Molecular Evidence for the Homologous Strains of Infectious Spleen and Kidney Necrosis Virus (ISKNV) Genotype I Infecting the Inland Freshwater Cultured Asian Sea Bass (*Lates Calcarifer*) in Thailand

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Abstract

Infectious spleen and kidney necrosis virus (ISKNV) is the fish pathogenic virus belonging to the genus *Megalocytivirus* of the family *Iridoviridae*. In 2018, disease occurrences (40–50% cumulative mortality) associated with ISKNV infection have been reported in grown-out Asian sea bass (*Lates calcarifer*) cultured in the inland freshwater system in Thailand. Clinical samples were collected from seven distinct farms located in the eastern and central regions of Thailand. The moribund fish showed various abnormal signs including lethargy, pale gill, darkened body, and skin hemorrhage, while the basophilic hypertrophied cell in gill, liver, and kidney tissue was observed microscopically. ISKNV infection was confirmed in 6 out of 7 farms using virus-specific semi-nested PCR. *MCP* and *ATPase* genes showed 100% identity among virus isolates which also classified the virus into ISKNV genotype I clade. Koch's postulates were later confirmed by challenge assay and the mortality of the experimentally infected fish at 21 days post-challenge was 50–90% depending on the challenge dose. The complete genome of two ISKNV isolates, namely KU1 and KU2, was recovered directly from the infected specimens using the shotgun metagenomics approach. The genome length of ISKNV KU1 and KU2 were 111,487 and 111,610 bp, respectively. In comparison to the closely related ISKNV strains, the ISKNV KU1 and KU2 harbored nine unique genes, including caspase recruitment domain-containing protein potentially involved in apoptosis inhibition. Collectively, this study indicated the homologous ISKNV strains affecting the inland cultured Asian sea bass which emphasized that the ISKNV genotype I should be prioritized for future vaccine research.

Introduction

Asian sea bass (*Lates calcarifer* Bloch, 1790), also known as barramundi, is a species native to Thailand and widely distributed in the Indo-West Pacific region from the Arabian Gulf to China, Taiwan, and northern Australia [14]. Asian sea bass is one of the most important marine cultured finfish in Australia and Asian countries including Indonesia, Singapore, Vietnam, and Thailand. According to its significant economic potential, this fish species has been expected by Thai governmental bodies and private sectors to be the major food fish products along with other fish species such as tilapia (*Oreochromis* spp.) and hybrid catfish (*Clarius gariepinus* ♀ *C. batrachus*). The total production of Asian sea bass in Thailand was approximately 39,500 tons in 2018, increased from the year 2017 (20,500 tons) by over 90% [9]. Formerly, Asian sea bass has been cultivated mainly in the coastal area along the Gulf of Thailand and the Andaman Sea, where the fish can grow in open sea cages or brackish/marine water earthen ponds. Presently, the main practice applied for Asian sea bass grow-out has been shifted to the inland freshwater pond system, owning to the catadromous nature of the fish and the advancement of pelleted feed quality, to align with the continual increase of consumer demand [14]. Asian sea bass farming in inland freshwater in Thailand is usually applied in an intensive system. Although maximum yield can be expected with high stocking density, this aquaculture system is undeniably posing a high risk for the emergence of infectious diseases due to the stressful conditions caused by the accumulation of metabolic waste, organic matters, and fluctuation of water qualities.

Mortalities and morbidities related to the distribution of infectious diseases have been reported as the major cause of economic losses for Asian sea bass farming industry worldwide. According to the literatures, Asian sea bass are susceptible to numerous pathogen organisms including bacteria, e.g., *Streptococcus iniae*, *Vibrio alginolyticus*, *Vibrio harveyi*, and *Photobacterium damselae* subsp. *damselae* [2, 4, 12] and viruses such as viral nervous necrosis, *Lates calcarifer* herpes virus, *Lates calcarifer* birnavirus, and megalocytiviruses [6, 36, 38]. Among the potential virus pathogens, the members of the genus *Megalocytivirus* (belonging to the family *Iridoviridae*), such as red sea beam iridovirus (RSIV), turbot reddish body iridovirus (TRBIV), infectious spleen and kidney necrosis virus (ISKNV), and scale drop disease virus (SDDV, another distant species within the same genus) have been associated with several disease outbreaks in marine fishes in many countries, e.g., red sea bream (*Pagrus major*), orange-spotted grouper (*Epinephelus*

coioides), olive (Japanese) flounder (*Paralichthys olivaceus*) and turbot (*Scophthalmus maximus*). The clinical signs and lesions presented in megalocytivirus-infected fish can be very diverse depending on pathogenic agents and host species. For example, fish infected with RSIV was reported showing lethargy, petechiae gills, and enlargement of the spleen [26], ISKNV-infected fish exhibited dark body, pale gills, and red eyes [11], whereas SDDV infection in Asian sea bass often correlated with extensive scale loss and skin hemorrhage lesions [29, 41]. The occurrence of megalocytivirus infection, namely ISKNV and SDDV, were also reported as the potential pathogens causing severe mortalities of 55–77% in Asian sea bass in Thailand [29, 48]. However, the epidemiological information and molecular characteristics of megalocytiviruses in Asian sea bass have been limited to only those observed in marine environments which is, to some extent, unsurprising since inland freshwater culture is not yet widely practiced on a global scale. To date, only one incidence of co-infection between a pathogenic bacterium *Flavobacterium columnare* and SDDV was reported in the freshwater system [29].

Recently, disease outbreaks occurred in the inland freshwater-based Asian sea bass grow-out farms located in the eastern and central parts of Thailand, causing mortalities ranged from 40–50%. The initial diagnoses indicated that a series of outbreaks may involve ISKNV infection. Therefore, this study aims to investigate the molecular characteristics of these ISKNV strains and their pathogenic roles on Asian sea bass reared in a freshwater system. In addition, the genome of the virus was also examined using a metagenomic approach which, to our knowledge, was the first ISKNV genome from a freshwater cultured Asian sea bass ever sequenced.

Materials And Methods

Disease history and sample collection

Fish farmers reported the occurrence of unknown diseases in Asian sea bass in seven freshwater grow-out farms located in Samut Sakhon, Samut Songkhram, and Chachoengsao provinces, Thailand, between February and November 2018. Diseased fish was ranged from 20 to 30 g in weight. The cumulative mortality varied from 40 to 50 percent (as monitored by farm's practitioners). Moribund fish was euthanized by decerebration and bacterial isolation was carried out on-site. Internal organs (kidney, spleen, and liver) of each individual were collected separately, preserved in 95% ethanol for PCR testing, and delivered on-ice to the laboratory within 4 hr. Ethanol-fixed tissues were maintained in a -20°C refrigerator until further PCR assay, whereas fresh tissues for virus isolation were preserved at -80°C. On the part of histopathology, collected tissue from sacrificed fish was immersed in 10% neutral buffered formalin at a ratio of 1:10 (w/v) for 24–36 hr, followed by replacement with the same volume of 70 % ethanol for long-term preservation. Preserved tissue samples were processed for standard histological analysis by dehydration, embedded with paraffin, and sectioned followed by staining with hematoxylin and eosin (H&E) [21]. The H&E-stained tissues were then examined under a light microscope equipped with a digital camera. The negative control used for further assays was the apparently healthy Asian sea bass obtained from a different location. The animal use protocol conducted in this study was approved by the Institutional Animal Care and Use Committee, Faculty of Fisheries, Kasetsart University (permit ID: ACKU61-FIS-055).

Bacterial isolation and identification

Direct isolation from external lesions (gills and skin) and internal organs (spleen, kidney, and liver) was conducted using two different media comprised of (i) tryptic soy agar (TSA, Himedia, India) supplemented with 5% sheep blood using as a generalized medium, (ii) Anacker and Ordal agar (AOA, tryptone 0.5g/L, yeast extract 0.5g/L, sodium acetate 0.2 g/L, beef extract 0.2 g/L, and agar 10 g/L) supplemented with 1 μg mL^{-1} tobramycin (Sigma-Aldrich, Singapore) using as a selective medium for *Flavobacterium* sp. [44]. Streaked plates were delivered to the laboratory

then inoculated at 28°C until bacterial colonies were visible (24–48 h). Colonies were sub-cultured using the same kind of medium until a pure colony was obtained. Pure colonies grown on either TSA or AOA medium were subjected to preliminary identification of bacterial identity using Gram staining and primary biochemical assays including oxidase, catalase, oxidation-fermentation, and motility tests. Bacterial taxonomy was determined to the genus level based on Cowan and Steel's manual [7].

