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Identification and characterization of a novel lymphocystis disease virus isolate from cultured grouper in China

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Abstract

Grouper *Epinephelus* spp. is one of the most important mariculture fish species in China and South-East Asian countries. The emerging viral diseases, evoked by iridovirus which belongs to genus *Megalocytivirus* and *Ranavirus,* have been well characterized in recent years. To date, few data on lymphocystis disease in grouper which caused by lymphocystis disease virus (LCDV) were described. Here, a novel LCDV isolate was identified and characterized. Based on the sequence of LCDV major capsid protein (MCP) and DNA polymerase gene, we found that the causative agents from different species of diseased groupers were the same one and herein were uniformly defined as grouper LCDV (GLCDV). Furthermore, H&E staining revealed that the nodules on the skin were composed of giant cells that contained inclusion bodies in the cytoplasm. Numerous virus particles with >210 nm in diameter and with hexagonal profiles were observed in the cytoplasm. In addition, phylogenetic analysis based on four iridovirus core genes, MCP, DNA polymerase, myristoylated membrane protein (MMP) and ribonucleotide reductase (RNR), consistently showed that GLCDV was mostly related to LCDV-C, followed by LCDV-1. Taken together, our data firstly provided the molecular evidence that GLCDV was a novel emerging iridovirus pathogen in grouper culture.

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Introduction

Grouper *Epinephelus* spp. is one of the most important mariculture fish species in China and South-East Asian countries because of the high value for food (Pierre *et al.* 2008). Furthermore, due to its ability to change sex from female to male during development, grouper was also addressed as an ideal model for studying sex differentiation and reproduction (Zhou & Gui 2010). However, rapid expansion and intensification of aquaculture resulted in the increased incidence of disease outbreaks in recent years. The emerging infectious virus pathogens, including iridovirus and nodavirus, caused great economic losses in grouper aquaculture (Qin *et al.* 2003; Chao *et al.* 2004). To date, increased iridovirus isolates were identified from different species of groupers in different geographical zones. Among them, Singapore grouper iridovirus (SGIV) isolated from brown-spotted grouper, *Epinephelus tauvina* Forskal, and grouper iridovirus (GIV) isolated from diseased yellow grouper, *Epinephelus awoara* (Temminck & Schlegel), were identified to belong to genus *Ranavirus* (Murali *et al.* 2002; Qin *et al.* 2003). In addition, numerous iridovirus isolates from grouper were classified into genus *Megalocytivirus*, including orange-spotted grouper iridovirus (OSGIV) isolated from orange-spotted grouper, *Epinephelus coioides* (Hamilton) (Lü *et al.* 2005), grouper sleepy disease iridovirus (GSDIV)

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from Malabar grouper, *Epinephelus malabaricus* (Bloch & Schneider 1801) (Sudthongkong, Miyata & Miyazaki 2002), king grouper iridovirus (KGIV) isolated from giant grouper, *Epinephelus lanceolatus* (Bloch) (Wang *et al.* 2009), and grouper iridovirus in Taiwan (TGIV) isolated from cultured groupers (Chao *et al.* 2004).

Lymphocystis disease virus (LCDV), which belongs to genus *Lymphocystivirus,* was the aetiological agent of lymphocystis disease (Alonso *et al.* 2005; Huang *et al.* 2007; Sheng, Zhan & Wang 2007). To date, the research progress of LCDV mostly focused on that isolated from Japanese flounder, *Paralichthys olivaceus* (Temminek & Sehlegel), in China (LCDV-C) and European flounder (*Platic*htys flesus L.) (LCDV-1) (Tidona & Darai 1997; Zhang *et al.* 2004). In addition, LCDV was also identified and characterized in other different fish species (Peters & Schmidt 1995; Alonso *et al.* 2005; Kitamura *et al.* 2006; Sheng *et al.* 2007). To explore the molecular mechanisms of LCDV pathogenesis, the LCDV-1 and LCDV-C were completely sequenced and annotated (Tidona & Darai 1997; Zhang *et al.* 2004). The significant difference in genome sequences between LCDV-C and LCDV-1 suggested that more gene information from different LCDV isolates will contribute greatly to our understanding of genetic variations and evolution of LCDVs.

