A MEGALOCYTIVIRUS INVOLVED IN DARK BODY DISEASE OF CLIMBING PERCH (*Anabas testudineus*) CULTURED IN VIETNAM

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ABSTRACT

Dark body disease has become a serious problem for climbing perch, Anabas testudineus, cultured in freshwater intensive farming systems in Vietnam. Outbreaks of the disease occur when the fish are about 20-65 days old with 40 - 100% mortality. The main clinical signs of the disease are dark body, haemorrhagic or yellowish liver, and little or no food in the intestines. The disease has recently been attributed to a virus of unknown type. Electron microscopy analysis detected the virus in liver and kidney tissues but not in brain tissues of infected fish. The virion has a symmetric shape with a size of about 150-160nm and is surrounded by a capsid layer. Using primers designed to sequence the major capsid protein (MCP) gene of red seabream iridovirus (RSIV) yielded PCR products from DNA of infected fish. In a phylogenetic analysis based on the partial MCP sequence, the virus clustered with viruses in the genus Megalocytivirus of the family Iridoviridae and it is closely related to infectious spleen and kidney necrosis virus (ISKNV). This study indicated the involvement of a Megalocytivirus in the dark body disease of climbing perch cultured in Vietnam.

Keywords: Dark body disease, climbing perch, Anabas testudineus, Megalocytivirus, Iridovirus

Megalocytivirus liên quan tới bệnh đen thân trên cá rô đồng nuôi thâm canh (*Anabas testudineus*) ở Việt Nam

TÓM TẮT

Bệnh đen thân đã và đang được xem là một trong những mối nguy đối với nghề nuôi cá rô đồng thâm canh ở Việt Nam. Bệnh thường xuất hiện ở cá trong giai đoạn từ 20 đến 65 ngày tuổi và tỷ lệ cá chết do bệnh gây ra từ 40 đến 100%. Dấu hiệu bệnh lý điển hình của cá bị bệnh là toàn thân cá chuyển màu đen, gan xuất huyết hoặc chuyển màu nhợt nhạt, ruột không có hoặc có rất ít thức ăn. Bệnh được xác định là có liên quan tới tác nhân vi rút. Kết quả phân tích dưới kính hiển vi điện tử đã phát hiện thấy sự có mặt của các tiểu phần vi rút trong gan và thận của cá bệnh nhưng không phát hiện thấy trong tổ chức não. Vi rút có dạng hình khối đa diện đường kính khoảng 150-160 nm và có vỏ capsid bao quanh. Vi rút cũng có thể nhận biết bằng kỹ thuật PCR khi sử dụng cặp mồi đặc hiệu cho protein MCP của Iriovirus RSIV gây bệnh trên cá tráp đỏ. Kết quả phân tích cây phả hệ dựa trên sự tương đồng của đoạn gen MCP đặc trưng cho nhóm Iridovirus đã xác định được vi rút ở cá rô đồng bị bệnh đen thân thuộc giống Megalocytivirus, họ Iridoviridae và gần gũi với vi rút gây hoại tử thận và lách truyền nhiễm ISKNV. Kết quả nghiên cứu này đã khẳng định sự liên quan của Megalocytivirus đối với bệnh đen thân trên cá rô đồng nuôi thâm canh ở Việt Nam.

Từ khóa: Bệnh đen thân, cá rô đồng, *Anabas testudineus*, Megalocytivirus, Iridovirus

1. INTRODUCTION

Climbing perch, *Anabas testudineus* (Bloch 1792), is considered a promising freshwater fish species for intensive farming in Vietnam (Le, 2003). However, outbreaks of dark body disease have recently been occurring in a large majority of farms. The disease appears when the fish are about 20 to 65 days old and usually results in 40-70% mortality, but mortality can be as high as 100%.

The main clinical signs of dark body disease are dark colour on the whole body, haemorrhagic or yellowish liver, and little or no food in the intestines. Moribund fish usually float on the water surface for 1-2 days before they die. The causative agent of the disease has recently been reported as a virus but it has been not classified into any group (Dang *et al.,* 2013). In this study, this virus was further identified using electron microscopy and PCR analyses, and classsified into the genus Megalocytivirus of the family Iridoviridae using phylogenetic analysis.

2. MATERIALS AND METHODS

2.1. Sampling

Both apparently healthy and dark body-diseased climbing perch were collected from intensive cultured fish farms throughout the country, including Hai Duong and Bac Giang provinces in Northern Vietnam, and Hau Giang and Dong Thap provinces in Southern Vietnam, during 2012 and 2013. Liver, kidney, and brain tissues were sampled from the diseased and the healthy fish for both ultrastructural and molecular analyses. These studies were conducted in the authors' laboratories in Vietnam and Japan.