Identification of ISKNV infection from tissue samples

DNA extraction from fish tissues

Screening of ISKNV infection was carried out for every individual fish collected in this study ($n = 26$). To extract DNA, tissue was removed from ethanol and homogenized using disposable polypropylene pestles. DNA was extracted using Tissue Genomic DNA Extraction Mini Kit (Geneaid, Taiwan) according to the manufacturer's instructions. DNA concentration was quantified using a Nanodrop analyzer (Titertek Berthold, Germany) and stored at -20°C.

PCR identification for ISKNV

ISKNV-specific primers (Table 1) were used for screening of the infection by one-tubed semi-nested PCR (snPCR) [11]. Each 25 μL PCR mixture contained 1X master Mix (Go-Taq®-Green, Promega USA), 10 nM of each working primers, and 100 ng of DNA template. The thermal condition was 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min and final extension at 72°C for 5 min. PCR products were analyzed by 1% agarose gel electrophoresis followed by staining with Red Safe (Chembio, UK) and visualized under UV light. Expected PCR products from positive ISKNV samples were either one amplicon of 164 bp, two amplicons at 517 and 164 bp, or three amplicons at 754, 517, and 164 bp representing light, moderate and heavy infections, respectively, as described in the original article [11]. We used a positive control from DNA template extracted from ISKNV-infected sample kindly provided by Centex, Mahidol University, whereas healthy Asian sea bass and nuclease-free water without DNA template served as the internal and negative control, respectively.

Table 1
Primers used in this study.

Primer name	Primer sequence (5'–3')	Feature	Product size (bp)	References
ISKNV screening by one-tubed semi-nested PCR				
Megalo-F	AGATGATTGGCATGCGCAGCG	Semi-nested PCR targeting *MCP* gene for ISKNV screening	754	[11]
Megalo-1R	TTGGACAGGCGGCCGTAGT			
Megalo-2Rsn	TACACGGGACTGGCCGC		517	
Megalo-3Rsn	CTTGAAGTGGATGCGCACCTC		164	
ISKNV sequencing				
MMCP-F	ATGTCTGCRATCTCAGGT	Sequencing of *MCP* gene	1362	[20]
MMCP-R	TYACAGGATAGGGAAGCCTG			
MATPase-F	ATGGAAATCMAAGAGTTGTCCYTG	Sequencing of *ATPase* gene	720	
MATPase-R	TTACRCCACGCCAGCCTTGTA			
ISKNV qPCR				
Meg-MCP160F	TCAAAACAGACTGGCCATGC	qPCR targeting MCP gene for quantifying ISKNV	190	Kawato 2021, submitted paper
Meg-MCP349R	TAAATGACACCGACACCTCCTC			
Meg-MCP239P	6-FAM-FAM-TGTGGCTGCGTGTTA AGATCCCCTCCA-BHQ-1			

Sequencing analysis of viral MCP and ATPase genes

DNA sequencing of MCP and ATPase genes

Asian sea bass DNA extracts giving positive PCR results for ISKNV screening based on the one-tubed snPCR were further used for phylogenetic analysis. ISKNV positive sample was selected randomly (only one sample per farm) and DNA was extracted from the liver. Two ISKNV genes, encoded for MCP and ATPase, were targeted for sequence analysis. The detail of each primer set is shown in Table 1. The PCR conditions followed those described in the original article [20]. Amplicons were purified following agarose gel electrophoresis using a Universal DNA purification kit (Tiangen, Beijing, China) according to the manufacturer's instruction. The purified DNA fragments were ligated into pGEM-T Easy cloning vector (Promega, WI, USA) and transformed to *Escherichia coli* JM109 competent cells as described by Russell and Sambrook [40]. Transformants were selected using Luria-Bertani agar (LB, Oxoid, UK) containing selective antibiotic and subsequently multiply in LB broth prior to plasmid isolation using NucleoSpin® Plasmid MiniPrep kit (Macherey-Nagel, Germany). The extracted plasmid was submitted for Sanger sequencing at sequencing laboratory service (1st BASE Pte Ltd., Malaysia) using pUC/M13 primers as described in Promega's pGEM-T vector manual.

Phylogenetic analysis

Low-quality bases were trimmed manually based on a chromatogram of the raw sequences. Trimmed sequences were then assembled into contig using ContigExpress. The homology search was performed using Megablast against NCBI's nucleotide database to align and compared to the other ISKNV strains. *MCP* and *ATPase* genes of the ISKNV of this study ($n = 6$, one sample per farm) were compared to other members of the genus *Megalocytivirus* including ISKNV ($n = 11–22$), RSIV, ($n = 22–43$), and TRBIV ($n = 7–14$). Nucleotide sequences were aligned using ClustalW, then the phylogenetic tree was constructed using the maximum likelihood method with GTR + G + I substitution model and 1000 bootstrap replicates. Multiple sequence alignment and phylogenetic reconstruction were carried out using MEGA-X software [31].

Genome analysis

Library preparation and next-generation sequencing

Two samples with possibly severe ISKNV infection (one-tubed snPCR yielded three distinct amplicons as described in the previous section) from Samut Sakhon province were used for the metagenomic shotgun sequence. The Nextera XT library preparation kit (Illumina, CA, USA) was used to construct a paired-end library from the extracted genomic DNA according to the manufacturer's instruction. Subsequently, high throughput sequencing was performed using Illumina HiSeq system with 150-bp read length. Library construction and sequencing were carried out using the service provided by Novogene (Beijing, China).

ISKNV genome reconstruction and annotation

Processing of raw reads and *de novo* assembly was conducted as described in our previous publication [28]. The adaptor sequences and low-quality reads were filtered out from raw reads using Trimmomatic v0.39 [3]. Then, host-derived reads were discarded by mapping trimmed reads against Asian sea bass reference genome (NCBI assemblies accession no. GCA_001640805.1) using -x function in the Bowtie2 program [33]. The obtained non-host reads were subjected to *de novo* metagenome assembly using MEGAHIT v 1.2.9 with a minimum length of output contigs of 1000 bp [34]. The generated assemblies were submitted to the web server version of the Kaiju program to predict the taxonomic identity of each contig [37]. Contigs being assigned as ISKNV were annotated using Prokka v1.14.0 with Viruses Annotation mode and the complete ISKNV reference genome (Genbank accession no. NC_003494.1) was selected as annotation template. The name 'ISKNV KU1 and KU2' were assigned to these ISKNV-like contigs observed in our two samples, respectively. Visualization of the ISKNV KU1 and KU2 as a circular genome map was performed by uploading the annotated genomes to the web server version of CGview (http://cgview.ca/) [16].

Phylogenomic analysis and genome distance

ISKNV KU1 and KU2 were aligned to the ISKNV reference genome using MAUVE progressive alignment [8]. The genome segments of ISKNV KUs were rearranged manually following with the ISKNV reference genome. To determine genetic distance, multiple alignment of the megalocytiviruses, including ISKNV KUs, was performed using the FFT-NS-i method in MAFFT v7 online service [25] and MEGA-X was used to calculate the distance based on the Maximum Composite Likelihood model. The phylogenomic network was generated using SplitsTree4 based on the alignment of megalocytivirus genomes [22].

Identification of orthologous groups

OrthoFinder [13] was used to determine the possible orthologs among four ISKNV genomes, including two ISKNV strains of this study (KU1 and KU2) and two closely related strains (RSIV-Ku and ISKNV reference strain). The coding

sequences (CDSs) of strain RSIV-Ku and reference genome were obtained from GenBank under the accession nos. KT781098 and NC_003494, respectively. OrthoFinder pipeline automatically categorizes the proteins from tested subjects into orthologous groups (also called orthogroups) based on sequence similarity. In this study, proteins of ISKNV KU1 and KU2 predicted as 'non-orthologous' comparing to the reference strain and RSIV-Ku were subjected for further protein Blast analysis.