In the current study, we identified the pathogen of lymphocystis disease in grouper using electron microscopy and DNA sequencing. Our data firstly provided the molecular evidence of lymphocystis disease virus in grouper. Further investigation on the virus morphogenesis and genome sequence annotation will provide new insights into understanding the mechanism of LCDV pathogenesis.

Material and methods

Samples collection and experiment infection

The diseased giant grouper, *E. lanceolatus* (Bloch), orange-spotted grouper, *E. coioides* (Hamilton), and tiger grouper, *Epinephelus fuscoguttatus* Forsskal, were collected from the farms in Hainan and Fujian provinces of China. The nodules on the skin or fins were cut for further analysis.

For experimental infection, orange-spotted groupers were acclimatized under laboratory conditions for 5 days, and then, three of them were killed to confirm the absence of LCDV by PCR. Each group of 20 fish was immersed for 1 h in sea water containing 5 mL of suspension of the lymphocystis tissue homogenates or PBS (control) and then maintained for 45 days. Clinical signs of disease were monitored everyday, and the incidence was calculated at 45 days post-challenge (p.c.).

Paraffin sectioning and histochemical staining

The nodules on the fins from diseased grouper were stripped from the skin and fixed with formaldehyde. Then, samples were processed for routine paraffin sectioning and stained with H&E for histopathological observation as described previously (Sheng *et al.* 2007).

Transmission electron microscopy

The skin nodules from diseased grouper were cut into ~2 × 2 mm thick pieces and fixed with 2.5% glutaraldehyde overnight. Sample preparation was performed as described previously (Huang *et al.* 2011). In brief, the samples were post-fixed in 1% osmium tetroxide (OsO4) for 1 h after washing with PBS and then dehydrated in graded ethanol. The samples were embedded in Epon resin. Sections were double-stained with uranyl acetate and lead citrate. The grids containing ultrathin sections were examined in a JEM-1400 electron microscopy (JEOL) at 120 kV, and micrographics were taken by CDD camera.

PCR amplification

Total DNA of skin nodules was extracted for further study using Genomic DNA Miniprep kit (Biomga). Specific primers for LCDV MCP, DNA polymerase, MMP and RNR genes were designed according to the nucleotide alignment of these genes from LCDV-1 and LCDV-C (Tidona & Darai 1997; Zhang *et al.* 2004). The primers used in this study are listed in Table 1. PCR amplification was performed using LA Taq® (TaKaRa) according to the manufacturer's instruction, and the procedure parameter was listed in the following: 95 °C for 3 min, followed by 35 cycles of 45 s at 95 °C, 45 s at 47 °C and 2 min at 72 °C. The PCR fragments were purified and sequenced using an ABI 3730 DNA Analyzer.

Table 1 Primers used in this study

Primer name	Primer sequence (5′-3′)
MCP-PF	ATGACTTCTG TAGCGGGTTCAAGTG
MCP-PR	AATTCAAARTTTTGWGCATCTTTATAACC
DNA Pol-PF	ATGATAGTTTTTATTTTTCAATGG
DNA Pol-PR	AGC TGATATTTTACATGCTAATTG
MMP-PF	CACAAGTAGCAGATATTAACAACA
MMP-PR	TCTTGAGCACAATCTTGAAATA
RNR-PF	TTCTGATGTAGTAGAATGTTGTGT
RNR-PR	ATAAACCTTTACAACCTAACTTCC

Bioinformatics analysis

The obtained sequences were assembled and analysed using BLASTN and BLASTP (NCBI database). Amino acid alignments were carried out using clustalX and edited by Gendoc (Larkin *et al.* 2007). The phylogenetic tree was constructed by the neighbour-joining method using Molecular Evolutionary Genetics Analysis (MEGA 4.0) (Tamura *et al.* 2007).

Results

Signs of lymphocystis disease in grouper

Diseased groupers were collected from the fish farms in Fujian and Hainan provinces. The majority of nodules were seen on the fins of the diseased fish, including giant grouper (Fig. 1a), orange-spotted grouper (Fig. 1b) and tiger grouper (data not shown). In addition, papilloma-like lesions were also observed on the surface of the skin.