2.2. Ultrastructural analysis

Liver, kidney, and brain tissues were immediately fixed in 2.5% glutaraldehyde solution diluted in 0.1 M cacodylate buffer solution (pH = 7.2 – 7.4) and stored at 4°C. The fixed samples were then transferred on ice to the National Institute of Hygiene and Epidemiology, Vietnam for electron microscopy (EM) analysis. A total of 134 test samples were collected from dark body diseased fish, including 51 liver samples, 45 kidney samples, and 38 brain samples, and 10 control samples were collected from the livers, kidneys and brains of healthy fish to be used for EM studies.

2.3. Detection of virus by PCR

In order to determine the presence of virus particles in the dark body diseased fish, we looked for the presence of the major capsid protein (MCP) gene. DNA was extracted from the liver and kidney samples according to methods described by Green and Sambrook (2012). Two sets of primers were used (MCP1 and MCP2; Table 1). MCP1 primers were similar to the sequences of specific primers used to amplify 429 bp of the RSIV MCP gene (Dang *et al.*, 2008). MCP2 primers were designed based on the full 1362 bp length of RSIV MCP (Accession No: AB109371.1) to amplify a longer partial MCP gene (986 bp) from nucleotide 91 to nucleotide 1076. The MCP1 primers were used to determine the presence of the virus while the MCP2 primers were used to amplify 986 bp of the MCP gene for use in sequence identity and phylogenetic analysis. The β-actin gene was amplified as an internal control using specific β-actin primers (Table 1).

Cycling parameters were 1 min denaturation step (95°C), 30 sec annealing (55°C), and 30 sec extension (72°C) for 30-35 cycles. The reactions began with a denaturation step (5 min at 95°C) and ended with a 5 min extension step at 72°C. PCR products were analyzed in 1% agarose gels containing ethidium bromide and visualized under UV light.

Table 1. PCR primers used in this study

Primer name	Primer sequence	PCR product	Source
MCP1	F 5'- CCCTATCAAAACAGACTGGC -3'	429 bp	Lua *et al*., 2008
	R 5'- TCATTGTACGGCAGAGACAC -3'		
MCP2	F 5'- AATGCCGTGACCTACTTTGC -3'	986 bp	This study
	R 5'- TCGACAGATGTGAAGTAGTCTA -3'		
β-actin	F 5'- TTTCCCTCCATTGTTGGTCG -3'	200 bp	Lua *et al*., 2008
	R 5'- GCGACTCTCAGCTCGTTGTA -3'		

2.4. Cloning, sequencing and phylogenetic analysis

Positive PCR products amplified by both MCP1 and MCP2 primer sets were ligated into the pGEM-T easy vector (Promega, Madison, USA) and then transformed into ECOS JM 109 competent cells. The colonies were sequenced via the Automated DNA Sequencer using the Big Dye Terminator from ABI Cycle Sequencing Kit. The sequences of MCP1 PCR products were used to confirm the name of the virus by assignment of gene identities with known sequences available in GenBank based on BLAST analysis. The sequences of MCP2 PCR products were used for sequence identity analysis and phylogenetic analysis. DNA sequences were aligned using ClustalW, and the phylogenetic relationship between MCP gene sequences was determined using the Neighbour Joining method in the MEGA 6 program with bootstrap values of 1000 replicates.

The sequences of 16 other isolates of the family Iridoviridae used to construct the phylogenetic tree were obtained from GenBank: FV3 (Frog virus 3, FJ459783.1); GIV (Grouper iridovirus, JF264365.1); IIV-9 (Invertebrate iridescent virus 9, AF025774.1); IIV-31 (Invertebrate iridescent virus 31, AB686463.1); IIV-3 (Invertebrate iridescent virus 3, NC008187.1); LCDV1-RC (Lymphocystis disease virus 1, China strain, EF103188.1); LCDV1 (AY823414.1); RBIV (Rock bream iridovirus, HQ105005.1) and RSIV (AB109371.1); DGIV (Dwarf gourami iridovirus, AB109369.1); MCIV (Murray cod iridovirus, AY936203.1); ISKNV (Infectious spleen and kidney necrosis virus, AF370008.1); ISKNV-QY (HQ317460.1); ISKNV-NZh (HQ317461.1); ISKNV-DW (HQ317465.1); and ISKNV-Seabass (AB666338.1).

