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Characterization of a ranavirus isolated from the Chinese giant salamander (*Andrias davidianus*, Blanchard, 1871) in China

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ABSTRACT

In this study, we confirmed that a novel ranavirus was related to mass die-offs of farmed Chinese giant salamanders (*Andrias davidianus*) in Leshan, Sichuan Province, China. The farmed Chinese giant salamanders presented typical clinical symptoms that included ecchymoses or swollen areas on the head and limbs, necrosis of the limbs, and skin ulceration. Bacteriological and pathologic examinations further proved the possibility of ranavirus infection of the Chinese giant salamanders. The virus was isolated via inoculation in the epithelioma papulosum cyprini (EPC) cells. The virus multiplied and caused cytopathogenic effects (CPE) at 25 °C in the EPC cells and it was sensitive to chloroform treatment, trypsinase treatment, heating at 56 °C for 30 min, acidity (pH 3.0), and alkalinity (pH = 10.0). Treatment with 5-bromouracil deoxyriboside (5-BrdU) inhibited viral replication, which suggested that the viral genome contained DNA. After the viral inoculation in the cell, samples were collected and made the ultrathin sections, and then the iridovirus-like particles could be observed in the cytoplasm and nuclei by electron microscopy. Using the isolated viral DNA as the template for PCR, we amplified, cloned, and analyzed target sequences in three different genomic regions, i.e., the major capsid protein (MCP), DNA polymerase, and neurofilament triplet H1-like protein (NF-H1). Subsequent multiple alignment and phylogenetic analysis showed that the newly isolated virus was closely related to KRV-1, soft-shelled turtle iridovirus, *Rana grylio* iridovirus, and FV3. In the pathogenicity tests, the challenged salamanders showed similar clinical symptoms to the natural infections. Our data suggested that the virus isolate was a novel member of the genus *Ranavirus*, family *Iridoviridae*. We tentatively named the virus as Chinese giant salamander virus (CGSV-L). CGSV-L was able to cause serious systemic disease and it was capable of killing 80% of Chinese giant salamanders (body weight = 300.0 ± 15.0 g).

1. Introduction

The family *Iridoviridae* currently contains five genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystivirus*, and *Megalocytivirus*. The virions are enveloped, measure 120 to 200 nm in diameter, and display icosahedral symmetry with an electron-dense core that contains a single linear, circularly permuted and terminally redundant double-stranded DNA molecule containing 140–303 kbp. The virions are stable in water at 4 °C for extended periods and resist desiccation at 42 °C for up to 6 weeks (Williams et al., 2005). Ranaviruses are important pathogens of ectotherms and have been associated with massive die-offs of wild and farmed populations of fish, frogs, and salamanders in many areas of the world (Whittington et al., 2010; Williams et al., 2005). Ranavirus infections are considered to be emerging diseases and these infections have been listed as notifiable by the World Organization for Animal Health (Duffus and Cunningham, 2010; Schoegel et al., 2010).

The Chinese giant salamander (*Andrias davidianus*, Blanchard, 1871) is the world's largest amphibian species and it is classified as critically endangered by the International Union for Conservation of Nature and Natural Resources. Because of its biological, medical, and nutritional value, it has been cultured widely in many provinces of China for more than 30 years (Zhang et al., 2002). The various diseases of farmed Chinese giant salamanders have become more frequent in recent years, especially ranavirus infections (Dong et al., 2011; Geng et al., 2011). Although ranavirus has emerged as an important pathogen in Chinese giant salamander (Dong et al., 2011; Geng et al., 2011), there are no reports of the viral biological characteristics. In May and June 2011, a serious disease outbreak appeared in a Chinese giant salamander farm in Leshan, Sichuan province, China. The typical symptoms of the sick giant salamanders were almost the same as the clinical lesions observed

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in ranavirus-infected amphibians, i.e., skin ulcerations and necrosis of the distal limbs (Bollinger et al., 1999; Docherty et al., 2003; Geng et al., 2011; Miller et al., 2007). The outbreak lasted several weeks and resulted in more than 60% mortality. The present work describes the isolation of the viral pathogen in cell culture, an investigation of the viral infectivity and pathogenicity, and characterization of the virus based on its biochemical, structural, and molecular properties.

