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DETECTION AND GENOTYPE DETERMINATION OF LYMPHOCYSTIS DISEASE VIRUS INFECTING ORANGE CLOWNFISH, *Amphiprion percula* FARMED IN BATAM, INDONESIA

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ABSTRACT

The orange clownfish, *Amphiprion percula,* is a popular fish in the global marine ornamental trade. In March 2021, several orange clown fish at Batam Mariculture Development Center exhibited lethargic behavior with wart-like nodules on the fins suggesting a viral disease infection. This study aimed to detect the suspected viral disease agent from the clownfish and determine its genotype based on the major capsid protein gene. The fish tissues with wart-like nodules were collected and fixed in 96% ethanol. The DNA was extracted from the tissues and used as the template for the amplification of the major capsid protein (MCP) and myristylated *membrane protein* (MMP) genes using polymerase chain reaction (PCR). The nucleotide sequences of the PCR products were analyzed for their homology using the Basic Local Alignment Search Tool (BLAST). Multiple alignments of the amino acid sequence of MCP were performed using MEGA-X to determine the genotype. The PCR amplification produced the expected bands for detecting MCP, MMP and DNAPol genes. These results indicated the presence of Lymphocystis disease virus (LCDV), designated as LCDV-Oc-Btm. The sequencing of MCP and MMP genes produced the 1221 and 407 nucleotides, respectively. The BLAST analysis showed the highest identity was obtained with the species of LCDV-1 (LCDV-Sa strain) at 91.04 % and 88.19 % for MCP and MMP, respectively. The UPGMA phylogenic tree showed LCDV-Oc-Btm differs from the existing genotype and can be assigned as a new genotype. This study concludes that LCDV-Oc-Btm is a novel species of lymphocystis disease virus.

KEYWORD: Genotype; LCDV; MCP; MMP

INTRODUCTION

Lymphocystis disease virus (LCDV) is the causative agent of lymphocystis disease, affecting more than 140 species of marine and freshwater fish worldwide, both wild and farmed fish. The pathognomonic signs of lymphocystis-infected fish are mostly external multinodular tumor-like or wart-like masses growths on the fins, skin, or gills (Hick *et al*., 2016; Lam *et al.,* 2020). Nodular lesions may also be observed on the surfaces of viscera. However, in some species, such as yellowtail, nodules do not form, and the affected fibroblasts are disguised by overlying melanocytes, resulting in pigmented lesions (Hick *et al.,* 2016).

The tropism of LCDV-1 is on fibroblasts, with cytopathological characteristic as a cell hypertrophy in pathognomonic lesions as a nodule. Each nodule's diameter is up to 1 mm and consists of a single enlarged dermal fibroblast visible macroscopically. This cell hypertrophy can be confirmed by a direct microscope observation using a wet mount or histopathology. The cytoplasm of lymphocystis-affected cells contains multiple, highly reticulate or basophilic inclusion bodies in the nucleus, which are hypertrophic (Hick *et al*., 2016). The LCDV has a tropism limited to the dermis rather than causing a systemic infection. The mortality associated with lymphocystis was once considered to be rare. However, LCDV has been associated with significant mortality events in farmed fish (Hick *et al*., 2016).

Lymphocystis disease causes severe economic losses in the aquaculture and ornamental fish industries. Affected fish has low market value due to disease-associated anemia, the appearance of external lesions, reduction in the growth rate, high susceptibility to secondary bacterial infections, and high rates of cannibalism (Hick *et al.,* 2016; Maganha, 2020)