Propagation of ISKNV using GF cell line

ISKNV isolate KU1 was used for virus isolation. One gram (1 g) of liver and spleen preserved at -80 ℃ was pooled and homogenized in 10 ml of L15 medium (Gibco, CA, USA) followed by centrifugation at 9600× g for 30 min at 4°C. After centrifugation, the supernatant was collected and filtered with a sterile 0.22 µm membrane filter. Filtrate (0.5 mL) was inoculated in a 5 mL flask of monolayer Grunt fin (GF) cells for 2 hours, and the medium was replaced with fresh L15 medium supplemented with 10% FBS (Gibco, CA, USA). The flasks were then incubated at 25°C and observed under a microscope daily for 10 days to monitor the cytopathic effect (CPE). The virus was harvested by centrifugation of cell culture supernatant at 1000× g for 5 minutes at 4°C. Cell debris was discarded and the supernatant containing ISKNV was collected and preserved in a -80°C refrigerator until used in the experimental challenge.

The calculation viral copy number of ISKNV in viral suspension was conducted using qPCR specific to ISKNV (Kawato 2021, submitted paper). One hundred and forty microlitres (140 µl) of the supernatant was used for DNA extraction using the phenol-chloroform method. The qPCR reaction consisted of 1X iTaq Universal Probes Supermix (Bio-Rad), 3 µL of DNA template, 900 nM of each forward and reverse primers (Meg-MCP160F and Meg-MCP349R) and 250 nM of Meg-MCP239P probe in a total volume of 20 µL. The qPCR condition was an initial denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 64°C for 1 min. Both viral detection protocols were run using Bio-Rad CFX Connect RealTime PCR machine, which product size from this qPCR is 190 bp.

Pathogenicity test

The healthy Asian sea bass (*n* = 150) was used in the experimental challenge test to fulfill Koch's postulate. The fish with an average weight of 22 g were purchased from a local grow-out farm. Five fish were randomly sampled to verify the ISKNV-free status from splenic tissue using virus-specific one-tube snPCR as described above. ISKNV free fish were used for pathogenicity tests following a week of acclimatization in a 3,000-L tank. Fish were divided into three groups (*n* = 50 per group) comprising one control group and two challenge groups. A group served as control received sterile cell culture medium, whereas challenge groups were injected intraperitoneally by a volume of 0.2 ml containing the propagated virus of different dilutions (low dose [10^{-2} dilution] and high dose [non-diluted]). Injected fish was transferred into aerated 500-L tanks (n = *50* per tank) with the water temperature and dissolved oxygen maintained at 30–32°C and 5–7 ppm, respectively. Freshly dead and moribund fish was removed from the tank on notice. The liver, spleen, and kidney of affected fish were collected for histopathological investigation and ISKNV screening using one-tubed snPCR. Daily mortality was recorded for 21 days and the Kaplan-Meier survival curve with the logrank statistical test was carried out using the IBM SPSS Statistics 25 program.

Results

Clinical manifestations of naturally infected fish

The observed losses in grow-out Asian sea bass varied from 40 to 50% within 1–2 weeks after the disease was noticed. The moribund fish manifested clinical signs such as lethargy and anorexia, while the external lesions including darkened body, pale gill, skin hemorrhage, and ascites were also observed. Internally, swollen spleen and hemorrhage at liver and trunk kidney were the most abundant lesions (Fig. 1). Histopathological manifestations

included severe hemorrhage, inflammatory cell infiltration, accumulation of melanocytes, extensive necrosis at the skin and gill, and moderate tubular degeneration in the trunk kidney (Fig. 2A). Interestingly, the typical microscopic lesions representing viral infection, namely eosinophilic inclusion, and the hallmark histological changes for ISKNV infection, i.e., basophilic hypertrophied cells, were also detected in gill, liver, and kidney.

Bacterial isolation was attempted from necrotic gills and skin lesions using *Flavobacterium*-selective medium, AOA, whereas generalized medium, TSA, was applied to internal organs with apparent lesions. Bacterial colonies grown on the AOA medium showed yellowish and rhizoid morphology. The detail of collected fish samples and the observed lesions, coupled with the disease diagnostic results, were summarized in Table 2.

Table 2
Summary of diseased Asian sea bass samples collected in this study.

Month (2018)	Province	Farm	Macroscopic lesions	Microscopic lesions	Bacterial identification[a]				ISKNV identification[b] (no. of infected samples)
					Ae	Fl	Str	Ed	
February	Chachoengsao	A	Darkened body, pale gill	Gill necrosis, inflammation and melanocyte aggregation in muscle	ND	ND	ND	ND	+ (6/7)
		B	Inapparent	ND	ND	ND	ND	ND	– (0/4)
June	Chachoengsao	C	Skin and liver hemorrhage, pale gill	Skin hemorrhage, muscle inflammation, tubular degeneration, and cytoplasmic inclusion bodies in kidney	+	+	-	-	+ (3/3)
		D	Skin hemorrhage, eye bleeding	Muscle inflammation, tubular degeneration at kidney	-	+	-	-	+ (3/4)
August	Samut Sakhon	E	Inapparent	ND	-	ND	-	-	+ (3/3)
		F	Inapparent	ND	-	ND	-	-	+ (1/1)
November	Samut Prakan	G	Ascites, darkened body, Scale loss, pale gill	Necrosis and cellular proliferation at gill, muscle inflammation, kidney degeneration	-	+	-	-	+ (4/4)

a *Flavobacterium* sp. was presumptively diagnosed based on the appearance of yellowish and rhizoid colonies grown on *Flavobacterium*-specific medium, AOA, whereas other bacteria were classified to the genus level based on biochemical analyses according to Cowan and Steel's manual [7].

b ISKNV identity was identified based on virus-specific one-tubed semi-nested PCR from liver and spleen specimens [11].

Abbreviation. ND, not determined; Ae, *Aeromonas* sp.; Fl, *Flavobacterium* sp.; Str, *Streptococcus* sp.; Ed, *Edwardsiella* sp.; ISKNV, infectious spleen and kidney disease virus

Identification of ISKNV

Infection of ISKNV in Asian sea bass samples was primarily screened using one-tubed snPCR targeting *MCP* gene. Out of 26 fish collected in this study, 20 were positive to ISKNV detection (6 out of 7 farms). Despite the samples of the same origin (farm), the degree of infection, as interpreted by the number of amplicons, varied among positive samples. The electrophoresis photographs are demonstrated in the supplementary Figure S1. According to the original study

mentioning the semi-quantitative nature of one-tubed snPCR [11], relatively high, moderate, and low viral copy numbers would appear as three, two, and one amplicon on agarose gel electrophoresis, respectively.

Phylogenetic analysis based on MCP and ATPase genes

Amplification of *MCP* and *ATPase* genes was conducted from 6 samples (one per farm) diagnosed as ISKNV positive (Supplementary figure S2). The nucleotide sequences of the almost complete *MCP* (1,275 bp) and complete *ATPase* (720 bp) genes were identical (100% identity) among the ISKNV isolates of this study. Therefore, we submitted both *MCP* and *ATPase* genes from only a single ISKNV isolate to the GenBank database under the accession nos. MW269579 and MW269580, respectively. Blast analysis of *MCP* and *ATPase* genes indicated that the ISKNV obtained in this study was also identical to several ISKNV strains available in NCBI's nucleotide database such as AFIV-16 (MK689685) and ISKNV strain RSIV-Ku (KT781098). The reconstruction of phylogenetic trees based on *MCP* and *ATPase* genes of ISKNV, RSIV, and TRBIV (virus species in the genus *Megalocytivirus*) was demonstrated in Figs. 3A and 3B. According to the *MCP*-based tree, ISKNV isolates were clustered into two different subclades, corresponding to the genotype I and II. Herein, the ISKNV of this study fell into genotype I. On the contrary, sub-clustering among ISKNV isolates were unclear in the *ATPase*-based tree which could be due to the lower number of taxa.

Genome features

Asian sea bass samples from farm E and F ($n = 2$) in Samut Sakhon province with relatively high viral load (as shown by one-tubed sn PCR) was selected for metagenomics shotgun sequencing. Non-host reads were *de novo* assembled into 1,482 and 1,030 contigs, respectively. Taxonomic identification by the Kaiju web server indicated that, from each sample, only the longest contig was predicted as ISKNV. The length (111 kb) and GC content (54.8%) of these contigs were almost identical to the ISKNV reference genome. These virus strains were named 'ISKNV KU1' and 'ISKNV KU2', and the summarization of their genomics characteristics is shown in Table 3. Both ISKNV genomes were submitted to GenBank under the accession nos. MT128666 and MT128667. Their genome map is visualized in Fig. 4.