Identification of the causative agents from different groupers as the same LCDV isolates

According to the similar exterior sign of these diseased groupers, we designed the specific primers to amplify the fragments of LCDV MCP and DNA polymerase. As shown in Fig. 2, the specific products of MCP and DNA polymerase gene were obtained in the nodules from giant grouper, orange-spotted grouper and tiger grouper.

To clarify whether the pathogens from these different samples were the same isolate, the specific gene fragments of MCP and DNA polymerase were sequenced. Nucleotide sequence alignment indicated that the identity among LCDV isolates from giant grouper, orange-spotted grouper and tiger grouper was 100%. Herein, we designated the virus agent from these diseased groupers as grouper LCDV (GLCDV). Further homology search revealed that GLCDV MCP gene shared 90% identity with LCDV-C, followed by 80% with LCDV-1. Moreover, DNA polymerase gene from GLCDV showed 90% identity to LCDV-C, followed by 71% to LCDV-1, suggesting that GLCDV was different from LCDV-C and LCDV-1 isolates.

To determine the pathogenicity of GLCDV in grouper, GLCDV was used for experimental infections of orange-spotted grouper. The results showed that lymphocystis cells were firstly observed on the fins and around the mouth of orange-spotted grouper at 23 days p.c., and the incidence of lymphocystis disease reached 55% at 45 days p.c., while no signs were observed in the control group. Together, GLCDV was proposed as the causative agent of lymphocystis disease in groupers.

Histopathology of nodules from diseased grouper

The nodules were composed of growing clusters of lymphocytic cells. After the histological sections stained by HE, many inclusion bodies were strongly stained by haematoxylin and observed peripherally near the membrane (Fig. 3).

Ultrastructure of the virus particles

To clarify whether the papilloma-like lesions contained virus particles, the nodules were cut and prepared for electron microscopy observation. As shown in Fig. 4, numerous virus particles with >210 nm in diameter and with hexagonal profiles were observed. Moreover, the virus particles are organized into crystalline arrays, which were the typical characteristics of iridovirus infection.

Phylogenetic position of GLCDV in family Iridoviridae

Given that the DNA polymerase is an essential enzyme of transcription of replicating systems cytoplasmic DNA viruses (Sonntag & Darai 1995), we cloned the full length of DNA polymerase encoded by GLCDV and elucidated its structure. Although significant sequence variations existed between GLCDV and other LCDV isolates, the amino acid alignment revealed that the functional multidomain structure in eukaryotic and prokaryotic DNA polymerase genes was also