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LCDV is a large iridovirus with virions of heterologous size characterized by an icosahedral capsid approximately 200-230 nm in diameter, a double-layered capsid with an outer envelope and a fringe of fibril-like external protrusions (Chinchar *et al.,* 2017). LCDV is distinguished from other iridoviruses by a genomic GC content of 29% compared with 48–55% (Hick *et al*., 2016). The International Committee on Taxonomy of Viruses (ICTV) recognizes three species of LCDV as Lymphocystis disease virus 1 (LCDV-1), Lymphocystis disease virus 2 (LCDV-C) and Lymphocystis disease virus 1 (LCDV-Sa) with their genome of 102 kbp, 186 kbp and 208 kbp, respectively (Chinchar *et al*., 2017). A novel lymphocystis disease virus from whitemouth croaker (*Micropogonias furnieri*) (LCDV-WM) is the largest vertebrate iridovirus with a 211 kbp genome and 148 genes compared with 108 kbp for LCDV-1 (Doszpoly *et al*., 2020). This LCDV-WM has been established as a novel species, the Lymphocystis disease virus 4 (Walker *et al*., 2021).

Phylogenetic and evolutionary analyses of the family Iridoviridae are mainly based on the highly conserved major capsid protein (MCP) gene (Hossain *et al*., 2008; Maganha, 2020). Several genotypes were present in the genus Lymphocystivirus, based on MCP gene sequence and the pathogenicity of lymphocystiviruses: genotype I (consisting of LCDV-1); genotype II (consisting of Japanese flounder isolates and sea bass isolate); genotype III (consisting of rockfish isolates) (Kitamura *et al*., 2006); genotype IV (for the sea bass isolate), genotype V (for painted glass fish isolates) and genotype VI (for gourami isolates) (Hossain *et al*., 2008). Nine genotypes of Lymphocystiviruses have been described in yellow perch, *Perca flavescens* (Palmer *et al*., 2012).

The clown anemone fish 'Nemo' *Amphiprion ocellaris* is the most popular fish species in the global marine ornamental fish trade. Its popularity leads to the exploitation of this species' wild population. The densities *A. ocellaris* are significantly lower in coral reefs with high exploitation than in reefs with low exploitation (Madduppa *et al*., 2014). A study by Lam *et al.* (2020) has confirmed the lymphocystis disease virus from the captive-bred clownfish *Amphiprion percula* from South Sulawesi by sequencing a portion of the DNA polymerase (DNAPol) gene. Breeding efforts of the orange clownfish *A. percula*, has been conducted at Batam Mariculture Development Center. In March 2021, several orange clownfish reared in the grow-out containers looked lethargic and anorexic and had tumor-like nodules on the body surface. These symptoms signaled a possible infection of LCDV. However, the LCDV genotype based on the sequence of MCP in Indonesia has not been determined yet. This study aimed to confirm the presence of lymphocystis disease virus by PCR with MCP and myristylated *membrane protein* (MMP) primer and to determine the LCDV genotype.

MATERIAL AND METHODS

Fish Sampling

The Ornamental Fish Unit of Batam Mariculture Development Center was established to produce several ornamental marine fishes, including the orange clownfish *Amphiprion percula.* The clown fish production involves breeding, larva rearing, nursery and grow-out stages. Possible LCDV-infected fish observed in March 2021 were collected for morphological observation in the laboratory. The tissues with nodules were removed and preserved in ethanol 96%.

DNA Genomic Isolation

The tissue samples were washed using PBS. Approximately 25 mg of tissue was ground in a 200 µL buffer and treated with Proteinase K (1 mg/mL) at 60 ^{o}C for 1.5 hours. DNA isolation of samples was done using FavorPrep™ Tissue Genomic DNA extraction Mini Kit following the protocol recommended by the manufacturer. In the final step, the DNA was eluted in a 50 µL elution buffer and stored at -20 ^{o}C until used.

DNA amplifications of the major capsid protein (MCP), myristylated *membrane protein* (MMP), and DNA polymerase (DNAPol) LCDV genes were conducted using PCR . The PCR used a thermal cycler TM 100 (Biorad, US) with the specific primer pairs listed in Table 1. The PCR mixtures were composed of 2 µL of each oligonucleotide primer, 2 µL DNA template, 20 µL nuclease-free water, and 24 µL PCR mix 2x (My Taq Hs Red Mix, Bioline, US). The amplification was conducted with the reaction of the initial denaturation at 95^{o}C for 120 sec, 37 cycles of denaturation at 95^{o} C for 45 sec, annealing at 50^{o}C for 45 sec and extension at 72^{o}C for 120 sec, following one cycle of final extension at 72^{o}C for 7 min.