Table 3
Genome features of ISKNV KU1 and 2 compared to those of reference genome

	ISKNV KU1	ISKNV KU2	ISKNV reference strain
Genbank accession numbers	MT128666	MT128667	NC_003494
Genome length	111,487 bp	111,610 bp	111,362 bp
Number of contigs (coverage depth)	1 (47×)	1 (56×)	1 (na)
GC content	54.8%	54.8%	54.8%
Number of genes	122[a]	123[a]	125
Host	Asian sea bass, *Lates calcarifer*	Asian sea bass, *Lates calcarifer*	Mandarin fish, *Siniperca chuatsi*
Nucleotide identity compared to the reference genome	99.98%	99.98%	na
a Number of genes were predicted by Prokka v 1.14.0			
na, Not applicable			

Phylogenomic analysis

The neighbor-net network analysis was able to differentiate 15 members of the genus *Megalocytivirus* into three distinct clusters, namely RSIV, TRBIV, and ISKNV groups (Fig. 3C). The genomes of ISKNV KU1 and KU2 were almost identical to those of the ISKNV reference strain and RSIV-Ku strain with minimal genome distances of 0.02% and 0.06%, respectively. On the contrary, the genome distance between distinct clusters can be as high as 5.5–9.4%. The reticulation pattern was clearly observed among the ISKNV and RSIV groups which indicated the possible genetic recombination within this cluster.

Orthology between ISKKNV KU1, KU2, and reference strains

OrthoFinder categorized a total of 499 genes, obtained from four distinct ISKNV strains (KU1, KU2, RSIV-Ku, and reference strain), into 124 orthogroups. Among these, 88 orthogroups (70.9%) were presented in all ISKNV strains. The RSIV-Ku and reference strains possessed 13 and 18 genes that were unassigned to any orthogroups (unique genes). Seven orthogroups can be found exclusively in the strain KU1 and KU2, but absent in the other two ISKNV strains. The strain KU1 also carried two genes that unique to its genome. Most of these KU1- and KU2-specific genes were predicted as hypothetical proteins-encoded and similar to other viruses in the genus *Megalocytivirus*, such as Angelfish iridovirus AFIV-16, scale drop disease virus, Banggai cardinalfish iridovirus, and Red sea bream iridovirus. There was one protein from KU1 (QQZ00456) and KU2 (QQZ00673) strain that almost identical to the caspase recruitment domain-containing (CARD) protein of the Angelfish iridovirus AFIV-16. The details of these nine genes presented only in the stains KU1 and KU2 are demonstrated in Table 4, coupled with the Blast protein analysis result.

Table 4

The ISKNV strain KU1 and KU2 protein non-orthologous[a] to the ISKNV RSIV-Ku and reference strain

Protein ID		Length (aa)	Best protein BLAST hit	Accession number	Query coverage, Identity (%)	E-value
KU1	KU2					
QQZ00456.1	QQZ00673.1	101	Caspase recruitment domain-containing protein [Angelfish iridovirus AFIV-16]	QIQ54447.1	100, 99.01	5.00E-68
QQZ00464.1	QQZ00681.1	80	Hypothetical protein [Scale drop disease virus]	QLI60734.1	86, 37.84	5.00E-06
QQZ00514.1	QQZ00609.1	122	Hypothetical protein [Banggai cardinalfish iridovirus]	QOE77200.1	100, 100	6.00E-84
QQZ00518.1	QQZ00613.1	97	Not found	-	-	-
QQZ00523.1	QQZ00618.1	158	ORF068 [Angelfish iridovirus AFIV-16]	QIQ54512.1	100, 100	1.00E-115
QQZ00536.1	QQZ00631.1	34	Not found	-	-	-
QQZ00568.1	QQZ00663.1	95	Not found	-	-	-
QQZ00479.1	-	316	ORF025 [Angelfish iridovirus AFIV-16]	QIQ54469.1	100, 61.59	3.00E-37
QQZ00505.1	-	63	Hypothetical protein ORF050 [Red seabream iridovirus]		100, 93.65	3.00E-30

a Categorization of the orthologous group was carried out using the OrthoFinder program

Virus isolation

In vitro replication of ISKNV was carried out by propagating the virus in the GF cell line. ISKNV KU1 was selected as the source of the virus seed. At 25°C, the on-set for CPE was 5 days in which focal degeneration of GF cell can be observed by the limited area of cell detachment and cell shrinkage, inferring pyknosis (Fig. 5, top-right panel). At 7 days, vacuolization and pyknosis were observed throughout the confluent monolayer suggesting severe degeneration of host cells (Fig. 5, bottom-right panel). On the contrary, no CPE change was found in GF cells propagated with sterile L15 medium until the end of the culture period of 10 days. Cell culture supernatant was harvested from both control and virus-infected cells at 7 days post-inoculation (dpi) and genomic DNA was extracted. This DNA was used as a template in ISKNV-specific one-tubed snPCR and the result indicated the specific amplification only from the ISKNV-infected cells (Supplementary Figure S3). The ISKNV-specific qPCR indicated that the viral copy number of viral stocks for challenge assay was 1.45×10^6 copies/mL.

Viral pathogenicity assay

The healthy Asian sea bass reared in freshwater was challenged intraperitoneally with GF-cell grown ISKNV at the dosage of 2.9×10^5 (high dose) or 2.9×10^3 (low dose) copies/fish. Mortality on-set for high dose and low dose groups was at 5- and 8-days post-challenge (dpc), respectively. Daily mortality in both high and low dose groups was 1–3 fish per day, except for 18 and 19 dpc in which 5–8 dead fish was monitored in the high dose group. In the low dose group, mortality has stopped at 19 dpc. The cumulative mortality at the end of the experiment (21 dpc) was 90% on the high dose group (45 out of 50 fish), significantly higher than those observed in the low dose group [50%, 25 out of 50 fish ($p < 0.05$)]. The experimentally infected fish was found positive to one-tubed snPCR. These infected fish samples yielded three specific amplicons suggesting relatively high viral load present in the liver (Supplementary Figure S4). Herein, two fish in the control group (4%) died a day after mock infection, which could be due to injection injury since one-tubed snPCR showed negative results. The survival curve is demonstrated in Fig. 6.

The challenged fish showed clear pathological changes similar to the naturally infected fish. Externally, moribund fish exhibited darkened bodies with pale gills. Microscopically, hematopoietic tissues showed apoptosis (pyknotic nuclei) and cytoplasmic inclusion bodies, whereas tubular degeneration was found in the kidney, and gill lamellar showed basophilic hypertrophied cell (Fig. 2C and 2D).

Discussion

Nowadays, most of the Asian sea bass culture in Thailand has been conducted using an inland freshwater culture system, in response to the continual increase of consumer demand. According to our observation (unpublished data), the former cage culture system in the estuary has been considered by Asian sea bass farmers as a risky practice due to the unmanageable biosecurity and water qualities. The major cultivation area of grown-out Asian sea bass in Thailand, to date, is in the central and eastern regions due to the availability of fingerling supplies and abundant water resources. Moreover, the Asian sea bass farming area is expected to be on the rise since this fish species has been appraised, by the governmental bodies and private sectors in Thailand, as valuable fisheries products for future exportation. However, intensive farming would induce stress responses due to the overstocking and overfeeding which resulted in the increase disease vulnerability, possess a risk for severe disease outbreaks. In this study, ISKNV was identified from diseased Asian sea bass cultured in the inland freshwater system. The most common histopathological lesions from ISKNV-infected Asian sea bass collected in this study, i.e., severe necrosis and the appearance of basophilic inclusion bodies in gills, liver, and kidney, was consistent with the pathognomonic lesions of megalocytivirus infection (hypertrophy/megalocytosis in gill and liver) reported previously [32, 45]. In this study, the histopathological manifestations in the spleen, kidney, and liver also suggested the viral tropisms in these hematopoietic organs which, to some extent, may result in immune incompetent/suppression and increased

susceptibility to opportunistic infections. Co-infection between *Flavobacterium columnare* and SDDV, another member of the genus *Megalocytivirus*, in grown-out Asian sea bass has been reported in our previous investigation [29]. Considering that ISKNV and the potential bacterial pathogens, such as *Aeromonas* sp., *Streptococcus* sp., and *Flavobacterium* sp., was co-identified from 3 out of 7 disease incidences in this study, it is feasible that simultaneous infection is relatively common among natural disease occurrences in Asian sea bass. Simultaneous infection, also called a concurrent infection, can be found often in farmed fish and could outweigh a single infection [10]. In our case, we speculated that simultaneous infection by ISKNV and other bacteria could influence the large diversity of clinical appearances, which range from inapparent signs to severe symptoms, e.g., extensive hemorrhage. In this study, it remains elusive whether ISKNV or bacterial pathogen is the primary pathogen, and the clear understanding of pathogens interrelation *in vivo* required further investigation.