3. RESULTS

3.1. Ultrastructural analysis

Electron micrographs of almost all the liver and kidney samples (47 of 51 liver samples and 38 of 45 kidney samples) from the dark body diseased fish showed degeneration and necrosis. Degenerated mitochondria formed spaces/cavities in which no pathogens could be observed. However, virus particles were observed in 27 of the 47 degenerated liver sections (Fig. 1A) and in 21 of the 38 degenerated kidney sections (Fig. 1B). Brain tissues from diseased fish showed no necrosis or viral particles and showed no differences from the controls (data not shown).

The viral particles detected in the liver and kidney cells were hexagonal, symmetrical with a diameter of about 150-160 nm, and were surrounded by a capsid layer. Virus particles were observed in the cytoplasm and not in the nucleus of the cells (Fig. 2A). Some of the virus particles were incomplete (black arrows in Fig. 2B). Complete virus particles had electron-dense cores that contained the genetic materials of the virus while incomplete virus particles consisted of only a capsid layer.

3.2. PCR virus detection

The MCP1 primer set yielded a product of the expected size (~429 bp) from DNA extracted from liver and kidney tissues from dark body diseased fish collected throughout Vietnam. No PCR products were obtained from the brain tissues of any of the diseased fish or from any tissues collected from the healthy controls (Table 2 and Fig. 3). The same sized PCR products were obtained from RSIV DNA as a positive control and the β-actin gene products confirmed the quality of the extracted DNA.

Some of the MCP1 PCR products were sequenced. A search of GenBank showed that the sequences were 95-100% identical to MCP sequences of viruses belonging to the family Iridoviridae, such as RSIV, RBIV, DGIV, ISKNV and GIV (data not shown). These findings indicate that the virus belongs to the family Iridoviridae. Accordingly, it was named *Anabas testudineus iridovirus* or ATIV.

3.3. Sequencing and phylogenetic analysis of MCP gene

The MCP gene of ATIV was amplified by PCR using MCP2 primers and sequenced. The sequence (986 bp) was registered to DDBJ (DNA Data Bank of Japan) with the Accession No. AB930172. The ATIV detected in this study were identical to MCIV, DGIV and ISKNV strains and amongst each other with nucleotide sequence identity that was ranging from 99% to 100% (Table 3).

A phylogenetic tree of the MCP gene of ATIV and MCPs of 16 other iridoviral isolates (Fig. 4) showed five major clusters with high bootstrap values indicating support for 5 genera within the family Iridoviridae.

Based on the phylogenetic tree generated, ATIV was clustered into the genus *Megalocytivirus* together with RSIV isolated from red seabream (*Pagrus major)* (Kurita *et al.*, 2002); RBIV isolated from rock bream (*Oplegnathus fasciatus*) (Do *et al.*, 2004); DGIV isolated from dwarf gourami (*Colisa lalia*) and MCIV isolated from Murray cod (*Maccullochella peelii peelii*) (Go *et al.*, 2006); and ISKNV strains isolated from brackish water and ornamental fish (Subramaniam *et al.*, 2014). This information along with the high homology between ATIV and DGIV and between MCIV and ISKNV-types (99% - 100%) shown in Table 3, suggest that ATIV should be considered as a Vietnamese type of ISKNV.