PCR products were evaluated using gel electrophoresis on a 1% agarose gel containing 0.03 % DNA stain (1st Base, Singapore) in the presence of a 100 bp DNA ladder (Geneaid, Taiwan). A commercial company (Genetika Science) performed the direct sequencing of PCR products.

Data Analysis

Obtained sequences were aligned to determine the consensus among the forward and reverse primer sequences. The consensus DNA sequences were analyzed using the Basic Local Alignment Search Tool

Table 1. Major capsid protein, myristylated membrane protein, and DNA polymerase primers for detection and sequencing of lymphocystis disease in orange clownfish, *Amphiprion percula* from Batam

Gene	Primer	Sequences	Reference
Major capsid protein	MCP-PF	ATGACTTCTGTAGCGGGTTCAAGTG	Huang *et al.* (2015)
	MCP-PR	AATTCAAARTTTTGWGCATCTTTATAACC	
Myristylated membrane protein	MMP-PF	CACAAGTAGCAGATATTAACAACA	
	MMP-PR	TCTTGAGCACAATCTTGAAATA	
DNA polymerase	DNA Pol-PF	ATGATAGTTTTTATTTTTCAATGG	
	DNA Pol-PR	AGC TGATATTTTACATGCTAATTG	

(BLAST) to look for the homology with the data on Genbank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignments for phylogenic analysis of this study sequences with the data in Genbank were conducted using the MEGA-X software (Kumar *et al*., 2016). The construction of phylogenetic trees based on the Maximum Parsimony analysis and bootstrap analysis was performed with resampling 1000 times.

RESULTS AND DISCUSSION

Clinical Signs

The sampled orange clown fishes reared in a conical fiberglass tank showed the clinical symptoms of lymphocystis. The four cm-long clownfish had white spots or small warts on the dorsal and caudal fins. The fully developed warts on the body surface were present in the caudal peduncle and abdomen (Figure 1). This clinical sign was similar to the white wart on the dorsal fins of LCDV-infected clownfish *A. percula* in Makassar (Lam *et al*., 2020). In this study, the lesions in the mouth were found in one fish (data not shown).

The genomic DNA from the wart-like tissue was used as a template for the amplification of MCP, DNA Pol, and MMP genes. The specific PCR product of MCP and MMP genes were detected with sizes of approximately 1200 bp and 400 bp, respectively. The amplification using DNA Pol primer produced a thin band of approximately 1600 bp as expected, but in the presence of several non-specific fine bands (Fig.2). These results using the three genes strongly confirmed that the orange clownfish from Batam was infected with LCDV and the virus was designated as LCDV-Oc-Btm. In several studies, the confirmation of LCDV only used one gene, such as Sihananto *et al.* (2019) on giant snakehead, and Lam *et al*. (2020) on orange clownfish.

The PCR products of MCP, MMP and DNA polymerase from LCDV-Oc-Btm were applied for sequencing. From the sequencing, the MCP and MPP genes were observed at 1221 and 407 nucleotides, respectively. The nucleotide sequences of the MCP and MPP genes are listed in Table 2 and have been deposited in Genbank with accession numbers OL606742 and