Apart from Asian sea bass, ISKNV has been reported in farmed Nile tilapia and ornamental fish in Thailand as well [1, 10, 47]. The clinical appearances of these ISKNV-infected fishes also similar to those observed in this study. The ISKNV from ornamental fish were genetically classified as genotype I [1], based on the *MCP* gene sequence, similar to the ISKNV of this study. In the previous studies, the area of disease outbreaks was also in the same region (central Thailand) as the ISKNV-positive Asian sea bass samples collected in this study. Therefore, the cross-species transmission between these freshwater farmed fishes, although not yet been documented officially, should be aware and the biosecurity-oriented management should be of concern (particularly in the area with outbreak history) since the sharing of water resources among farms is hardly evitable.

ISKNV was also identified from various marine fish species in other Southeast Asian countries including Indonesia, Vietnam, Malaysia, and Singapore [11, 23, 39, 46]. Most of the ISKNV found in these countries were genotype I, similar to our study, which indicated the widespread of this ISKNV genotype in Southeast Asia. According to the original article describing ISKNV genotyping [15], ISKNV can be classified into three genotypes (I, II, and III) based on the diversity in the *MCP* gene. These genotypes later assigned into three clusters comprised of RSIV, ISKNV, and TRBIV groups under the same virus species, as described by the International Committee on Taxonomy of Viruses (ICTV, https://talk.ictvonline.org/ictv-reports/ictv_online_report/dsdna-viruses/w/iridoviridae/615/genus-megalocytivirus). To date, the ISKNV genotype II was reported from orbiculate batfish (*Platax orbicularis*), Banggai cardinalfish (*Pterapogon kauderni)*, and marble goby (*Oxyeleotris marmorata*) in Indonesia, Japan, USA, and China [32, 43, 49], while Asian sea bass mortality associated with ISKNV genotype II infection was found in Southern China and Vietnam [11, 50]. According to the phylogenetic analysis conducted in this study, the genetic diversity among the current ISKNV isolates was rather small as all the samples are classified as genotype I and shared 100% sequence homology in both *MCP* and *ATPase* genes. *MCP* and *ATPase*, as well as *DNA polymerase*, genes were generally used to determine the genetic relationship of megalocytivirus due to their evolutionary conservation nature [30, 32]. However, the relationship between the genetically similar strains cannot be inferred, at least for the current collection of virus isolates, relying on these conserved genes alone. To date, the in-depth epidemiological regarding ISKNV genotype distributed in Thailand and neighboring countries in Southeast Asia is scarce. Thus, the development of an efficacious ‘regional’ vaccine against ISKNV distributed in Southeast Asia is hardly accomplished unless sufficient epidemiological insight is established. For further investigation, other molecular markers, such as four ankyrin repeat domains [30], offering higher discriminatory power would be added to the phylogenetic comparisons which will determine the intra-genotype diversity of ISKNV on a finer scale.

Isolation of ISKNV from the infected Asian sea bass specimens using GF cell line was successful in this study. Application of GF cell for the propagation of ISKNV simultaneously with nervous necrosis virus has been reported in our previous study [24]. However, the result of this study was slightly inconsistent with another publication in terms of CPE on-set [48]. In the former study, CPE cannot be observed clearly until 14 dpi, whereas 5 dpi on-set was found in

this study and clearly discerned at 7 dpi. The variation in CPE development possibly due to the difference in the initial inoculation dose since this study intentionally selected a specimen with potentially high viral titer (indicated by one-tubed snPCR). Recently, the alternative cell line GS-1 originated from orange-spotted grouper (*Epinephelus coioides*) fibroblast was reported to be ISKNV susceptible and allowed the virus to reach $10^{5.2}$ $TCID_{50}$/ml titer within 7 days, though direct comparison of ISKNV replication kinetic to GF cell has not been described yet [19].

The pathogenicity assay conducted in this study was able to fulfill Koch's postulates and proved that ISKNV was pathogenic to freshwater reared Asian sea bass. The result suggested that the pathogenicity of ISKNV and on-set of mortality depend on the infection dose (Fig. 6). The cumulative mortalities were similar to the previous investigations (90, 77, and 85.89%), as well as the on-set of mortalities at 5–9 dpc [48, 50]. It should be mentioned that the size of the experimental animal used in this study (juvenile, 22 g weight) was different from the previous report (fingerling, 3.5 g weight) [48], which suggested that ISKNV was able to cause severe mortality in Asian sea bass at various life stages. Pertaining to clinical appearances, it is worth mentioning that the experimental animals exhibited only the typical darkened body and pale gill, in agreement with those described in the recent study [50]. On the other hand, the obvious external lesions such as scale loss, muscle necrosis, and hemorrhage were observed only from the naturally infected fish. The difference in the clinical manifestations could be due to bacterial pathogens co-infected with ISKNV in natural cases (Table 2). This emphasized that diagnosis of field outbreaks should be performed cautiously and multiple approaches, including pathogen isolation, PCR, and histopathology, should be applied when possible, to get a comprehensive understanding of the disease scenario.

The metagenomic shotgun sequence was able to unveil the draft genome sequence of SDDV directly from the infected specimen, without the need of virus culture, in our recent investigation [28]. In this study, the same analytical approach has been implemented and the complete genomes of ISKNV KU1 and KU2 were reconstructed with the acceptable coverage depth at 47–56×. The genome-scale phylogenetic network (Fig. 3C) showed the reticulation pattern within ISKNV and RSIV groups which implied the possible genetic recombination between the members of these groups. In fact, one of the ISKNV group members, RSIV-Ku, harbored 7% of genome contents similar to the GSIV-K1 (belonging to RSIV group) which indicated that this strain was ISKNV/RSIV recombinant [42]. Herein, possible recombination between ISKNV KU1/KU2 and other megalocytiviruses was screened using the RDP4 program [35] but no evidence of recombination was found in their genomes (data not shown). Orthology analysis showed a surprisingly high number of unassigned orthogroups (unique genes) among the genomes of ISKNN KU1/KU2, RSIV-Ku, and reference strain. It is predictable that the strain RSIV-Ku, a natural recombinant virus, may possess numerous unique genes since its genome carried a 7.8-kb-long region similar to RSIV genotype II rather than ISKNV [42]. In the case of the ISKNV reference strain, 18 genes were unassigned to any orthologous groups, though the core genome similarity comparing to the ISKNV KU1/KU2 was as high as 99.98%. This could be explained by the difference in genome annotation methods used for CDS prediction in the ISKNV reference strain and KU1/KU2. Protein-coding sequences of the ISKNV reference strain were identified by querying sequences through a protein domain database [17], whereas the unsupervised machine learning algorithm-based program (Prokka) was employed in the case of ISKNV KU1/KU2. Among the non-orthologous genes presented in ISKNV KU1/KU2, the caspase recruitment domain-containing (CARD) protein was identified. This protein was highly similar to those of Angelfish iridovirus AFIV-16, also belongs to ISKNV genotype I [27], isolated from angelfish *Pterophyllum scalare* in Southeast Asia. CARDs are well-known interaction motifs involved in inflammation and apoptosis regulation [18]. CARD protein has been demonstrated *in vitro* for apoptosis inhibitory effects in Grouper iridovirus, a member of the genus *Ranavirus* of the family *Iridoviridae* [5]. Nevertheless, the role of CARD protein on the molecular pathogenesis of ISKNV, whether it is involved in apoptosis inhibition, remains to be further elucidated.

Summarily, the homologous strains of ISKNV genotype I have been identified as a causative agent of mass mortalities in freshwater cultured Asian sea bass in the eastern and central Thailand by a combination of histopathology, molecular analyses, and pathogenicity assay. The complete genome of two ISKNV isolates, KU1 and KU2, was obtained using a metagenomics approach. The genome information, as well as the virus archive collected in this study, could be useful for evolutionary analysis and selection of potential genotype I vaccine candidates for the sustainable prevention of ISKNV outbreaks in the future.

Declarations

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Conflicts of interest/Competing interests

The authors declared no conflict of interest.

Availability of data and material

This manuscript has data included as electronic supplementary material.