2. Materials and methods

2.1. Sample collection

In May and June 2011, nine diseased Chinese giant salamanders (weight = 200–3000 g) were collected from a Chinese giant salamander farm located in Leshan (Sichuan Province, China) and they were transferred alive to the Key Laboratory of Animal Disease and Human Health of Sichuan Province. Symptoms included anorexia, lethargy and small pale raised foci in the skin, and cutaneous erosions and ulcers. Ecchymosis and swollen areas were noted on the head, ventral surface, and limbs, and in some cases necrosis and rotting occurred in the limbs.

2.2. Pathologic and bacteriological examination

Postmortem examinations were performed on all diseased Chinese giant salamanders. Samples for histopathologic examination, including liver, lung, kidney, spleen, heart, stomach, intestine and brain, and gross lesions when present, were collected and fixed in 10% neutral buffered formalin, then tissues were processed routinely and pathological sections were accordingly prepared which were stained with hematoxylin and eosin (HE).

To isolate the bacteria present, the body surface of each sick Chinese giant salamander was swabbed with 70% ethyl alcohol to prevent contamination. Next, samples obtained from the kidney, liver, spleen, and ascites of each sick Chinese giant salamanders were directly inoculated onto sheep blood tryptone soy agar (TSA) and inoculated at 28 °C. All isolates were identified using the API 20E system (BioMerieux, La Balm, France).

2.3. Virus isolation

Liver, kidney, and spleen tissues were collected from moribund Chinese giant salamanders and prepared as blend homogenates, which were triturated in a mortar and diluted 1:5 in Hanks solution containing 100 IU/ml penicillin and 100 μg/ml streptomycin. The homogenate was centrifuged at 3000 rpm for 10 min at 4 °C and the supernatant was filtered through a 0.22 μm filter. The supernatant was inoculated onto monolayer of epithelium apapulosum cyprini (EPC) cells and the inoculated cell cultures were incubated at 25 °C in M199 supplemented with 2% fetal calf serum and penicillin–streptomycin to observe the cytopathic effects (CPE) on a daily basis. After the appearance of CPE, the inoculated cells were frozen at −20 °C and thawed at least three times. The mixture was clarified by centrifugation at 3000 rpm for 10 min at 4 °C. The virus (tentatively named Chinese giant salamander virus, CGSV-L) was re-inoculated onto the EPC cell line for further passage, and the second generation of virus was used to the followed studies.

2.4. Physicochemical characterization of the isolated virus

2.4.1. $TCID_{50}$ assay

Using the Reed and Muench (1938) method, the 50% tissue culture infectious dose ($TCID_{50}$/ml) of the isolated virus was calculated in the EPC monolayers of 96-well microculture plates. The stock virus suspensions were serially diluted 10-fold and triplicate wells were inoculated with 0.1 ml of each virus dilution, before the cells were incubated at 25 °C for 7 days. Each viral dilution of inoculated cells was fixed with formalin and stained using crystal violet, before the titers were determined.

2.5. Sensitivity to chloroform and trypsin

The chloroform sensitivity of the virus was determined using the method of Feldman and Wang (1961). A 0.5% trypsin solution (diluted with M199, pH 7.2, 1 ml) was added to 1 ml of virus suspension in the trypsinase treatment. A control virus suspension received 1 ml of PBS. These mixtures were incubated at 25 °C for 1 h before testing.

2.6. Temperature sensitivity

A temperature sensitivity test was performed on the virus. The virus was heated at 56 °C for 30 min and the residual virus titers were determined in EPC cell culture.

2.7. pH sensitivity

To determine the sensitivity of the virus at pH 3 and pH 10, the pH of virus suspensions was adjusted to pH 3 using HCl or pH 10 using NaOH. Samples were incubated at 25 °C for 1 h and the pH was adjusted to pH 7 using $NaHCO_3$. The corresponding untreated control and treated suspensions were titrated using the $TCID_{50}$ method with EPC cells.