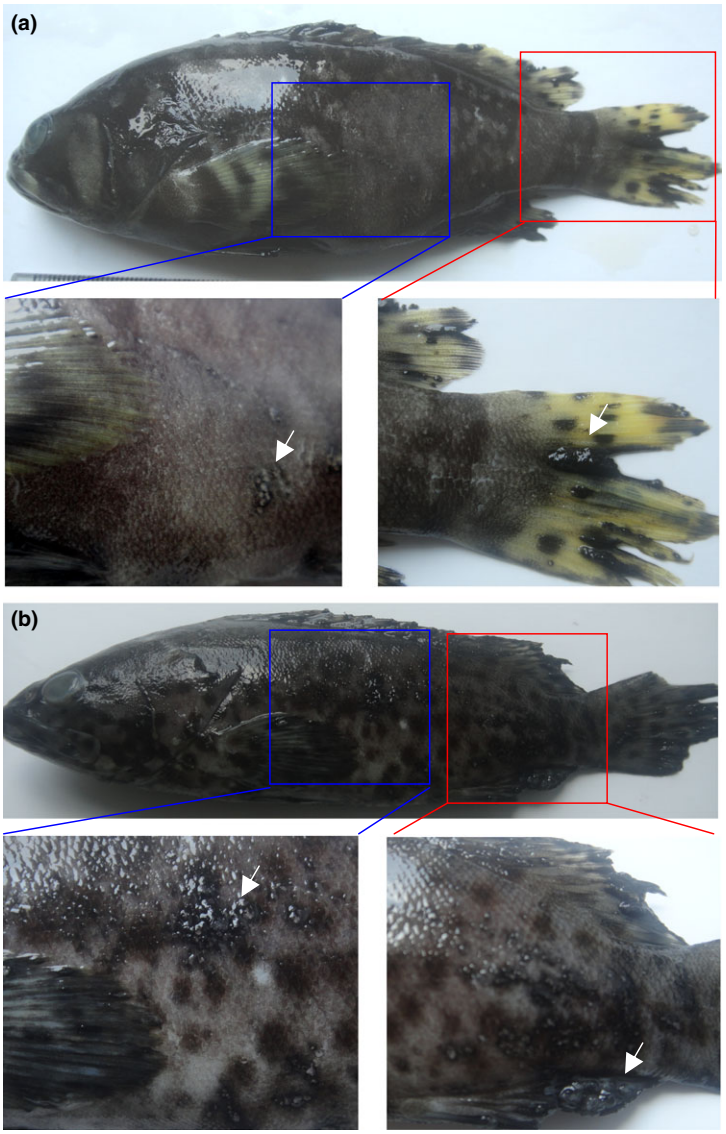

Figure 1 Typical signs of lymphocystis diseased grouper. The nodules (arrows) were observed on the skin and fin of orange-spotted grouper (a) and giant grouper (b).

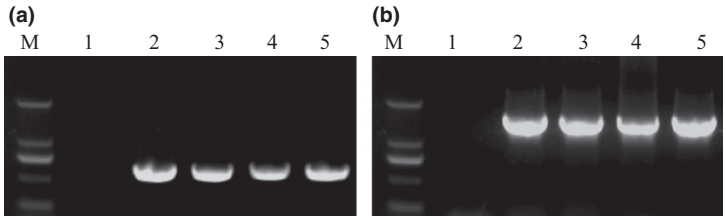

Figure 2 PCR amplification of MCP (a) and DNA polymerase (b) from different diseased groupers. Genomic DNA was extracted from the skin of normal groupers (lane 1) and nodules of diseased orange-spotted grouper from Hainan (lane 2) and Fujian (lane 3), diseased giant grouper from Hainan (lane 4) and diseased tiger grouper from Fujian (lane 5). The specific products were electrophoresed and photographed.

present in GLCDV DNA polymerase. Moreover, three other conserved regions, ExoI, ExoII and ExoIII that associated with 3′ and 5′ exonuclease activity, were also present in DNA polymerase encoded by GLCDV and other iridoviruses (Fig. 5).

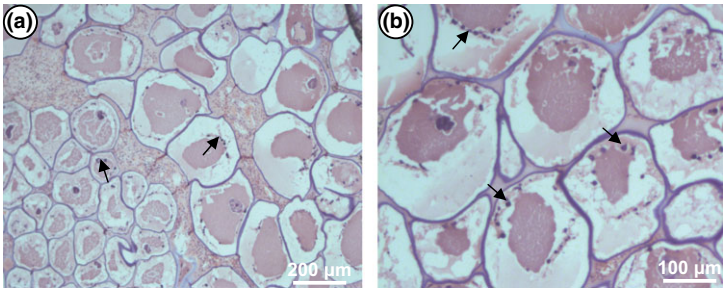

Figure 3 Histological sections of the nodules from the diseased grouper. Arrows indicated the inclusion bodies in the cytoplasm.

To further ascertain the phylogenetic relationship between GLCDV and other iridovirus isolates, we also amplified another four iridovirus core genes, MCP, DNA polymerase, myristoylated membrane protein (MMP) and ribonucleotide reductase (RNR). Phylogenetic trees were constructed using MEGA 4.0 based on the amino acid sequences of these genes. As shown in Fig. 6, phylogenetic analysis consistently revealed that GLCDV shared the closest relationship to LCDV-C, followed by LCDV-1 and other iridovirus isolates. Taken together, we proposed that GLCDV was a novel LCDV isolate in family Iridoviridae.

