and reference ISKNV, 97% to 100% (Zainathan *et al.,* 2017). The ISKNV strains were closely related to ISKNV and ISKNV strain RSIV-Ku and can be classified into *Megalocytivirus* genotype I (Zainathan *et al.,* 2017). The impact and extent of disease caused by *Megalocytivirus* in different species of ornamental fish Malaysia is unknown and further exacerbated by lack of knowledge on the host range, geographical distribution and the differences between strains if any. Thus, this study describes the molecular screening of Infectious Kidney and Spleen Necrosis Virus (*Megalocytivirus*) in four species of farmed freshwater ornamental fish in Malaysia based on pooled samples: *Xiphophorus maculatus, Poecilia reticulata, Trichogaster leeri* and *Apistogramma ramirezi*.

MATERIALS AND METHODS

Sampling

A total of 175 samples of ornamental fish (*X. maculatus,* n=50, *P. reticulata*, n=25, *T. leeri*, n=50 and *A. ramirezi*, n=50) were collected from an ornamental fish farm from Southern Malaysia in December 2017. The samples were collected randomly from the farm and transported alive to the Aquatic Animal Health laboratory in Universiti Malaysia Terengganu (UMT). The macroscopic examination was conducted including the weight, length and clinical signs of the samples. The selected organs such as the gill, stomach, intestine, kidney and spleen were isolated. The sample processing was conducted according to Zainathan *et al.* (2017). The organs were kept in viral transport media (VTM) until DNA extraction.

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, where 1 pool consisted of 5 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' – GGGTT CATCGACATCTCCGCG – 3') and reverse primer C1106 (5' – AGGTCGCTGCGCATGCCAATC – 3') for the primary reaction was conducted followed by forward primer, C1073 (5' – AATGCCGTGA CCTACTTTGC – 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 (10 µM) 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 seconds, 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 seconds, 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) 7.0.9.

RESULTS AND DISCUSSION

Gross observation

None of the samples demonstrated any gross observation except for *X. maculatus*. A total of 11 samples of *X. maculatus* demonstrated darkened body (Figure 1). *X. maculatus* sampled in Germany demonstrated anorexia, lethargy, swollen gills, and skin ulcerations (Jung-Schroers *et al*., 2016). In contradiction to this study, the *X. maculatus* samples tested positive for the presence of ISKNV without any clinical signs (Jeong *et al.,* 2008; Subramaniam *et al.,* 2014; Mohr *et al.,* 2015; Zainathan *et al.,* 2017). Ornamental fish infected with *Megalocytivirus* developed non-specific clinical signs, which could be similar to clinical signs observed with many other diseases. These 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 (Yanong & Waltzek, 2013). The affected red piranhas (*Pygocentrus nattereri*) in Brazil also demonstrated non-specific clinical signs such as loss of appetite, irregular swimming and positioning on the surface of the water, lethargy and respiratory movements; typical of ISKNV infection (Cardoso *et al.,* 2017).

PCR analyses

The conventional PCR analysis demonstrated positive results for 3 pooled samples (3, 4 and 5 (n=15) (Figure 2) of *A. ramirezi* and 1 pooled sample (n=5) of *T. leeri* for the presence of *Megalocytivirus* at expected size of 430 bp. Whereas, no bands were visualized at the primary reaction for the other species including *X. maculatus* and *P. reticulata*. Sample 3 from *A. ramirezi* was sent for sequencing analysis to determine the genotype of *Megalocytivirus*.

The secondary reaction of conventional PCR analysis of pooled samples demonstrated positive results for the presence of *Megalocytivirus* at the expected size of 167 bp. These results include *X. maculatus* (n=7 pooled samples) (Figure 3A), *P. reticulata* (n=1 pooled samples) (Figure 3B), *T. leeri* (n=5 pooled samples) (Figure 3C) and *A. ramirezi* (9 pooled samples) (Figure 3D). Sample 2, 7 and 10 from *T. leeri, P. reticulata* and *X. maculatus*, respectively, were excised and sent for sequencing analysis to determine the genotype of *Megalocytivirus*. In total, 22 pooled samples (n=110) of ornamental fish were found to be positive for the presence of *Megalocytivirus* based on both PCR analyses.

