Arch Virol (2014) 159:2445–2449
DOI 10.1007/s00705-014-2060-0

BRIEF REPORT

Identification of lymphocystis disease virus from paradise fish *Macropodus opercularis* (LCDV-PF)

Liwen Xu · Juan Feng · Youhua Huang

Received: 26 December 2013 / Accepted: 17 March 2014 / Published online: 6 April 2014

Abstract Iridoviruses are large DNA viruses that are subdivided into five genera: *Ranavirus*, *Megalocytivirus*, *Lymphocystivirus*, *Chloriridovirus* and *Iridovirus*. The iridovirus lymphocystis disease virus (LCDV) is an important fish pathogen that can infect marine and freshwater fish worldwide. In this study, we have identified the pathogen in paradise fish (*Macropodus opercularis*) with lymphocystis. On the skin and fins of diseased paradise fish, a large number of nodules were observed. H&E staining showed that the nodules were composed of encapsulated hypertrophied cells. Using electron microscopy, numerous virus particles with a diameter of >210 nm and with hexagonal profiles were observed in the cytoplasm. Phylogenetic analysis based on the major capsid protein (MCP), DNA polymerase and myristylated membrane protein (MMP) genes revealed that LCDV from paradise fish (LCDV-PF) was closely related to lymphocystis disease virus from China (LCDV-C), followed by lymphocystis disease virus 1 (LCDV-1). Taken together, our data provide the first molecular evidence that, in addition to megalocytivirus, LCDV is an important iridoviral pathogen in paradise fish besides megalocytivirus.

Iridoviruses are large DNA viruses that infect invertebrates and lower vertebrates, such as insects, fish, amphibians, reptiles, crustaceans and mollusks. The family *Iridoviridae* is currently divided into five genera: *Ranavirus, Lymphocystivirus, Megalocytivirus, Iridovirus* and *Chloriridovirus* [7, 16]. Lymphocystis disease virus (LCDV), which belongs to the genus *Lymphocystivirus*, is the etiological agent of lymphocystis disease, which is characterized by the appearance of pearl-like nodules, formed by hypertrophic fibroblastic cells, located on skin and fins of fish [6, 10, 13]. Lymphocystis disease has been reported in over 125 different fish species from 34 different families [11–13]. Although many attempts have been made to propagate LCDV *in vitro*, the complete replication cycle and pathogenesis process of this virus are not well understood. To explore the molecular mechanism of LCDV pathogenesis, the complete genome sequence of two LCDV isolates, including LCDV-C from Japanese flounder (*Paralichthys olivaceus*) in China and LCDV-1 from flounder (*Platichtys flesus L.*) in Europe, have been determined [14, 17]. Comparative genome analysis indicated that LCDV-C shared low similarity to LCDV-1, based on genome size, gene organization and gene product identity [17]. Notably, when compared to members of other genera in family *Iridoviridae*, the MCP sequences of different LCDV isolates exhibited a low degree of identity [2]. Further studies focusing on identification and genome sequencing of different LCDV isolates will contribute to understanding of genetic variations and evolution of LCDV.

Paradise fish (*Macropodus opercularis*) are not only important tropical ornamental fish but are also an ideal experimental system for behavioral genetic studies [3, 8]. To our knowledge, only megalocytivirus has been detected and identified as a viral agent in paradise fish [8]. Although lymphocystis in paradise fish has been described by Ashburner [1], no molecular data or morphological features of the pathogen were presented.

L. Xu · J. Feng
South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Guangzhou 510300, Guangdong, China

Y. Huang (✉)
Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China
e-mail: huangyh@scsio.ac.cn

Here, we have investigated a lymphocystis outbreak in cultured paradise fish at a local farm in Guangdong Province, China, in which about 20 % of the fish displayed symptoms of lymphocystis, including a decrease in mobility and loss of appetite. Moreover, wart-like nodules were diffusely distributed over the whole body but predominantly located on the caudal and pectoral fins and opercular region (Fig. 1A and B). Further observation under light microscopy showed that the nodules were composed of growing clusters of lymphocystic cells. The fibroblasts exhibited cytomegalia with spheroidal shapes surrounded by an unusually thick outer membrane, forming a hyaline capsule (Fig. 1C). The nodules were fixed, processed for routine paraffin sectioning, and stained with H&E for histopathological observation as described previously [13]. We observed that many inclusion bodies were strongly stained by hematoxylin and were observed peripherally near the membrane (Fig. 1D). Similar symptoms were observed previously in LCDV infected white-spotted puffer [13].