Discussion

Iridoviruses are large DNA viruses that were subdivided into five genera, *Ranavirus*, *Megalocytivirus*, *Lymphocystivirus*, *Chloriridovirus* and *Iridovirus* (Williams, Barbosa-Solomieu & Chinchar 2005). In recent years, iridovirus infection has caused great economic losses in grouper aquaculture. Notably, increased attention was focused on the iridovirus isolates from groupers which belonged to genus *Ranavirus* and *Megalocytivirus* to date (Qin *et al.* 2003; Lü *et al.* 2005). In our study, we observed numerous nodules on the skin or fins of different species of diseased groupers

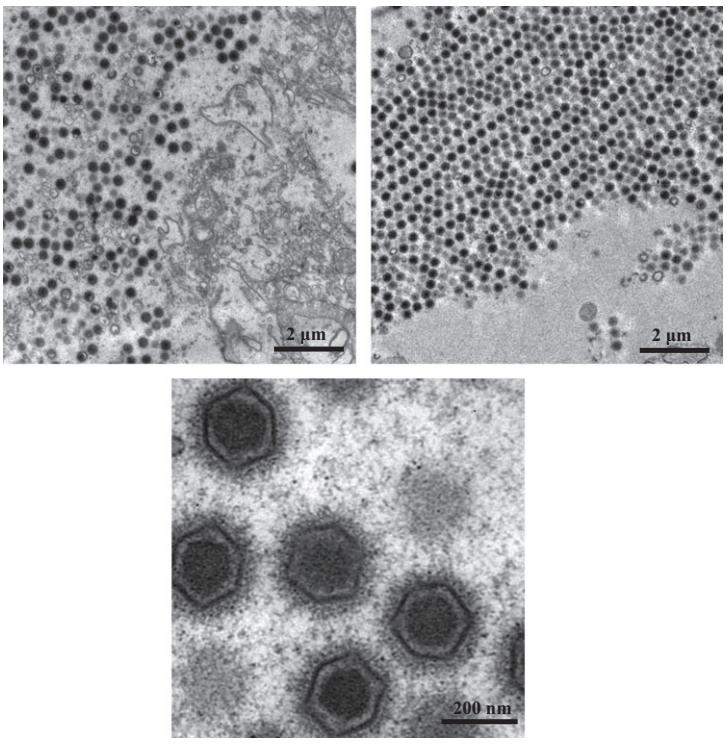

Figure 4 Transmission electron microscopy observation of the skin nodules from diseased grouper. Numerous viral particles with >210 nm in diameter and with hexagonal profiles were observed in the cytoplasm.