A total of 22 pooled samples of *X. maculatus, P. reticulata, T. leeri* and *A. ramirezi* demonstrated positive results for the presence of ISKNV. The result from this study is consistent with previous studies which showed *Megalocytivirus* infection in freshwater ornamental fish (Subramaniam *et al.,* 2014; Nolan *et al.,* 2015; Rimmer *et al.,* 2015; Mohr *et al.,* 2015; Jung-Schroers *et al.,* 2016). In Malaysia, ISKNV was present in ornamental fish species from four different families' including common platy (Poeciliidae), swordtail (Poeciliidae), pearl gourami (Osphronemidae), ram cichlid (Cichlidae) and

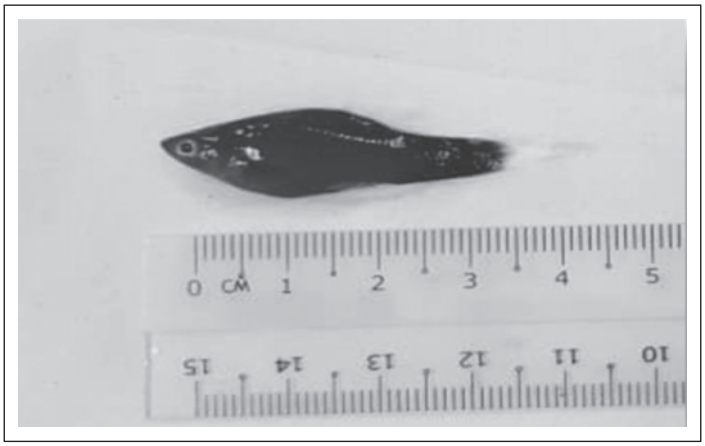

Fig. 1. Darkened body was observed in *Xiphophorus maculatus*.

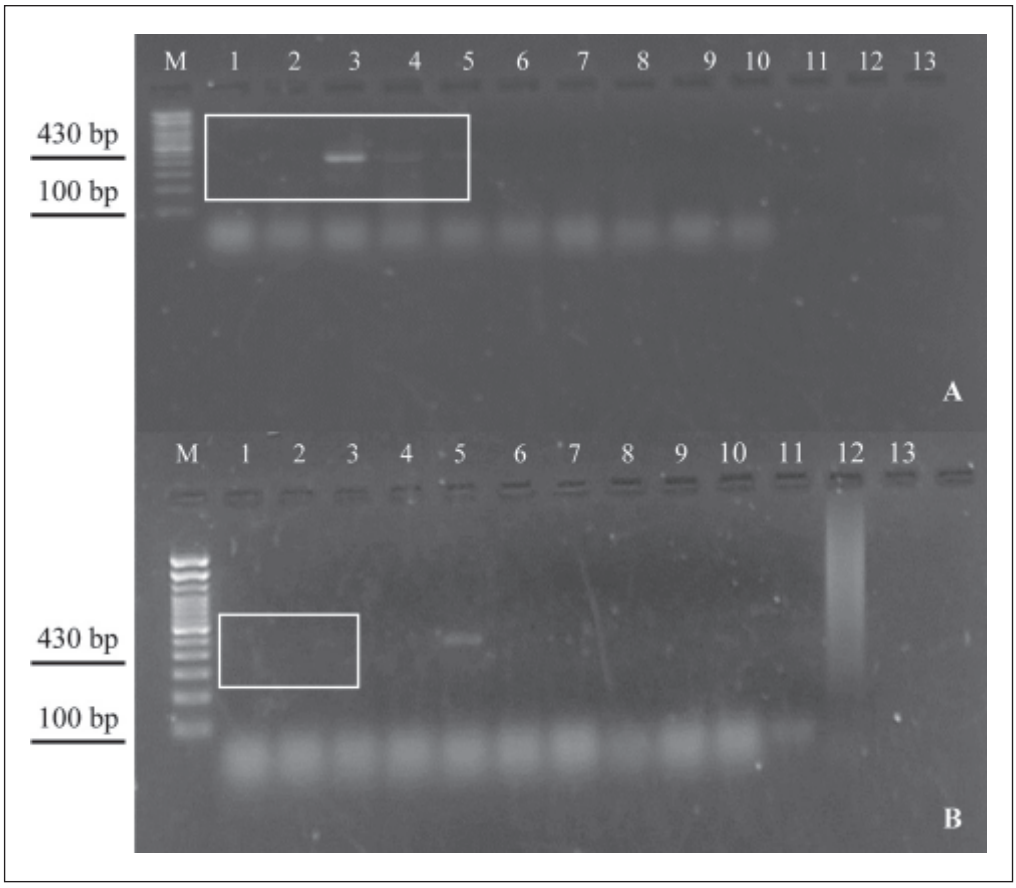

Fig. 2. Conventional Primary PCR amplification of *A. ramirezi* (A) and *T. leeri* (B) from Southern Malaysia according to the primer that designed by Rimmer *et al.* (2012). M: 100 bp DNA ladder as a molecular weight marker, Lane 1-10: Sample 1 to 10, Lane 11-12: Water as a PCR negative control for reaction, Lane 13: Synthetic positive control, *Megalocytivirus*.