To determine whether the wart-like lesions contained virus particles, the nodules were fixed with 2.5 % glutaraldehyde overnight and prepared for electron microscopy observation as described previously [5]. As shown in Fig. 2, numerous virus particles with a diameter of >200 nm in and with hexagonal profiles were observed,

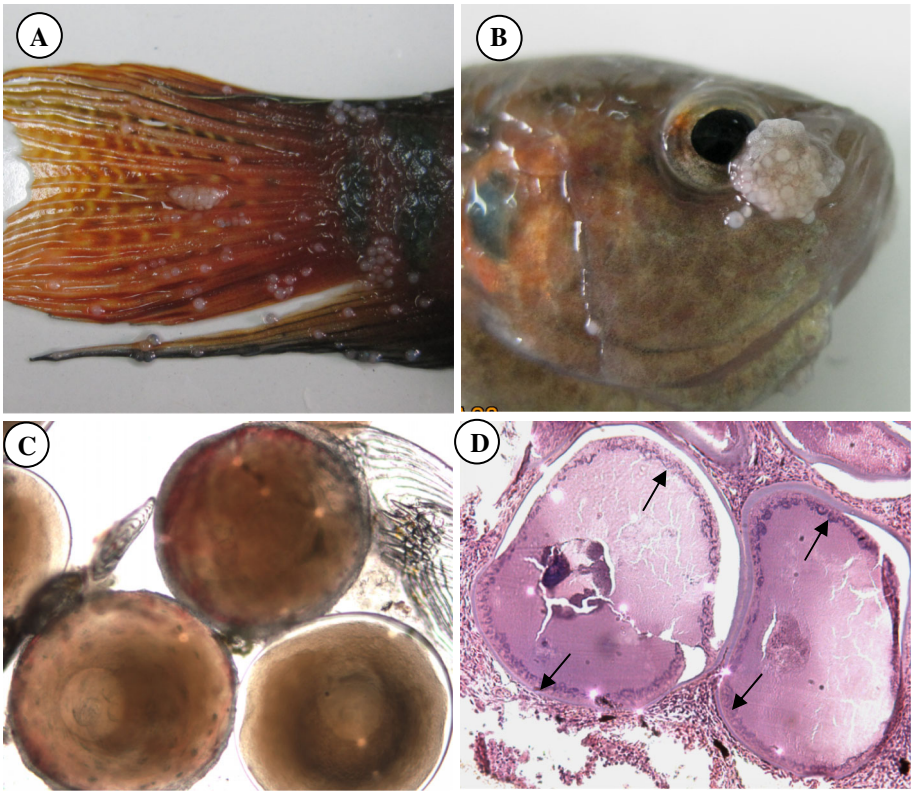

Fig. 1 Characterization of lymphocystis disease in paradise fish. Typical symptoms on the fin (**A**) and snout (**B**) of diseased paradise fish are shown. Nodules are indicated by arrows. (**C**) Many lymphocystis cells are seen on the cephalic skin of diseased paradise fish by light microscopy. (**D**) Histological sections of the nodules on the skin