2.8. Sensitivity to 5-BrdU

The inhibition of viral replication was tested by adding 10 μl 5-bromouracil deoxyriboside (5-BrdU) to 5 ml of virus suspension. A control virus suspension received 10 μl of PBS. The untreated control and treated suspensions were also titrated using the $TCID_{50}$ method with EPC cells.

2.9. Electron microscopy

Monolayers of EPC cells were infected with the virus suspension before electron microscope observations. The infected cells were collected in the 5th day by centrifugation at 3000 ×*g* for 30 min. The collected cells were fixed with 2% glutaraldehyde in phosphate buffer (pH 7.3, 0.1 M) at 4 °C for 1 h. After post-fixing with 1% osmium tetroxide (OsO_4), the specimens were dehydrated using a series of graded alcohols and embedded in epoxy resin. The blocks were sectioned at 50 nm, stained with uranyl acetate and lead citrate, and observed using a transmission electron microscope (TEM; JEM-1200EX, JEOL, Tokyo, Japan).

2.10. Polymerase chain reaction (PCR) amplification of the major capsid protein (MCP), DNA polymerase, and neurofilament triplet H1-like protein (NF-H1) genes

PCR tests were performed to analyze the molecular differences between the Chinese giant salamander virus and other iridoviruses. The viral DNA used as the template for PCR amplification was extracted from CGSV-L-infected EPC cells with 100% CPE using a commercially available kit (TaKaRa, Dalian, China). The extracted DNA was stored at −80 °C. Three different genomic regions (MCP gene, DNA polymerase gene, and NF-H1gene) were selected as target sequences to be amplified, cloned, and sequenced. The primers (Table 1) for the MCP, DNA polymerase (Zhang et al., 2006), and NF-H1 genes (Holopainen et al., 2009) were used in PCR reactions to amplify specific regions from the Chinese giant salamander virus genomes. The amplification reactions for all genes were performed as follows: 1 cycle at 94 °C for 4 min, followed by 30 cycles at 95 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, with a final elongation at 72 °C for 10 min. For each reaction, the mixture contained 0.5 μM of each primer, 50 μM of each nucleotide

Table 1
Primers used in PCR reactions.

Target gene	Primer (5′ to 3′)	Amplicon size (bp)	Reference
MCP	Forward primer: ACAGTCACCGTGTATCTTA Reverse primer: GGAAAAGACTTTGCGCTGA A	1500	Zhang et al. (2006)
DNA polymerase	Forward primer: GTGTAYCAGTGGTTTTGCGAC Reverse primer: TCGTCTCCGGGYCTGTCTTT	560	Holopainen et al. (2009)
NF-H1	Forward primer: CCA AAG ACC AAAGACCAG Reverse primer: GTTGGTCTTTGGTCTCGCTC	639	Holopainen et al. (2009)

(dATP, dTTP, dGTP, dCTP), 10× PCR buffer (100 mM Tris–HCl, 500 mM KCl, pH 8.3), 2 mM $MgCl_2$, 2.5 U Taq polymerase (TaKaRa, Dalian, China), 2 μl of DNA template, and distilled water to make up a 25 μl total volume. The PCR products were determined by electrophoresis using 1% (w/v) agarose gels that were stained with 0.5 μl/ml ethidium bromide before visualization under a UV transilluminator.

2.11. Sequencing and phylogenetic analysis

The PCR-positive bands were purified using a Gel DNA Purification Kit (TaKaRa, Dalian, China). The pMD19-T vector and the inserts in the 1:3 proportion were ligated using T4 DNA ligase (TaKaRa, Dalian, China) at 16 °C for 16 h. The ligation mixture was transformed into *Escherichia coli* DH5a using the heat shock method. The bacteria were plated on LB Ager containing 100 mg/ml ampicillin for 37 °C, 24 h and colonies were then cultured in LB broth with 100 mg/ml ampicillin for 37 °C, 24 h. The positive clones that were confirmed by colony PCR were sequenced using an AMI PRISM 377 automated sequencer (Shanghai Invitrogen Biotechnology Co. Ltd., Shanghai, China). Multiple sequence alignments (ClustalX 1.81) and phylogenetic analysis (MEGA 4.1) were respectively conducted for the three genes.