Code availability

Not applicable

Author contributions

Authors	Contributions
Pattarawit Kerddee	Carried out the experiment, wrote the manuscript
Nguyen Dinh-Hung	Performed analysis (disease diagnosis)
Ha Thanh Dong	Performed the analysis (histopathology, cell culture and qPCR)
Ikuo Hirono	Contributed analysis tool (GF cell line)
Chayanit Soontara	Performed analysis (disease diagnosis and experimental challenge)
Nontawith Areechon	Conceived of overall direction and planning
Prapansak Srisapoome	Collected the samples from natural outbreaks
Pattanapon Kayansamruaj	Designed experiment, wrote the manuscript

Ethics approval

The animal use protocol conducted in this study was approved by the Institutional Animal Care and Use Committee, Faculty of Fisheries, Kasetsart University (permit ID: ACKU61-FIS-055).

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Figures

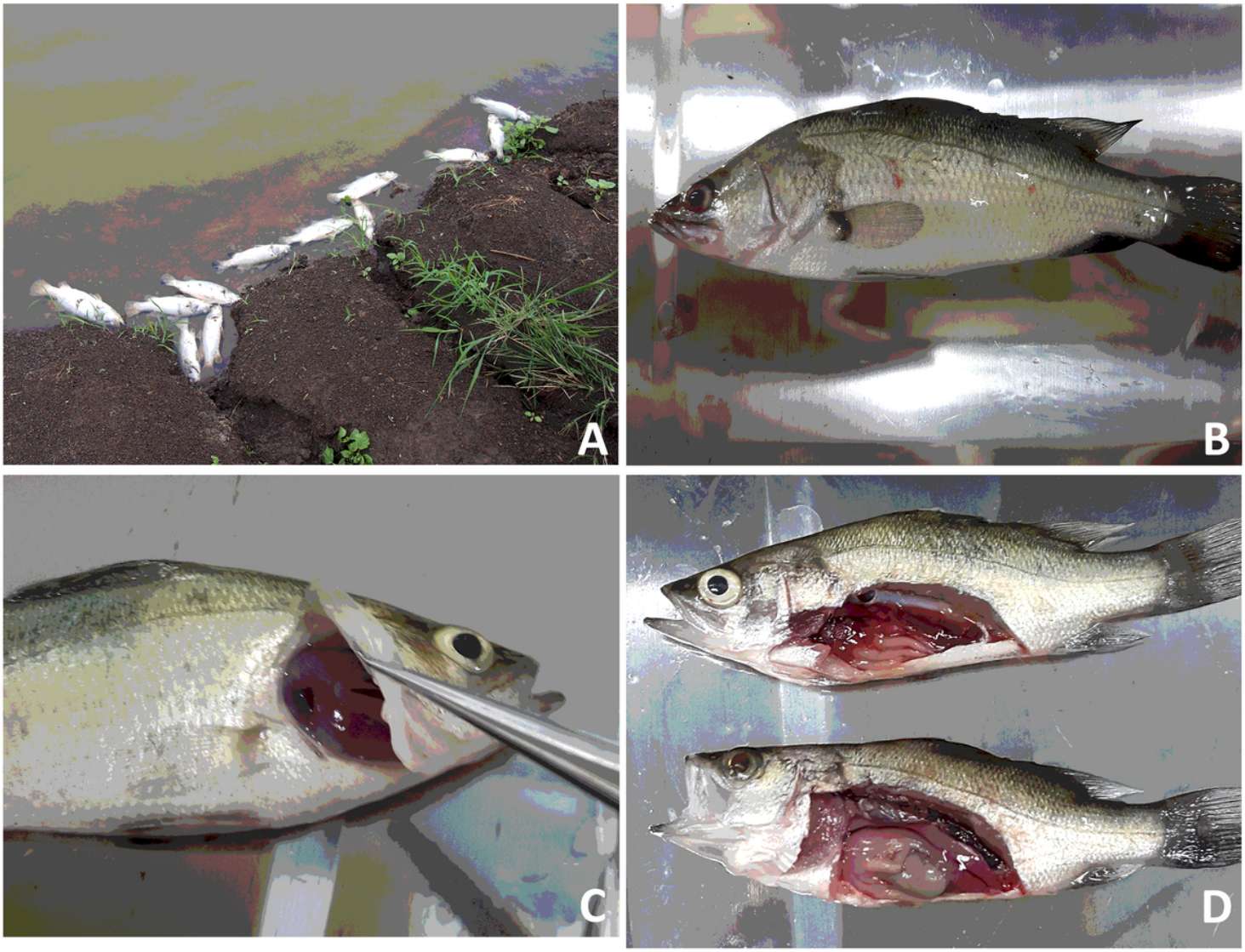

Figure 1

Macroscopic appearances of Asian sea bass naturally infected with ISKNV. (A) Aggregation of dead fish around the bank was commonly seen in the affected pond. (B) Affected fish exhibited darkened body with hemorrhage at skin and eyes, (C) pale gills. (D) Internally, swollen spleen and hemorrhage at liver and trunk kidney were observed often in moribund fish.

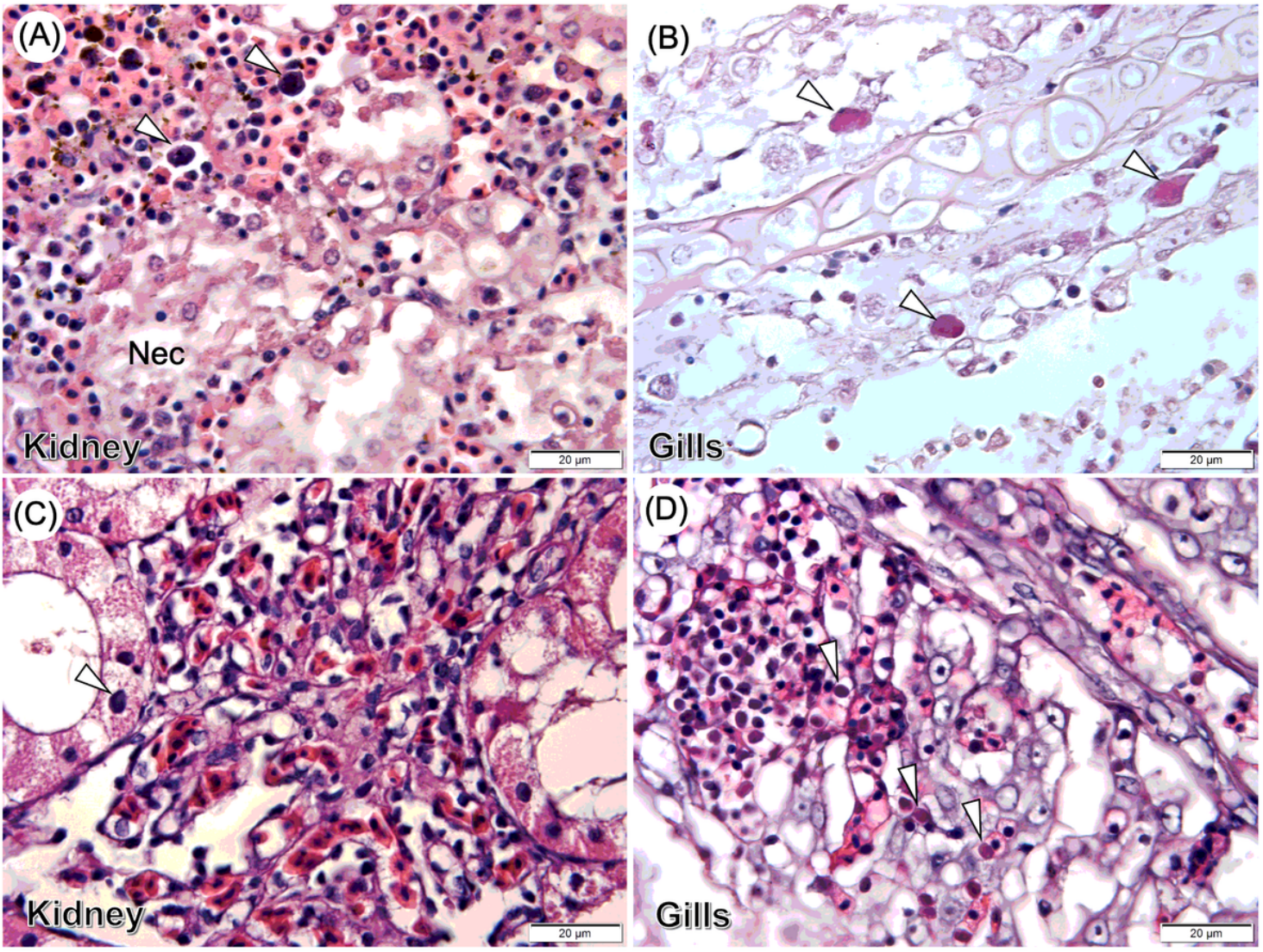

Figure 2

Histopathological appearances in trunk kidney and gill of Asian sea bass naturally infected with ISKNV (A, B) similar to those observed in experimentally infected fish (C, D). Head arrows, basophilic inclusion bodies (megalocytosis); Nec, necrosis of kidney tubules.