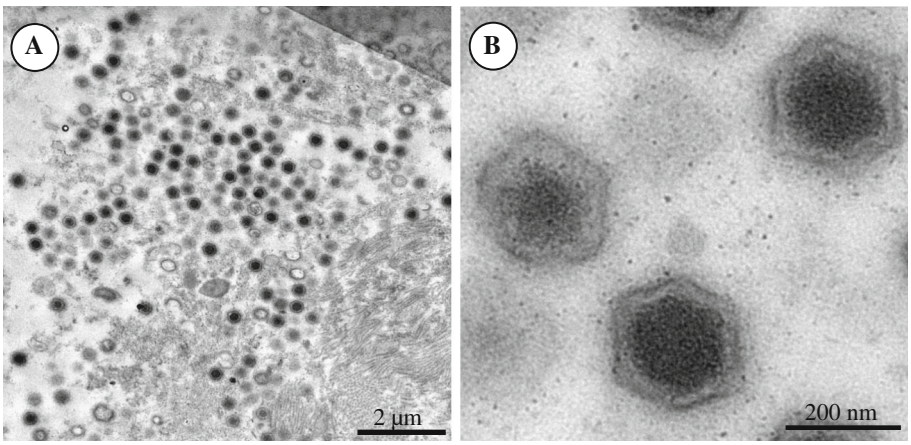

Fig. 2 Ultrastructure of viral particles from nodules

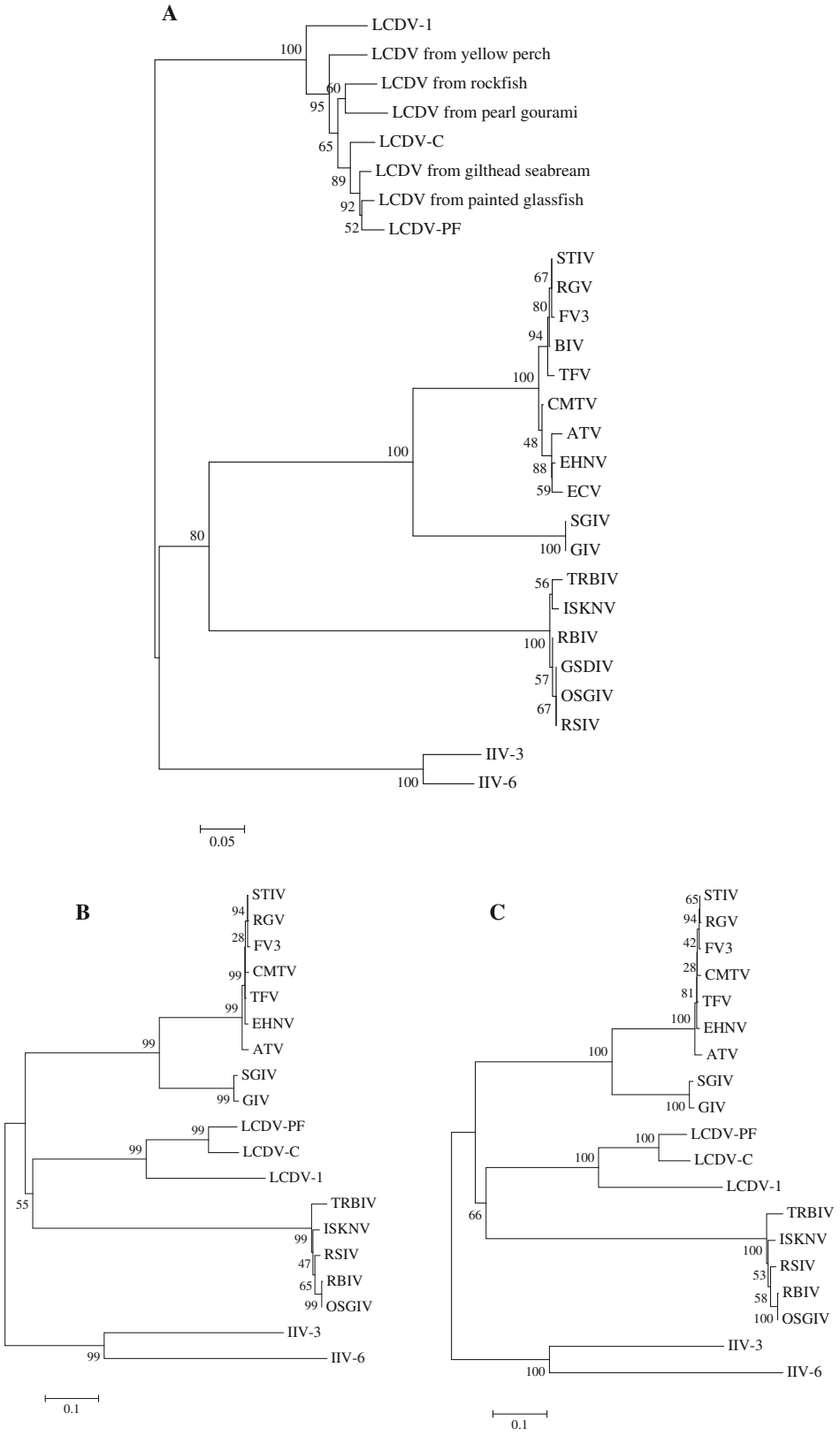

Fig. 3 Phylogenetic analysis of three core genes from different iridovirus isolates. Phylogenetic trees of LCDV-PF and other iridovirus isolates were constructed based on the MCP (**A**), DNA polymerase (**B**) and MMP genes (**C**). The bootstrap confidence values are shown at the nodes of the tree

indicating that the white nodules in paradise fish were lymphocystis lesions.

Given that the major capsid protein (MCP) of iridoviruses is a suitable target gene for the study of iridovirus evolution and their classification [4, 15], we designed primers based on the MCP gene sequences of LCDV-1 and LCDV-C as well as two other iridovirus core genes, DNA polymerase (DNA Pol) and myristylated membrane protein (MMP). Three pairs of primers were used in this study, including MCP-F/R (MCP-F, ATGACTTCTGTAGCGGG TTCAAGTG; MCP-R, CTAAAGTACAG GAAATCCCA TTGAAC), DNA Pol-F/R (Pol-F, ATGATAGTTTTTATT TTTCAAT GG; Pol-R, AGCTGATATTTTACATGCTA ATTG), and MMP-F/R (MMP-F, CACAAGTAGCAGAT ATTAACAACA; MMP-R, TCTTGAGCACAATCTTG AAATA). After extraction of genomic DNA from the nodules of five fish, PCR amplification was carried out using LA Taq® (TaKaRa) according to the manufacturer's instructions, with the following cycling conditions: 95 °C for 3 min, followed by 35 cycles of 45 s at 95 °C, 45 s at 48 °C, and 2 min at 72 °C. The PCR products were subcloned into the pMD18-T vector for sequencing, and the data that were obtained were assembled and submitted to the GenBank database (accession numbers of MCP, DNA Pol and MMP: KJ408271, KJ408272 and KJ408273, respectively). A homology search revealed that the LCDV-PF MCP shared 90 % sequence identity with LCDV from painted glassfish, followed