zebrafish (Cyprinidae) (Subramaniam *et al.,* 2014). Out of 32 positive samples, 12 positives were *A. ramirezi,* 14 positives of *X. maculatus* and 4 samples of *T. leeri* (Subramaniam *et al.,* 2014). Yanong and Waltzek (2010) listed *A. ramirezi* as susceptible to *Megalocytivirus* infection. Higher number of *X. maculatus* samples (n=40) were positive for *Megalocytivirus* based on Zainathan *et al.* (2017). Nine samples from ornamental fish of the species *Poecilia reticulata* and *Pygocentrus nattereri* demonstrated positive results for *Megalocytivirus* (Cordoso *et al.,* 2017). *T. leeri* has been shown to be positive for *Megalocytivirus* using PCR assays (Go *et al.,* 2006; Jeong *et al.,* 2008, Subramaniam *et al.,* 2014). Go *et al.* (2006) reported that 8% of *T. leeri* (n=39) tested positive for DGIV, using PCR analysis on samples of fish. These results demonstrate that the presence of ISKNV is high in healthy ornamental fish in Malaysia and may exist as carriers due to the absence of clinical signs.

Sequence and phylogenetic analysis

The sequencing and phylogenetic analysis were conducted to verify any similarities and variation of the *Megalocytivirus* based on the major capsid protein (MCP) region. 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 96% to 99% (Table 1). Similarly, the nucleotide sequence identity between the ISKNV strains in this study and reference ISKNV was within 96 to 100%. Based on the phylogenetic tree (Figure 4), the reference ISKNV and all ISKNV strains that were detected in this study were grouped within genotype 1. The ISKNV strains from this study demonstrated high similarity to ISKNV strain RSIV-Ku (KT781098.1) and ISKNV major capsid protein based on phylogenetic tree.