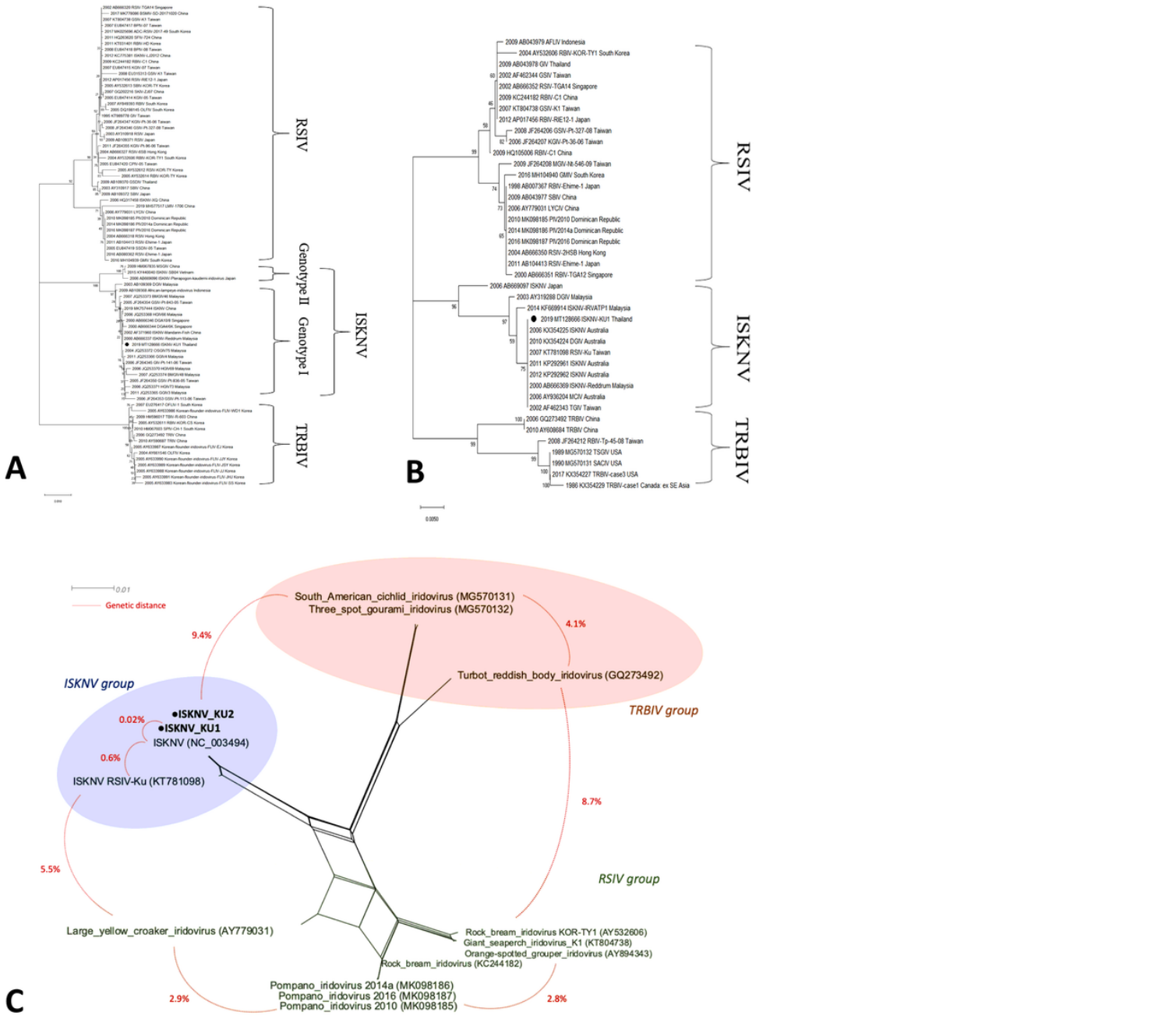

Figure 3

Phylogenetic analyses of megalocytiviruses comprised of ISKNV, RSIV and, TRBIV. Maximum likelihood tree generated from (A) MCP and (B) ATPase genes of the viruses. (C) Neighbor-net split graph generated from whole-genome alignment. Genetic distance between genomes was demonstrated as percent substitution per nucleotide (labeled in red and dashed line). The ISKNV recovered in this study was marked by a black circle. The number at the nodes of the tree represent the bootstrap value, whereas scale represent the rate of substitution per nucleotide.

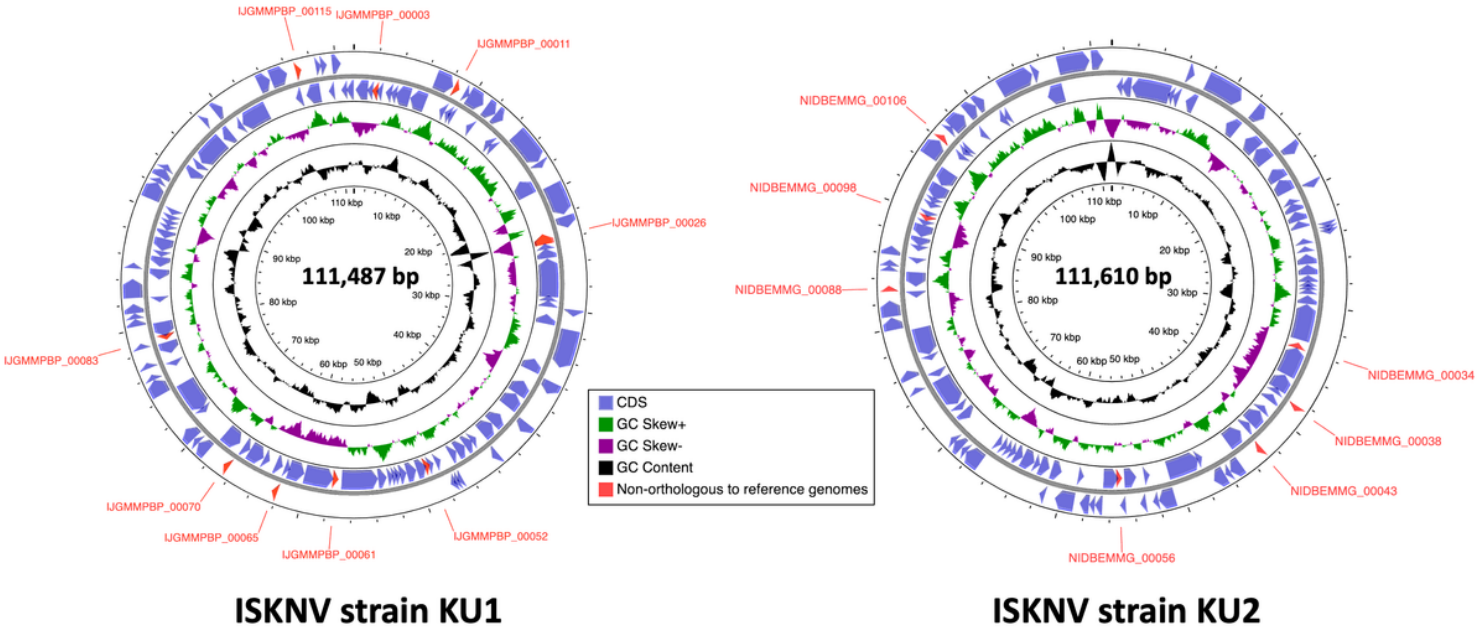

Figure 4

Circular map of ISKNV KU1, KU2 created by CGView. Red arrows in CDS rings represent non-orthologs, comparing to the ISKNV reference strain and ISKNV strain RSIV-Ku, with locus-tag labeled.