2.12. Pathogenicity tests

Experimental infection of Chinese giant salamanders was performed by intraperitoneal injection (i.p.). Eight healthy Chinese giant salamanders (body weight = 300.0 ± 15.0 g) were collected from a culture farm that had no previous history of disease and they were negative of ranavirus by RCR detection. All Chinese giant salamanders housed at the College of Veterinary Medicine (Sichuan Agricultural University, Sichuan, China) for six weeks prior to inoculation. EPC-grown Chinese giant salamander virus (CGSV-L) solution was used to inoculate five healthy Chinese giant salamanders, where each salamander was injected with 0.5 ml virus solution at a concentration of 2000 $TCID_{50}ml^{-1}$, whereas the other three Chinese giant salamanders were inoculated with the same amount of non-infected cell culture medium as controls. The salamanders were monitored daily for signs of disease. After the death of the salamanders, necropsies were performed and the tissues (liver, kidney, and spleen) were collected to re-isolate the virus.

3. Results

3.1. Gross pathology, histopathology and bacteriological examination

The typical clinical pathological changes in sick Chinese giant salamanders from Leshan (Sichuan Province, China) included cutaneous erosions and ulcers, necrosis and rotting of the limbs (Fig. 1).At necropsy examination, clear or bloody fluid was markedly in the coelomic cavity; there were much ecchymosis distributed on the liver, kidney and lung (Fig. 2). In addition, the obvious disease symptom included the mottled and friable liver, necrosis in the kidney.

Histopathologic examination revealed that the cell degeneration and necrosis were evident in the liver, spleen, kidney and lung similar to those described and summarized for ranavirus-infected pathology in salamanders (Dong et al., 2011; Geng et al., 2011; Gray et al., 2009). Minimal to mild lymphocytolysis, lymphoid depletion appeared in the spleen. Vacuolar degeneration of the renal tubular epithelial cells, focal peracute tubular necrosis, and acute glomerulonephritis were present in the kidney. Hepatocellular swelling and vacuolar degeneration were common in the liver and consisted of occasional focal areas of hepatocyte necrosis. Hemorrhage and focal areas of necrosis showed in lung with the inflammatory cells infiltration. Eosinophilic inclusions were found in the renal tubular epithelium and the hepatocyte (Fig. 3).

The bacteria including *Aeromonas hydrophila*, *Acinetobacter* spp., *Plesiomonas shigelloides* and *Citrobacter freundii* were isolated from the different diseased Chinese giant salamanders, however there were no bacteria found in 3 Chinese giant salamanders and antimicrobial drug treatment did not successfully improve the situation after the outbreak in the Chinese giant salamander farm.

3.2. Viral isolates and the cytopathology of infected cells

The viral isolates from the filtered liver, kidney, and spleen tissue homogenates could grow on EPC cells at 25 °C and CPE were apparent on the 3rd day after viral infection (Fig. 4A). Compared with non-infected cells, the virus produced small foci in the monolayers with some cells rounding and floating in the cell maintenance solution, before more cells began to detach from the bottom of the flask and the foci became enlarged. On the 7th day, the monolayers of EPC cells infected with virus had numerous plaques as the cells died (Fig. 4B). The re-inoculated virus yielded the same CPE in less time.

3.3. Physicochemical properties of isolated virus

In the EPC cells at 25 °C, the titer of isolated virus from sick Chinese giant salamanders was $10^{5.3}$ $TCID_{50}$/0.1 ml. The virus was sensitive to chloroform, pH 3, pH 10, 0.5% trypsin solution, and heat treatment, while virus replication was inhibited by BrdU (Table 2).

3.4. Morphological study by electron microscopy

Examination of ultrathin sections showed the presence of cytoplasmic viruses that appeared to form paracrystalline arrays and/or aggregates. The paracrystalline arrays were located within rarified areas of the cytoplasm or in the surrounding cytoplasm (Fig. 5A). The cytoplasmic viruses were observed at different stages of assembly ranging from complete icosahedral particles containing electron-dense cores to incomplete particles containing empty to partially full cores. All were enveloped viral particles with a diameter of approximately 160 nm. Viruses were also observed budding from the infected cells, where they acquired a host-derived membrane that encapsulated the virus. Some hexagonal viral particles were also observed inside the cell nuclei of infected cells (Fig. 5B).