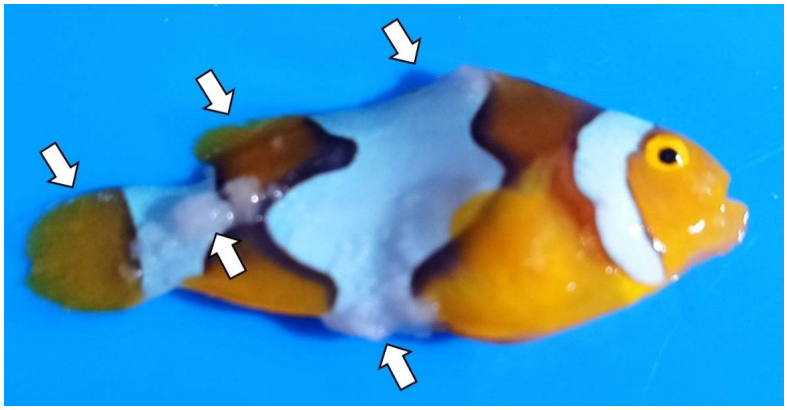

Figure 1. Orange clownfish, *Amphiprion percula* from Batam showing the presence of the whitish wart on the dorsal fin, caudal fin, and ventral skin.

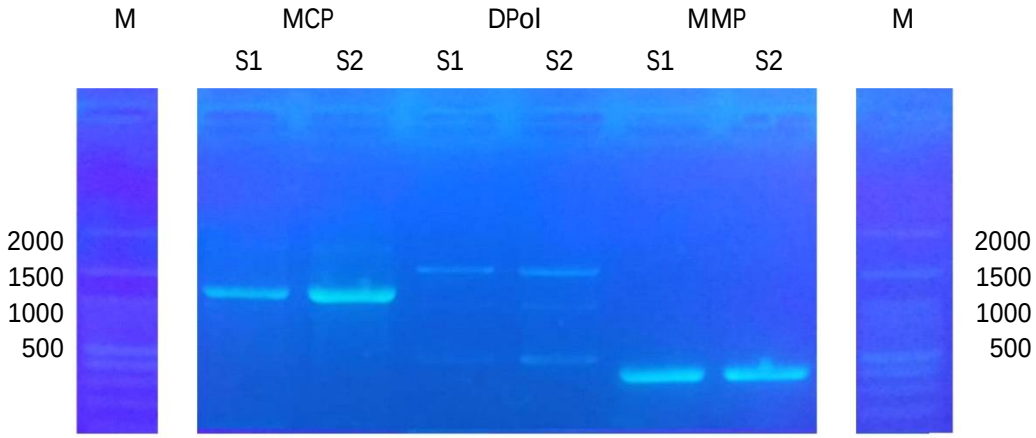

Figure 2. Agarose gel electrophoresis amplification product of major capsid protein (MCP), myristylated membrane protein (MMP) and DNA polymerase (MMP) of LCDV from orange clownfish *Amphiprion percula* in Batam with DNA marker as indicated size.

OL606743, respectively. Since multi bands were present in the agarose electrophoretic of the PCR product, DNA sequences of the polymerase gene of this LCDV could not be read.

The alignment analysis on the MMP using BLAST showed that only six entries have high similarity on nucleotides sequence. The highest identity of the MMP LCDV-Oc was at 90.53 % with the LCDV-PF (ac-

Table 2. The nucleotide sequences of the major capsid protein and myristylated membrane protein genes of LCDV-Oc-Btm from orange clownfish *Amphiprion percula*, Batam