by LCDV from gilthead sea bream (89 %), LCDV-C (86 %) and LCDV-1 (84 %). In addition, DNA polymerase and MMP of LCDV-PF shared 84 % and 86 % identity, respectively, with that of LCDV-C. The MCP sequences of different LCDV isolates exhibited lower identity (as low as 22 % nucleotide sequence identity) when compared to those of members of other genera in family *Iridoviridae* [2].

A phylogenetic tree based on the amino acid sequences of these three genes was constructed by the neighbour-joining method using Molecular Evolutionary Genetics Analysis (MEGA 4.0). Based on the MCP gene, LCDV-PF showed the closest relationship to LCDV from painted glassfish, followed by that from gilthead seabream and LCDV-C. Due to the lack of sequence data from other LCDV isolates, the phylogenetic analysis based on iridovirus core genes, including DNA polymerase and MMP consistently indicated that LCDV-PF is closely related to LCDV-C, followed by LCDV-1 and other iridoviruses (Fig. 3). Together, our data provide the first molecular evidence that LCDV-PF is a novel member of the genus *Lymphocystivirus* in the family *Iridoviridae*. Phylogenetic analysis of LCDV isolates based on major capsid protein (MCP) sequences has strongly supported the existence of three genotypes in the genus *Lymphocystivirus* [10]. Kitamura et al. [9] have proposed that the genetic similarity among LCDV isolates is more closely associated with the host species than with geographic distribution. Interestingly, although LCDV-C and LCDV-1 were both isolated from flounder, LCDV-C showed a closer relationship to LCDV-PF than LCDV-1 based on the phylogenetic tree. Therefore, the question whether genetic relationships between LCDV isolates are influenced more by geographic location or virus/host specificity needs further investigation.