3.5. MCP gene, DNA polymerase gene, and NF-H1 gene sequencing and phylogenetic analysis

In this study, the complete MCP gene was amplified, cloned, and analyzed. The 1453 bp MCP sequence obtained was registered in GenBank under the accession number HQ684746. According to the Basic Local Alignment Search Tool (BLAST) algorithm, the sequence shared 98–100% similarity with other *Ranavirus* species MCP genes, e.g., common midwife toad ranavirus (JQ231222.1), epizootic hematopoietic

Fig. 1. Gross lesions in infected Chinese giant salamanders. (A) Systemic swelling and ulcer on the ventral surface. (B) Necrosis in the anterior limb. (C) Necrosis in the posterior limb.

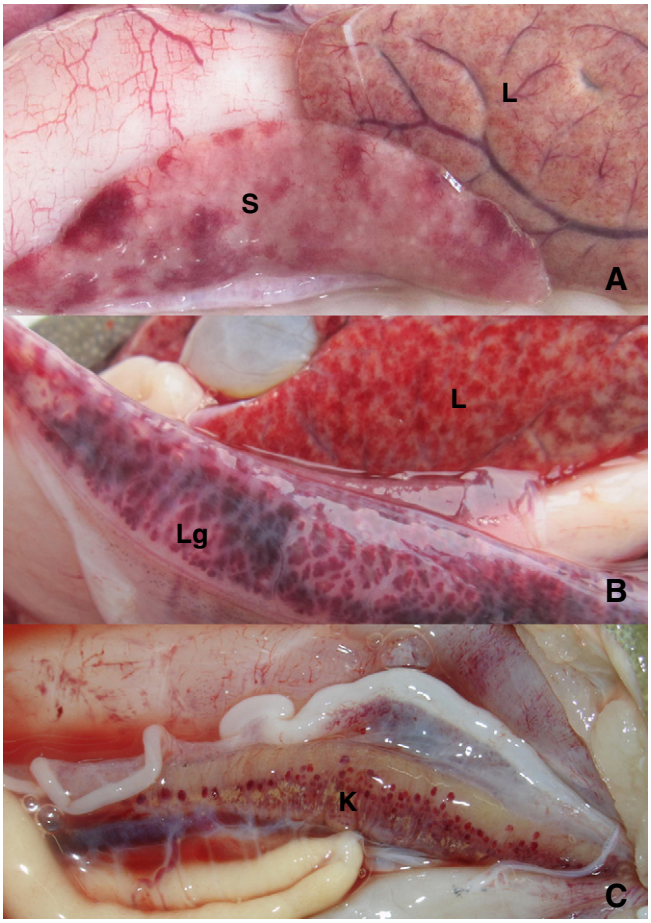

Fig. 2. Gross lesions in infected Chinese giant salamanders. (A) The liver and spleen were pale, with the diffused ecchymosis. (B)The liver and lung were swollen with multifocal hemorrhages. (C) The diffused ecchymosis and necrosis in the spleen (L: liver, S: spleen, Lg: lung, K: kidney).

necrosis virus (EHNV) (FJ433873.1), *Rana grylio* iridovirus (JQ654586.1), and frog virus 3 (AY548484.1).

The GenBank BLAST search for the 560 bp DNA polymerase gene (HQ684747) showed that it shared 98–99% sequence identity with soft-shelled turtle iridovirus (EU627010.1), European catfish virus (JQ724856.1), and frog virus 3 (AY548484.1).

A 606 bp gene fragment was amplified from the sequence encoding protein NH-F1 (HQ684748) in the isolated virus and it formed a cluster with 99–100% sequence similarity with ranavirus KRV-1 (HM133595.1), soft-shelled turtle iridovirus (EU627010.1), *R. grylio* iridovirus (JQ654586.1), and tiger frog virus (AF389451.1).