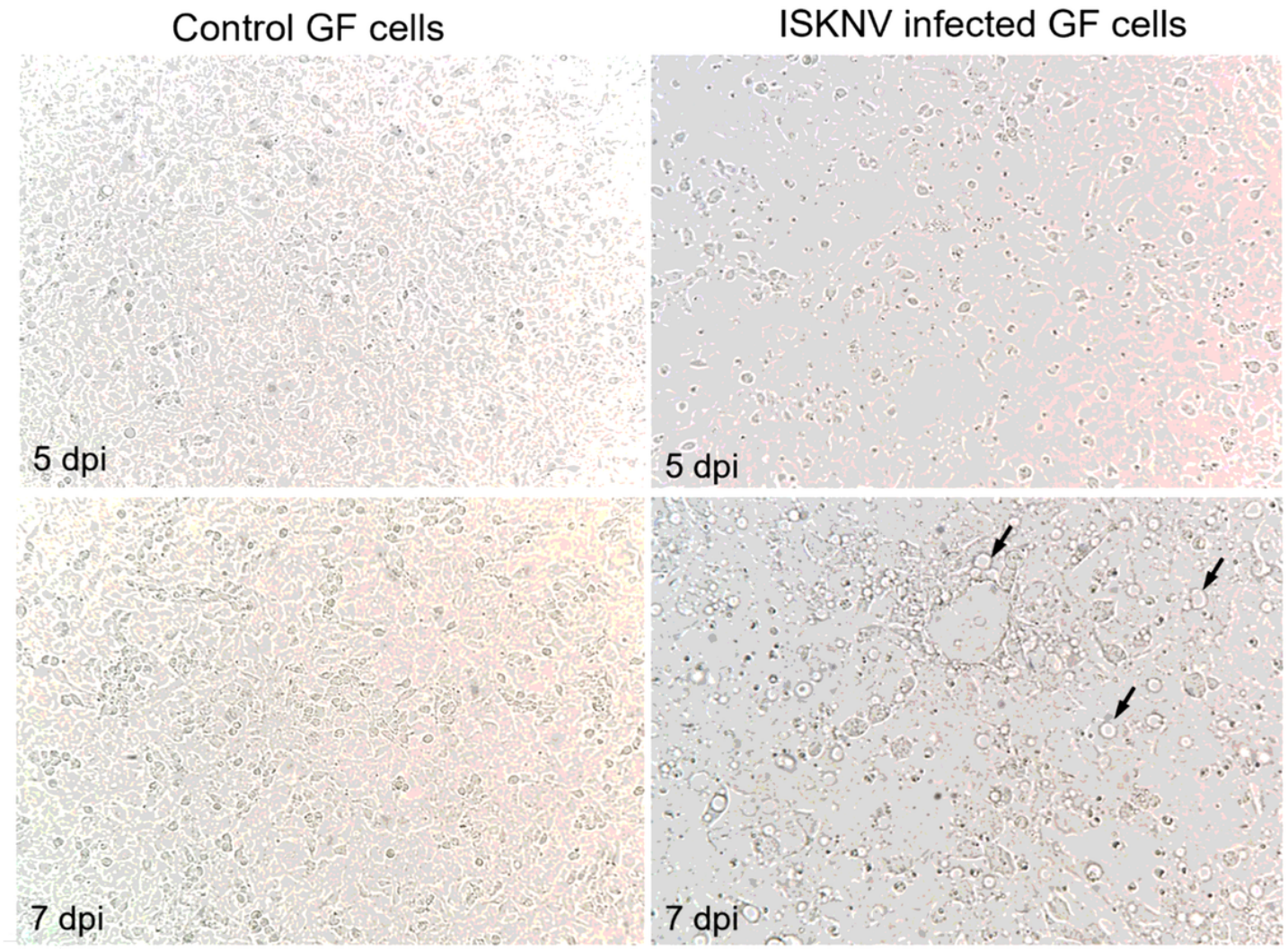

Figure 5

Photomicrograph of Grunt fin (GF) cell monolayer inoculated with filtrate extracted from the tissue of ISKNV-infected (right panel) Asian sea bass. Control GF cells (left panel) were culture with conditions identical to those of ISKNV-infected cells. Arrows indicated cytopathic effect (vacuolization) observed in the ISKNV-infected cells at 7 days post-inoculation.

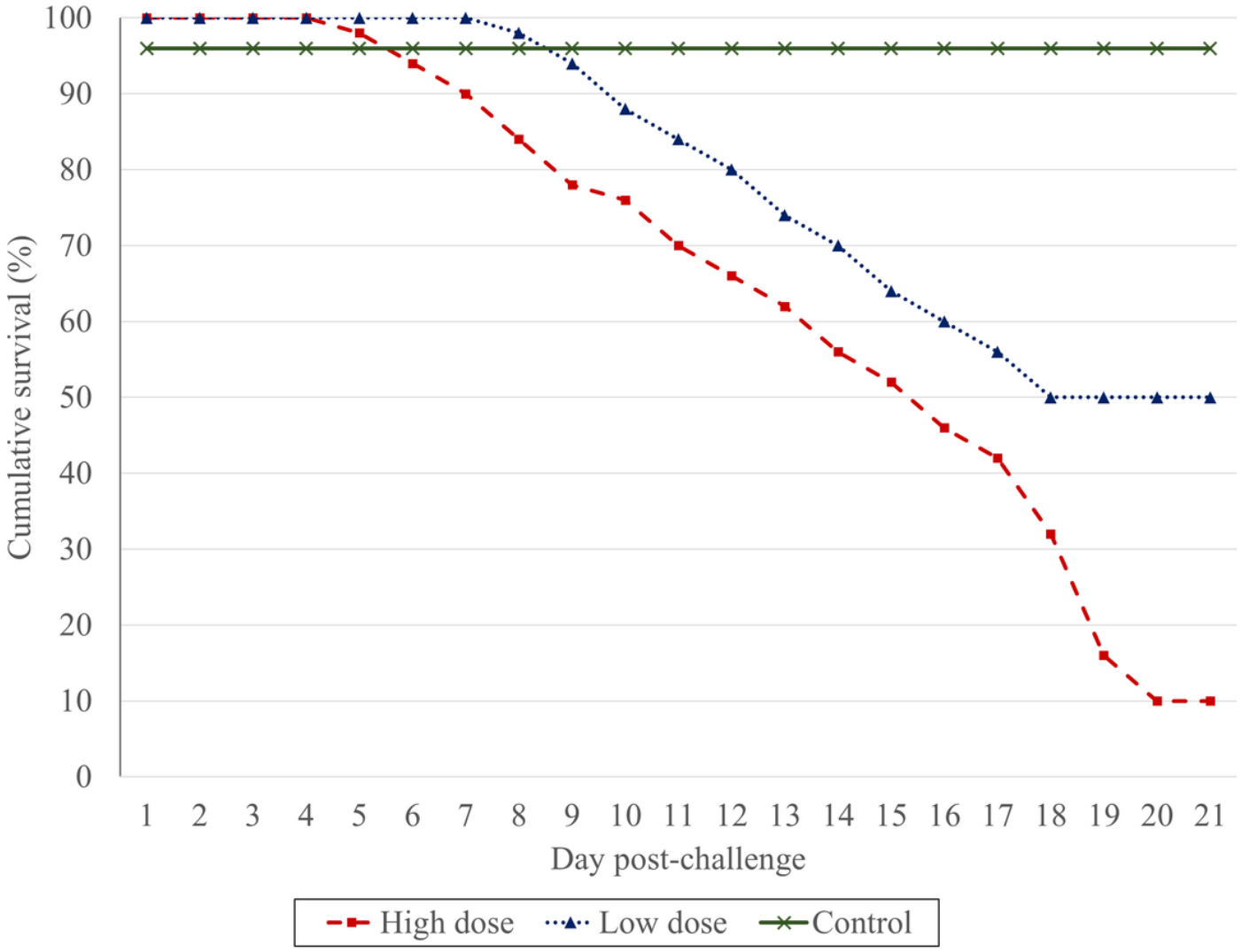

Figure 6

Cumulative survival of Asian sea bass experimentally infected with ISKNV high dose (2.9 × 105 virus copy numbers per fish) and low dose (red line, 2.9 × 103 virus copy numbers per fish). Fish in the control group were inoculated with a sterile cell culture medium.

Supplementary Files

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