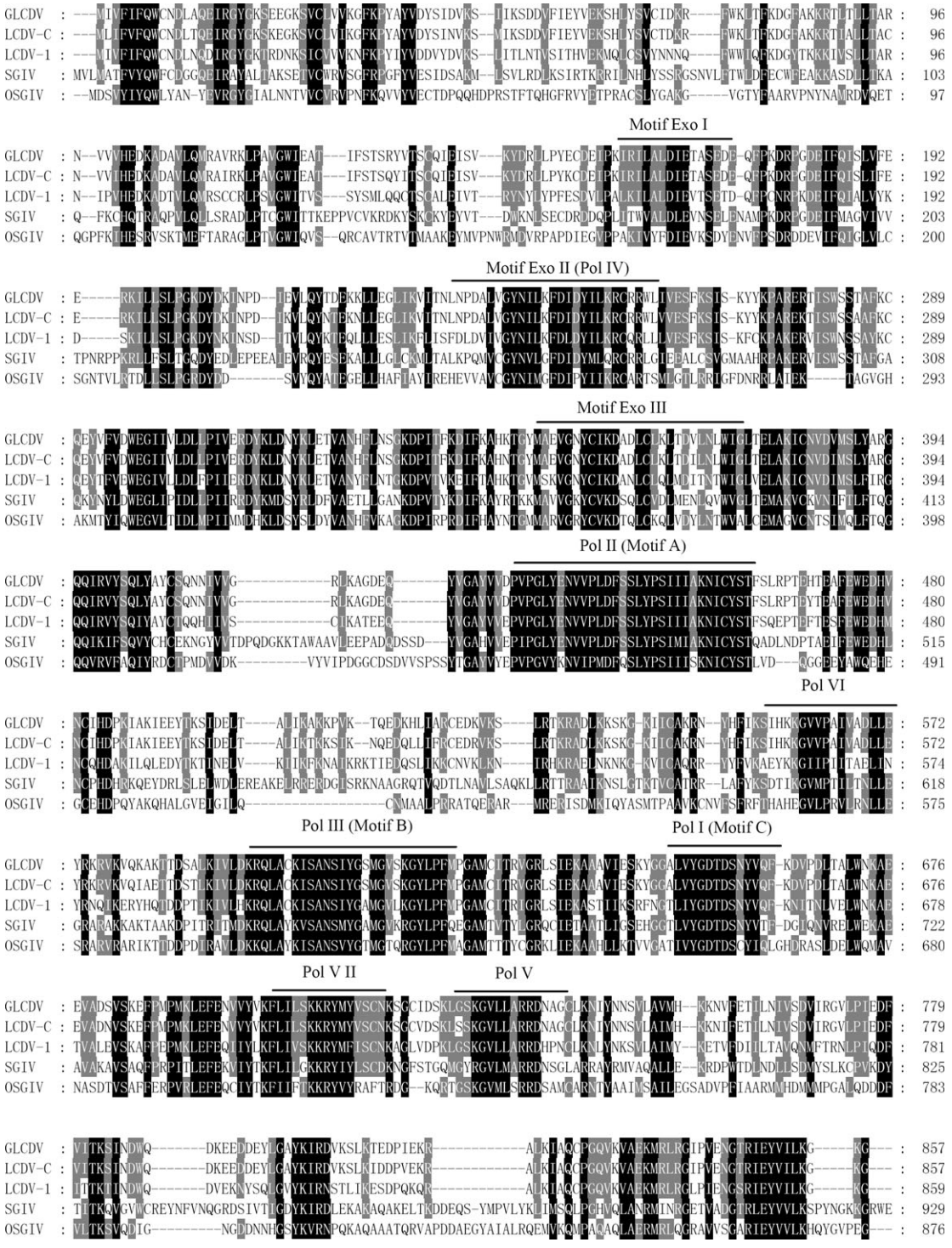

Figure 5 Amino acid alignment of DNA polymerase from different fish iridovirus. Putative homologous regions and motifs, including Exo I, Exo II (Pol IV), Exo III A, Pol II (Motif A), Pol VI, Pol III (Motif B), Pol I (Motif C), Pol VII and Pol V are indicated by rectangles.