Gene	Nucleotide sequence	Size (nt)
Major capsid proteian (MCP)	CCAGCGCTTTTATAGATTTAGCCACTTACGATACTATTGAAAAACATCTTTACGGCG GCGATTCAGCCGTGGCTTATTTTGTACGAGAAACTAAAAAATGTACTTGGTTCAGCA AATTACCGGTGCTTTTAACGCGATGCTCGGGATCGCCCAATTTTGACCAAGAATTTT CCGTTAACGTTTCTCGGGGCGGAGATTACGTCATCAACGCCTGGATGACGGTGCGTA TACCCGCCGTTAAATTGAAAAATAACAATAGAATGAACGCCAACGGCACCATTCGAT GGTGTAAAAATTTGTTTCACAATCTGGTCAAACAAACGTCCGTTCAATTCAACGATT TGGTGGCTCAAAAATTCGAGAGTTACTTCCTCGATTTCTGGTCCGCCTTTGGCATGT GCGGATCTAAACGAACGGGCTACGACAACATGATCGGAAATACCGCCGATATGACGC AACCCGTCGATTCCAACGGTCAACTACCCGAAAAAGTGTTGGTATTACCTTTACCTT ATTTCTTTTCCCGGGACAGCGGCGTAGCTTTACCCAGCGCCGCTCTGCCTTATAACG AAATAAGATTAACCTTTCATCTCAGAGATTGGACCGAATTGCTTATTTTTCAAAACA AAAACGACTCTACCATCATGCCTCTAACGGCCGGCGATTTAGAATGGGGTAAACCCG ATTTAGAGGACGTACAAGTATGGATCACCAACGTCGTGGTAACCAACGAAGAACGTC GATTGATGGGTACGGTACCCAGAGATATTTTGGTGGAACAAGTACAAACGGCGCCGA AACACGTGTTTCAACCGTTGACCATCCCCAGTCCCAATTATGACATCAGATTTTCTC ACGCCATCAAAACTCTTTTTTTCGGCGTGCGCAACGTGACTCATCAAGCCGTACAAT CCAATTATACCAGTTCTTCTCCGGTTATTTTTGACGGCGGAATAGCCAGCGATTTGC CGGGTATCGCCGCCGATCCTATTTCGGACGTGACCTTGGTGTACGAAAACAGCGCGC GTCTCAACGAAATGGGCAGCGAATATTATTCTTTGATTCAACCTTATTATTTCGGGG GTTCTATTCCTATAGAAACGGGTTATCACATGTATTGTTATTCTCTCAACATGATGG ACGTCGTTCCTATGGGATCCACCAATTACGGTCGATTGTCCAACGTCAGCATTAAAT TGAAAACGTCTCCTAAAGCGGTAA	1221
Myristylated membrane protein (MMP)	CACAAGTAGCAGATATTAACAACAGTCAAATCGTTACCGTTTCGGACGTAGACGGCG ACGTGGATGTTTCGGACGTGTATTTTGTTCAACAAATCGACGTCAACGTAAAAAGTT TGATGAACGTATTGTCTCAGCAAAAAGCTCAACAAGATTTATTCGAAGCCGTAGCTC AAAACGCTAAATCCGTCACTTCAGGTTTAAACGCCGCGCAATACGCTTACGCCGTTA ATCAAATCAGCGCTATAGCAACGGCGTGCGTTCAAGCTGCCGTACGCATCGACGATT CGTGTTCGTTGGTCGACGCTATGACGCAAGAAATCGTTTTACAAAACGTAGGCGGTT CCGTTAAAATAAAAGATTTAAATCTGTCTCAAATACAACGCGTATTTCAAGATTGTG CTCAAGAA	407

cession number KJ408273), which was isolated from paradise fish *Macropodus opercularis* in China (Xu *et al*., 2014). The second highest identity was at 88.19 % to LCDV-Pa (accession number KX643370) isolated from gilthead seabream (*Sparus aurata*) in Spain (López-Bueno *et al.,* 2016). The MMP of LCDV-Oc-Btm has identity at 84% to the MMP of LCDV-C, LCDV-JP-Oita, and LCDV-WC (Table 3).

The MCP gene is highly conserved in the family of Iridoviridae and has been used for phylogenetic and evolutionary analyses (Hossain *et al*., 2008; Maganha, 2020). The alignment analysis on the MCP using BLAST showed a high nucleotide sequence identity to 51 entries of Lymphocystis disease virus MCP genes. This MCP LCDV-Oc-Btm has the highest identity at 92.15 % to LCDV-PF (accession number KJ408271) isolated from paradise fish *M. opercularis* in China (Xu *et al*., 2014). The second highest identity was at 91.24 % to LCDV isolated from painted glass fish collected from an aquarist shop in Yeosu City, Korea (Hossain *et al*., 2008). This MCP sequence showed 91.04 % identity to LCDV-Sa isolated from gilthead seabream *Sparus aurata* in Spain (López-Bueno *et al*., 2016). The MCP of LCDV-Oc-Btm has identity at approximately 84% to the MCP of LCDV-C, LCDV-JP-Oita, and LCDV-WC. The MMP LCDV-1 had the lowest identity at 76.80 % to MCP LCDV-Oc-Btm (Table 3).