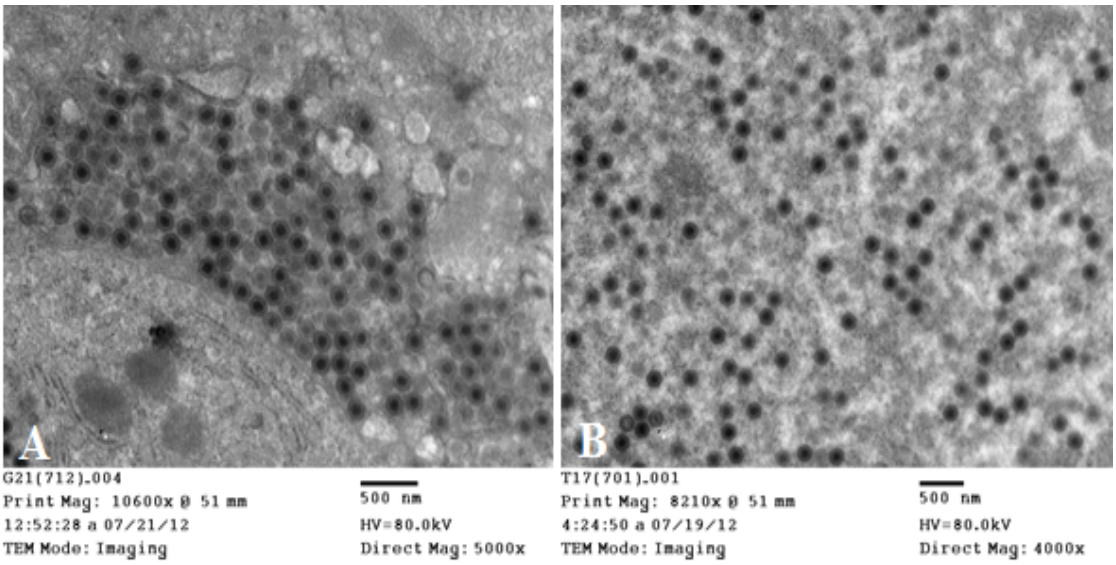

Figure 1. Virus particles in liver cells (A) and kidney cells (B)

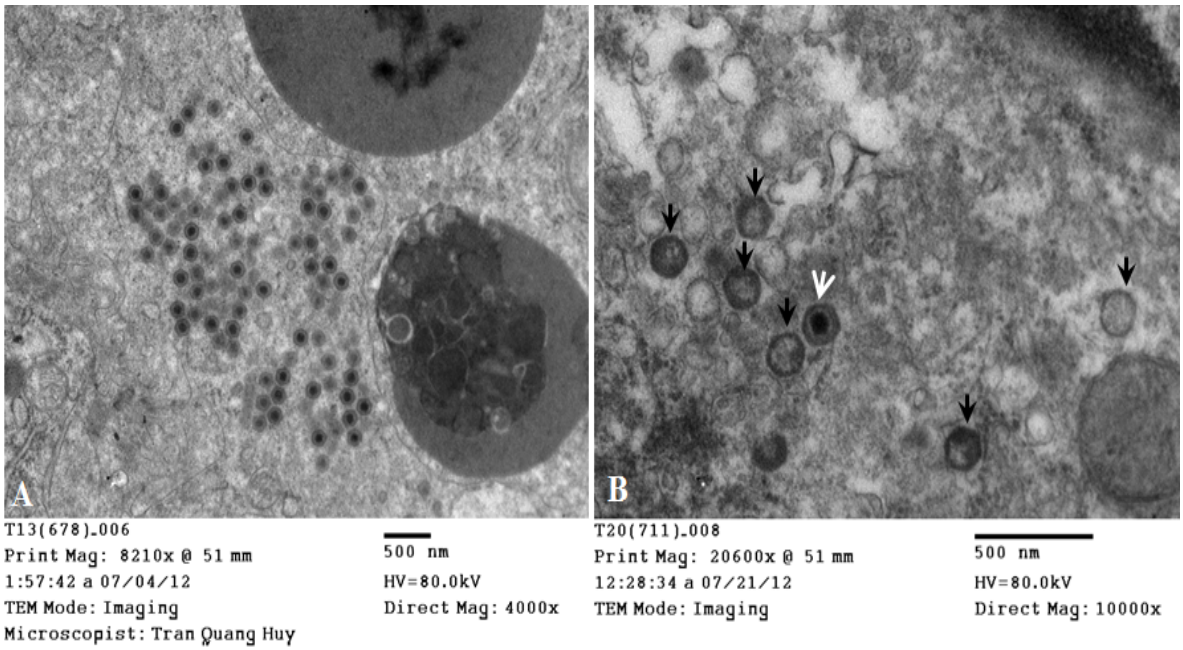

Figure 2. Virus particles in the cytoplasm of a kidney cell (A) of dark body diseased climbing perch

Note: Complete (white arrows) and incomplete (black arrows) virus particles inside a cell (B)

Table 2. PCR analysis utilizing primer set MCP1 on diseased fish and controls

Tissue	No. of PCR positive samples/No. of samples examined					%	Control (Healthy fish)
	Province						
	Hai Duong	Bac Giang	Hau Giang	Dong Thap	Total		
Liver	21/27	13/18	11/15	6/10	51/57	73	0/10
Kidney	-	-	5/10	6/10	11/20	55	0/10
Brain	0/15	0/18	0/10	0/10	0/53	0	0/10

3. DISCUSSION

Ultrastructural analysis by EM has long been used to detect and describe viruses (Doane and Anderson, 1987; Goldsmith and Miller, 2009). This method is particularly important in the diagnosis of unknown diseases. Ultrastructural analysis has been used to diagnose viral infections in humans (Biel *et al.*, 2004; Chua *et al.*, 2007), terrestrial animals (Hyatt and Selleck, 1996; Bayer-Garner, 2005), and aquatic animals such as fish and shrimp (Do *et al.*, 2004). In this study, EM observations revealed the changes in liver and kidney tissues, including symptoms of degeneration and necrosis, as well as revealed the presence of viral particles in the livers and kidneys of diseased fish (Figures 1 & 2).