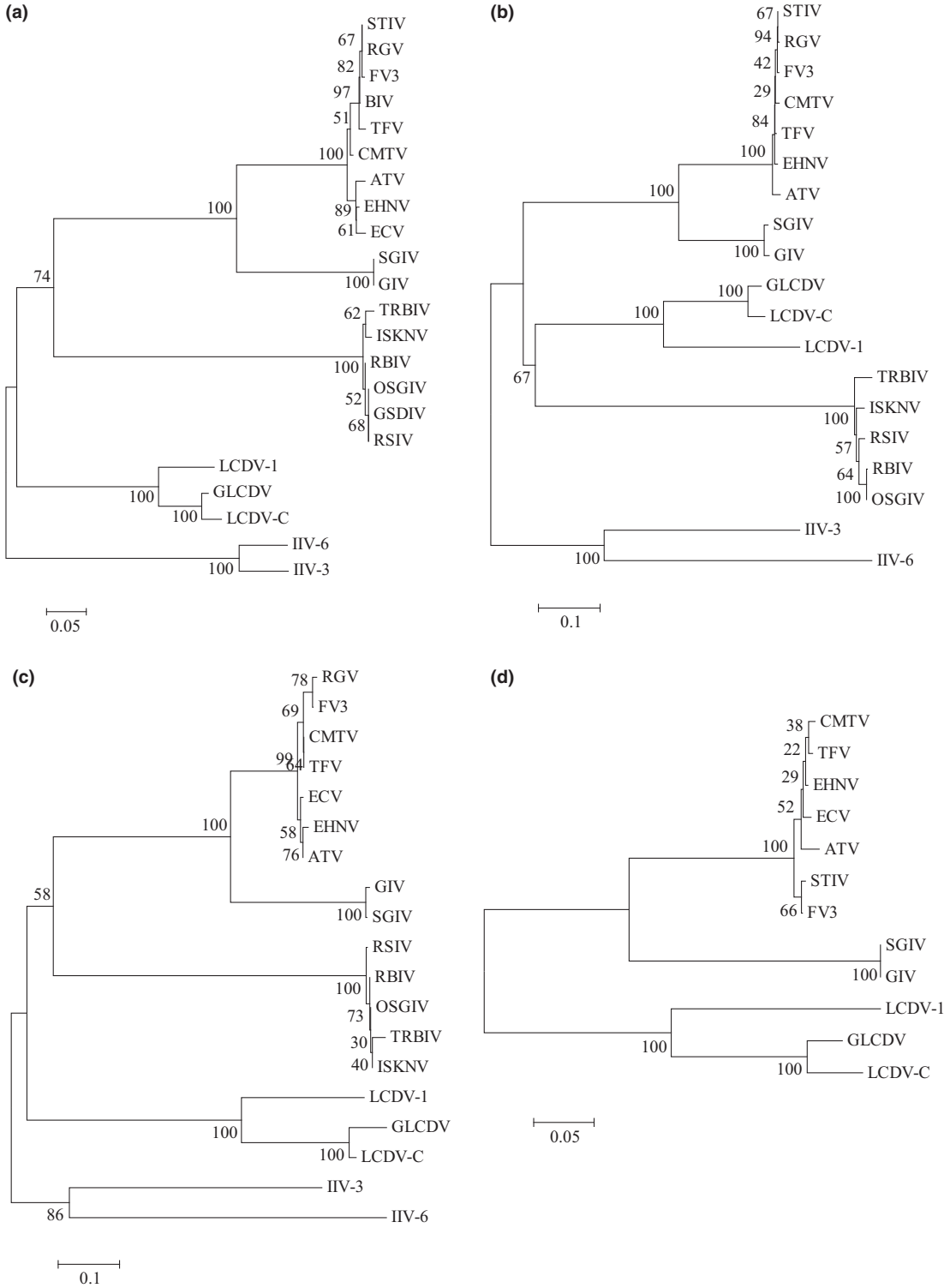

Figure 6 Phylogenetic analysis of four core genes from different iridovirus isolates. Phylogenetic trees based on MCP (a), DNA polymerase (b), MMP (c) and RNR gene (d) were constructed using MEGA 4.0.

and the existence of intracytoplasmic inclusions in the lymphocytic cells, which were the typical characteristics of lymphocystis disease (Alonso *et al.* 2005; Sheng *et al.* 2007). Moreover, numerous hexagonal virus particles about 200 nm in diameter and the crystalline array were observed in the nodules, providing the morphological evidence that GLCDV virus particles were the causative agents. In addition, GLCDV MCP gene showed 90% identity to LCDV-C, followed by 80% to LCDV-1, and GLCDV DNA polymerase showed 90% identity to LCDV-C, followed by 71% to LCDV-1. It has been reported that the gene sequence from different LCDV isolates usually shared lower identity (Cano *et al.* 2007). Significant difference in these gene structures revealed the diversity of LCDV isolates from different fish lymphocystis disease.

Hossain *et al.* (2008) proposed a hypothesis that genetic similarity of lymphocystiviruses might be dependent on their host fish species rather than on their geographical distributions. In our study, the phylogenetic analysis based on MCP gene and three other iridovirus core genes consistently revealed that LCDV-C was closely related to GLCDV, but not LCDV-1. However, the hosts of LCDV-C and LCDV-1 both belong to family Pleuronectidae, and the hosts of GLCDV belong to family Serranidae. We proposed that different LCDV isolates and their hosts might evolve independently, although genetic variation usually occurs in many DNA viruses during their co-evolution with hosts (Yan *et al.* 2011).

In summary, we identified the causative agents of lymphocystis disease from different species of groupers as a novel LCDV isolate, GLCDV. Phylogenetic analysis based on four iridovirus core genes revealed that GLCDV shared the closest relationship to LCDV-C. Our results firstly provided the molecular evidence of lymphocystis disease virus in grouper and might contribute greatly to diagnosis and control of grouper viral diseases.

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