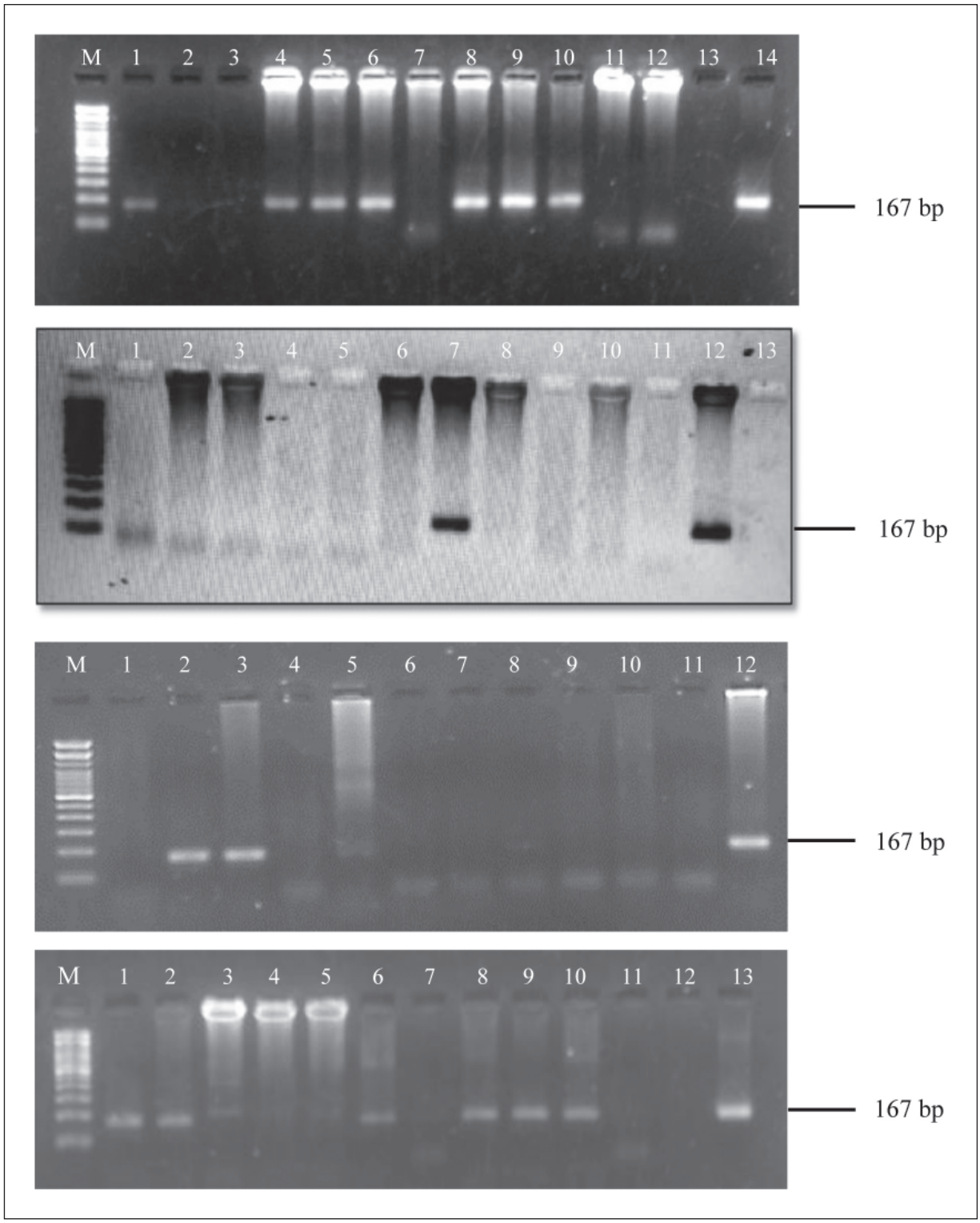

Fig. 3. Nested PCR amplification of pooled samples in *X. maculatus* (A), *P. reticulata* (B), *T. leeri* (C) and *A. ramirezi* (D. M: 100 bp ladder (A-D). (A) Lane 1 – 10: Samples 1 to 10. Lane 11-12: Negative control. Lane 13: Extracted negative control and Lane 14: Synthetic positive control, *Megalocytivirus*. (B) Lane 1-5: Sample 1-5 pooled gills, Lane 6-10: Sample 6-10 pooled internal organs, Lane 11: Negative control and Lane 12: Synthetic positive control, *Megalocytivirus*. (C) Lane 1 – 10: samples 1 to 10. Lane 12: Negative control and Lane 13: Synthetic positive control, *Megalocytivirus*. (D) Lane 1-10: Sample 1 to 10, Lane 11: Negative control and Lane 13: Synthetic positive control, *Megalocytivirus*.

Table 1. 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
1		96	96	96	97	97	97	97	97	97
2			97	98	98	98	98	98	98	98
3				99	99	99	99	99	99	99
4					99	99	99	100	99	99
5						99	99	99	99	99
6							100	100	99	100
7								100	99	99
8									99	99
9										99
10										

1 = ISKNV (*P. reticulata*/Johor/2017/PR7), 2 = ISKNV (*X. maculatus*/Johor/2017/XM10), 3 = ISKNV (*T. leeri*/Johor/2017/TL2), 4 = ISKNV (*A. ramirezi*/Johor/2017/AR3), 5 = ISKNV (KY440040), 6 = ISKNV (KX354220.1), 7 = ISKNV RSIV-Ku (KT781098.1), 8 = *Anabas Iridovirus* (AB930172), 9 = *Megalocytivirus* Sabah (JQ253374.1), 10 = ISKNV strain DGIV (AB666344.1).