In summary, we describe for the first time the ultrastructure and genome sequence of an LCDV isolate from a paradise fish. Phylogenetic analysis based on three core genes indicates that LCDV-PF is a novel isolate that, like megalocytivirus, can cause disease in paradise fish. To better understand the differences between LCDV-PF and other LCDV isolates, more sequences of complete genomes of LCDV-PF isolates are needed.

Acknowledgments We thank Fanmei Zeng from the Administration of Ocean and Fisheries of Guangdong Province for the sample collection. This work was supported by grants from the National Basic Research Program of China (973) (2012CB114402) and Special Scientific Research Funds for Central Non-Profit Institutes, Chinese Academy of Fishery Sciences (2012A0503; 2013A0604).

References

1. Ashburner LD (1975) Letter: Lymphocystis in paradise fish (*Macropodus opercularis*) in Australia. Aust Vet J 51:448–449
2. Cano I, Ferro P, Alonso MC, Bergmann SM, Römer-Oberdörfer A, Garcia-Rosado E, Castro D, Borrego JJ (2007) Development of molecular techniques for detection of lymphocystis disease virus in different marine fish species. J Appl Microbiol 102:32–40
3. Gerlai R, Crusio WE (1995) Organization of motor and posture patterns in paradise fish (*Macropodus opercularis*): environmental and genetic components of phenotypical correlation structures. Behav Genet 25:385–396
4. Hossain M, Song JY, Kitamura SI, Jung SJ, Oh MJ (2008) Phylogenetic analysis of lymphocystis disease virus from tropical ornamental fish species based on a major capsid protein gene. J Fish Dis 31:473–479
5. Huang XH, Huang YH, Yuan XP, Zhang QY (2006) Electron microscopic examination of the viromatrix of Rana grylio virus in a fish cell line. J Virol Methods 133:117–123
6. Huang Y, Huang X, Zhang J, Gui J, Zhang Q (2007) Subcellular localization and characterization of G protein-coupled receptor homolog from lymphocystis disease virus isolated in China. Viral Immunol 20:150–159
7. Jancovich JK, Chinchar VG, Hyatt A, Miyazaki T, Williams T, Zhang QY (2011) Family Iridoviridae virus taxonomy. 9th report of the ICTV. Elsevier, San Diego, pp 193–210
8. Kim WS, Oh MJ, Kim JO, Kim D, Jeon CH, Kim JH (2010) Detection of megalocytivirus from imported tropical ornamental fish, paradise fish *Macropodus opercularis*. Dis Aquat Organ 90:235–239
9. Kitamura SI, Jung SJ, Kim WS, Nishizawa T, Yoshimizu M, Oh MJ (2006) A new genotype of lymphocystivirus, LCDV-RF, from lymphocystis diseased rockfish. Arch Virol 151:607–615

10. Kvitt H, Heinisch G, Diamant A (2008) Detection and phylogeny of Lymphocystivirus in sea bream Sparus aurata based on the DNA polymerase gene and major capsid protein sequences. Aquaculture 275:58–63
11. Noga EJ (2010) Problem 40. Lymphocystis. In Fish disease: diagnosis and treatment. 2nd edition. Wiley-Blackwell. Ames, Iowa. pp 171–173
12. Pirarat N, Pratakpiriya W, Jongnimitpaiboon K, Sajjawiriyakul K, Rodkhum C, Chansue N (2011) Lymphocystis disease in cultured false clown anemonefish (*Amphiprion ocellaris*). Aquaculture 315:414–416
13. Sheng XZ, Zhan WB, Wang Y (2007) Whitespotted puffer Arothron hispidus, a new host for lymphocystis in Qingdao Aquarium of China. Dis Aquat Organ 75:23–28
14. Tidona CA, Darai G (1997) The complete DNA sequence of lymphocystis disease virus. Virology 230:207–216
15. Tidona CA, Schnitzler P, Kehm R, Darai G (1998) Is the major capsid protein of iridoviruses a suitable target for the study of viral evolution? Virus Genes 16:59–66
16. Williams T, Barbosa-Solomieu V, Chinchar VG (2005) A decade of advances in iridovirus research. Adv Virus Res 65:173–248
17. Zhang QY, Xiao F, Xie J, Li ZQ, Gui JF (2004) Complete genome sequence of lymphocystis disease virus isolated from China. J Virol 78:6982–6994