The virus was also detected in liver and kidney tissues of the dark body diseased fish by PCR analysis using MCP1 primers (Fig. 3). The MCP1 primers are specific to the MCP gene of RSIV, a virus belonging to the family Iridoviridae. This indicated that the virus found in the liver and kidney of diseased climbing perch should be grouped with this family and it was named as ATIV in this study.

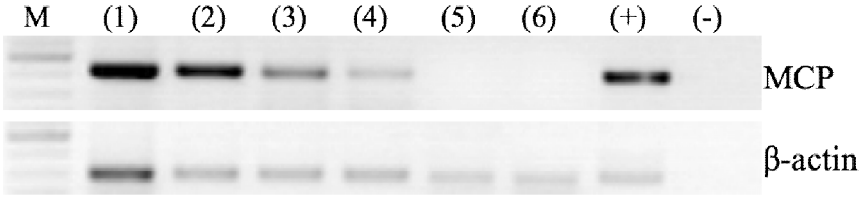

Figure 3. PCR analysis of the MCP gene of the virus isolated from liver and kidney tissues of dark body diseased and healthy climbing perch

Note: Lanes 1, 2: liver of diseased fish; lanes 3, 4: kidney of diseased fish; lane 5, 6: liver and kidney of healthy fish, respectively; (+): DNA extracted from RSIV-infected red seabream spleen (Positive control); (--): Distilled water (Negative control))

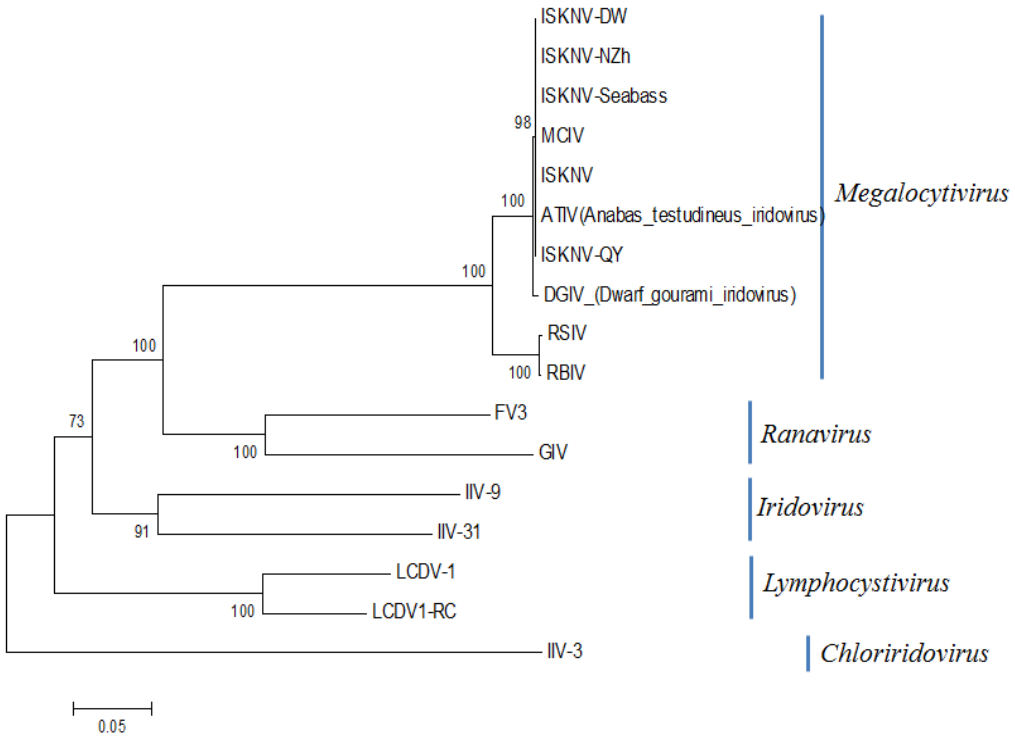

Figure 4. Phylogenetic analysis of ATIV and 16 other isolates of the family *Iridoviridae* based on partial MCP sequences

Note: The numbers at each node indicate bootstrapped percentages values.