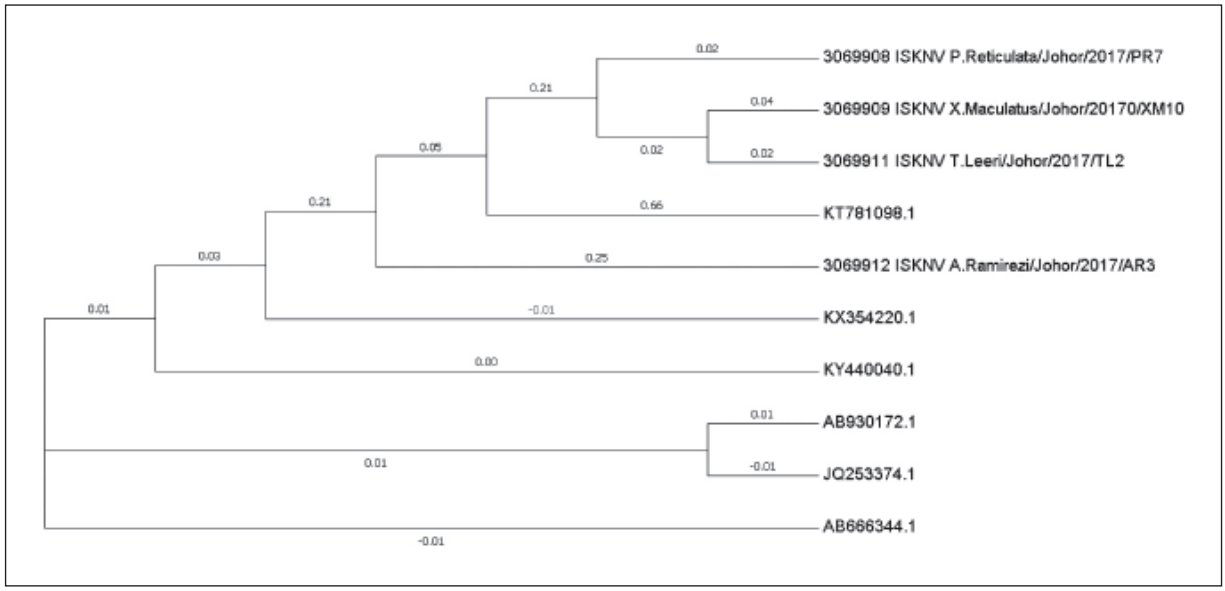

Fig. 4. The phylogenetic tree, based on MCP gene sequences of *Megalocytivirus* (ISKNV) detected in ornamental fish from Malaysia and reference viruses from genus *Megalocytivirus*.
The scale bar represents distance values. Note: XM = *X. maculatus*, PR = *P. reticulata*, TL = *T. leeri*, AR = *A. ramirezi*, published Genbank sequences: ISKNV (KY440040), ISKNV (KX354220.1), ISKNV RSIV-Ku (KT781098.1), *Anabas Iridovirus* (AB930172), *Megalocytivirus* Sabah (JQ253374.1) and ISKNV strain DGIV (AB666344.1).

The positive samples were from the same member of *Megalocytivirus* under ISKNV strain and shared high nucleotide identity, 96 to 100% and fit under genotype 1. These findings are similar to the studies by Subramaniam *et al.* (2014) and Zainathan *et al.* (2017) in different species of ornamental fish in Malaysia. The phylogenetic analysis indicated that the strains in both study were closely related to the reference ISKNV; 99.8 to 100 (Subramaniam *et al.,* 2014) and 97% to 100% nucleotide sequence identity (Zainathan *et al.,* 2017). MCP sequences of *Megalocytivirus* in ornamental fish samples had almost complete identity to each other (99.9-100%) and to ISKNV providing further evidence that these *Megalocytiviruses* were genetically distinct (Go *et al.,* 2016). The sequencing and phylogenetic analyses showed that the samples were genetically related with ISKNV strain RSIV-Ku, complete genome (GenBank accession number KT781098.1) and belonged to Genotype 1. Song *et al.* (2008) revealed three clusters: genotype I including nine Japanese isolates, thirteen Korean isolates, one Chinese isolate, one Thailand isolate and one South China Sea isolate; genotype II including five freshwater fish isolates in Southeast Asian countries and Australia; and genotype III mainly consisted

of flatfish isolate in Korea and China (Song *et al.,* 2008). This proposes that viruses belonging to the genotype I are widely distributed among various fish species in many Asian countries (Song *et al.,* 2008). Furthermore, in 2009, detailed study of ISKNV infection in *P. kauderni* with the presence and systemic distribution of enlarged virus-infected cells, demonstrated a close relationship with ISKNV (Weber *et al.,* 2009).

CONCLUSIONS

A total of 175 samples of farmed freshwater ornamental fish from four species were positive for the presence of *Megalocytivirus* and confirmed as ISKNV strain, genotype 1. Thus, it is evident that these species: *X. maculatus, P. reticulata, T. leeri* and *A. ramirezi* are susceptible to ISKNV infection. It is vital to tackle this issue to confirm the source of infection. In order to do so, an epidemiology study is needed to confirm the source of infection, to further determine the genotype and phenotype of ISKNV in Malaysia proceeded with early stages of vaccine development.

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