Based on the complete genome data, the ICTV recognizes four species of LCDV as *Lymphocystis disease virus 1* (LCDV-1), *Lymphocystis disease virus 2* (LCDV-C), *Lymphocystis disease virus 3* (LCDV-Sa) and *Lymphocystis disease virus 4* (LCDV-WM) (Walker *et al*., 2021). The identity of LCDV-Oc-Btm to those established LCDV species is presented in Table 3. The LCDV-Oc-Btm has the highest identity to the LCDV-Sa at 91.04 % and 88.19 % for MCP and MMP sequences, respectively. These results indicate that this Indonesian LCDV is close to the species of *Lymphocystis disease virus 3*.

The identity of MCP intra-species of LCDVs is very high, at least at 98.77 % among the *LCDV-3* species and at 99.70 % among the *LCDV-2* species (data not shown). In this study, the highest identity is only approximately 91.04 % which therefore gives the possibility that this Indonesian LCDV is a novel species of LCDV. Lam et al. (2020) also suggested a new strain LCDV from A. percula in Makassar South Sulawesi using the DNA polymerase sequence analysis. These DNAPol sequences showed the highest identity of nucleotides at 91.2 % to LCDV-PF from Paradise Fish in China. This LCDV-Oc-Btm also showed the highest identity to LCDV-PF. These results suggested that the LCDVs from Batam and Makassar are the same species.

The phylogenetic and evolutionary analyses of the family Iridoviridae are mainly based on the highly conserved MCP gene. The determination of Iridoviridae genotypes based on MCP genes has been widely used, from which nine genotypes existed in the genus Lymphocystivirus (Palmer *et al.*, 2012). The MCP amino acid sequences were used to construct the phylogenic tree of this LCDV-Oc-Btm with other MCP lymphocystis viruses (Fig 3). The LCDV-Oc-Btm is located at a distinct branch indicating that the genotypes of LCDV-Oc-Btm differ from the existing genotypes. There are 23 of 403 amino acid residue differ-

Table 3. Blast analysis of major capsid protein and myristylated membrane protein genes of LCDV-Oc-Btm from orange clown fish *A. percula* in Batam with established LCDV species

Virus strain Acession number	Species	Identity	
		MCP	MMP
LCDV-PF KJ408271.1, KJ408273.1	n.a	91.15	90.53
LCDV-Sa KX643370.1	LCDV-3	91.03	88.19
LCDV-C AY380826.1	LCDV-2	83.73	84,75
LCDV-JP_Oita LC534415.1	LCDV-2	83.73	84.75
LCDV-WC NC_055603.1	LCDV-4	84.76	84.92
LCDV-1 L63545.1	LCDV-1	76.8	69.4