Iridoviruses are nuclear and cytoplasmic large DNA viruses whose genomes are encapsidated by an icosahedral shell ranging between 120 and 200 nm in diameter and comprised of about 50 kDa MCP (Wolf, 1988; He *et al.,* 2002). In agreement with these characteristics, ATIV has a symmetric shape and a size of about 150 - 160 nm (Fig. 2). Major capsid proteins account for about 45% of the total protein of the iridoviral isolates and are needed for the cleavage and packaging of viral DNA to form viable virions (Williams, 1996). The MCP gene is considered the most suitable gene for detection and measurement of Iridoviruses (Caipang *et al.,* 2003; Dang *et al.,* 2008). The MCP gene has also been used to analyse the phylogenetic relationships of Iridoviruses (Lu et al., 2005; Go *et al.,* 2006; Imajoh *et al.,* 2007).

Table 3. Percentages of sequence identity of the MCP gene between ATIV and reference viruses from genus *Megalocytivirus*

	1	2	3	4	5	6	7	8	9	10
1		99	100	100	100	100	100	99	95	95
2			99	99	99	99	99	99	95	95
3				99	99	99	99	99	95	95
4					100	100	100	99	99	99
5						100	100	99	95	95
6							100	99	95	95
7								99	95	95
8									95	95
9										99
10										

Note: 1 = ATIV; 2 = DGIV (AB109369.1); 3 = MCIV (AY936203.1); 4 = ISKNV (AF370008.1); 5 = ISKNV-Seabass (AB666338.1); 6 = ISKNV-DW (HQ317465.1); 7 = ISKNV-NZh (HQ317461.1); 8 = ISKNV-QY (HQ317460.1); 9 = RSIV (AB109371.1); and 10 = RBIV (AB109371.1)

In the phylogenetic tree (Fig. 4), the MCPs of different iridoviruses formed 5 clusters. One cluster included IIV-3, which is a Chloriridovirus (Delhon *et al.*, 2006); a second cluster included IIV-9 and IIV-31, which are iridoviruses (Chinchar *et al.*, 2005; Wong *et al.*, 2011); a third cluster included LCDV-1 and LCDV1-RC which are lymphocystiviruses (Tidona and Darai, 1997; Zhang *et al.*, 2004); a fourth cluster included FV3 and GIV, which are ranaviruses (Tan *et al.*, 2004; Tsai *et al.*, 2005); and a fifth cluster included RSIV (Kurita *et al.*, 2002), RBIV (Do *et al.*, 2004), DGIV and MCIV (Go *et al.*, 2006), ISKNV types (Subramaniam *et al.*, 2014), and ATIV, all of which are megalocytiviruses.

A previous study also supports the family Iridoviridae being organized into five genera (Chinchar *et al.*, 2005). Two of the five genera, Iridovirus and Chloriridovirus, usually infect invertebrates (primarily insects) while the other three genera (Ranavirus, Megalocytivirus and Lymphocystivirus) usually infect cold-blooded vertebrates (Williams *et al.*, 2005). Megalocytiviruses cause systemic infections that can result in moderate to heavy losses in many species of freshwater and marine fish in both cultured and wild stocks. In some disease outbreaks, the mortality rate can be as high as 100% during one week (Eaton *et al.*, 2007; Yanong and Waltzek, 2010). Clinical signs of diseases caused by Megalocytivirus are non-specific, including lethargy, loss of appetite, darkening, abnormal swimming, increased respiration, and hemorrhage (Yanong and Waltzek, 2010). Similarly, the disease caused by ATIV showed clinical signs of dark colour on the body, hemorrhage, and abnormal swimming.

The MCP sequence of ATIV is 99% identical to that of DGIV and 100% identical to the MCP sequences of MCIV and ISKNV (Table 3). The finding that DGIV and MCIV are closely related to ISKNV (Go *et al.*, 2006; Subramaniam *et al.*, 2014) strongly suggests that ATIV is closely related to ISKNV.

4. CONCLUSIONS

In summary, our findings indicate that ATIV belongs to the genus Megalocytivirus of the family Iridoviridae and it should be considered as an ISKNV-Vietnamese type. This is the first report for a Megalocytivirus in climbing perch, a freshwater cultured fish in Vietnam.

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