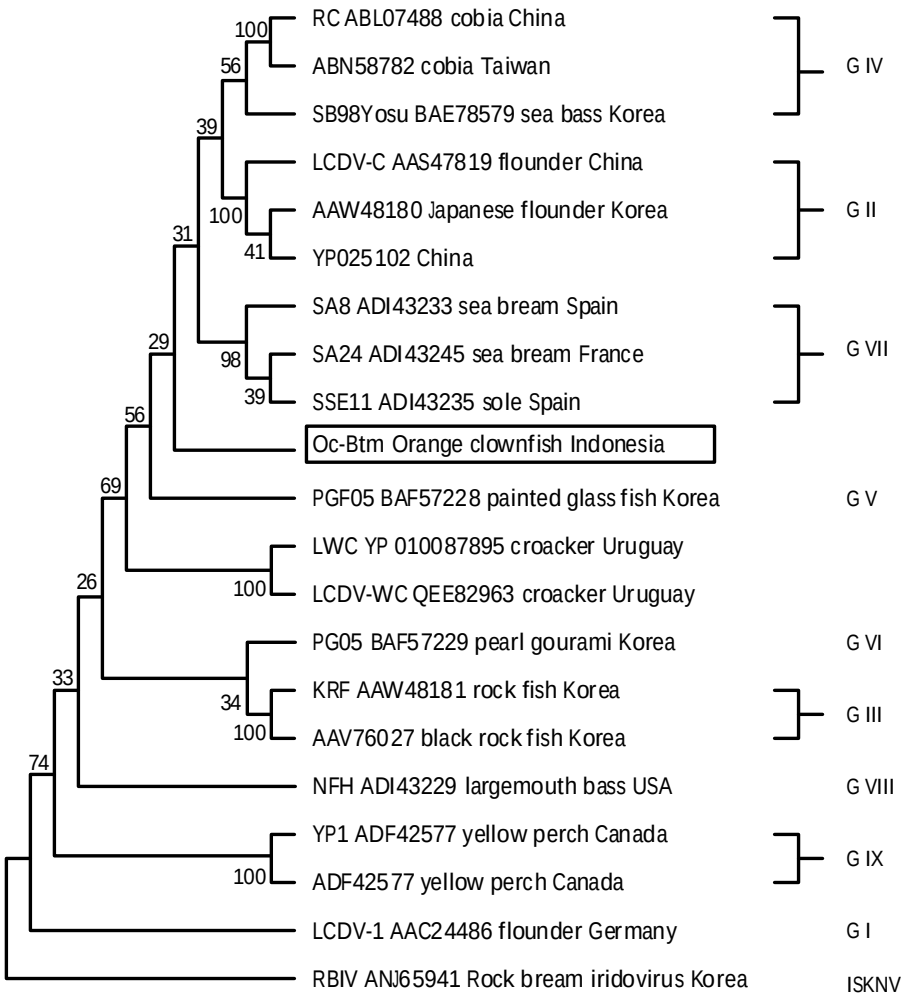

Figure 3. Phylogenic three on the amino acid sequence of major capsid protein of LCDV with the virus strain, Genbank accession number, host and country are indicated. The box highlights the position of LCDV-Oc-Btm. G I–G IX are existing genotypes I to IX. The Infectious Spleen and Kidney Necrosis Virus (ISKNV) member of Megalocytivirus are used as the outgroup.

ences between LCDV-Oc-Btm and the nearest genotype of YP025012-China. Therefore, LCDV-Oc-Btm could be assigned as a new genotype (10th genotype). The other branch also appeared as the LCDV in the croaker from Uruguay, where the genotype was not determined.

There are a limited number of Lymphocystis disease studies in Indonesia. One of the studies was by Lam *et al*. (2020), who used a DNA polymerase gene to identify a lymphocystis disease virus (LCDV) using PCR, construct its phylogenetic, and develop detection assays. The LCDV is found at 19.4% among the PCR-tested clownfish but not in seahorse *Hippocampus* spp., mandarin fish *Synchiropus* spp., and pajama cardinal fish *Sphaeremia nepatoptera* from several hatcheries in Makassar. A study by Sihananto *et al.* (2019) reported the presence of Lymphocystis virus in farmed giant snakehead *Channa striata* in Mandiangin South Kalimantan through histopathological and PCR investigations. Such limited studies, coupled with the increasing number of farmed and potentially farmed species in Indonesia (Albasri *et al*., 2020), imply the need to study the LCDV and its potential hosts in Indonesia. The results of this study and that of Lam *et al*. (2020) suggest that the Indonesian LCDV differs from the other LCDV species.

CONCLUSION

The orange clownfish *Amphiprion percula* from Batam with wart-like nodules was infected by the lymphocystis disease virus. Based on the major capsid protein, myristylated *membrane protein* genes, this study suggests that the LCDV-Oc-Btm is a new species of lymphocystis disease virus. The genotype of LCDV-Oc-Btm differs from the existing genotypes and can be assigned as a new genotype (10^{th} genotype). A more comprehensive genetic study on this LCDV is needed to verify the result of this study further.

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