Phylogenetic trees (Figs. 6–8) were constructed based on the MCP, DNA polymerase, and NH-F1 sequences, which included those investigated in this study and the homologous sequences of other viruses from the family *Iridoviridae*. CGSV-L formed a different cluster from other *Iridoviridae* members in the phylogenetic analysis, such as frog virus 3 (AY548484) and *Ambystoma tigrinum stebbensi* virus (AY150217), but they all shared the same basal branch. Based on the phylogenetic analysis of NH-F1, we found that CGSV-L, *R. grylio* iridovirus, *Ranavirus* KRV-1 HM, and soft-shelled turtle iridovirus formed a separate cluster from the other ranaviruses.

3.6. Pathogenicity tests

The virus was shown to be highly pathogenic in Chinese giant salamanders. The salamanders began to die within 7 days of infection with the CGSV-L virus solution. The clinical signs and postmortem lesions in infected salamanders were similar to those described in naturally infected specimens. Challenged salamanders had a cumulative mortality of 80% at 20 days after injection, whereas the negative control salamanders were alive and showed no clinical symptoms. Homogenates of the liver, spleen, or kidney from each dead salamander were used to isolate the virus by culture in EPC cells and the same CPE were observed, and the virus was re-isolated from the challenged salamanders.

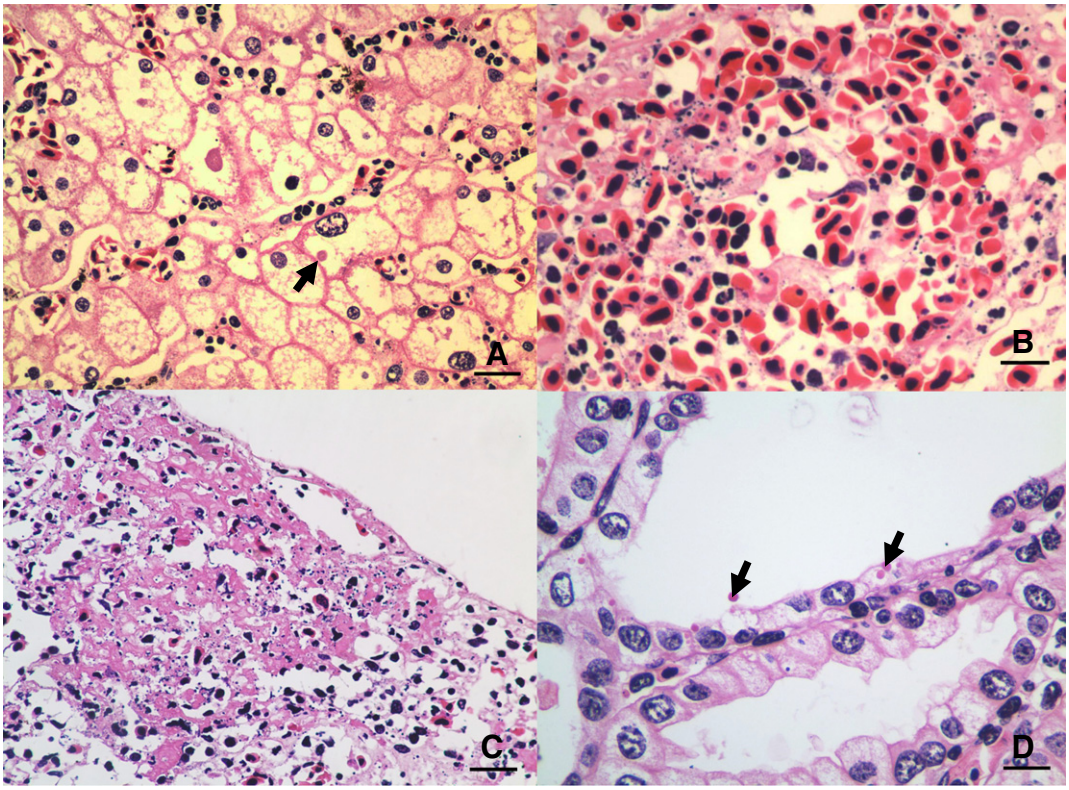

Fig. 3. Microscopial lesions in infected Chinese giant salamanders. (A) Vacuolar degeneration and eosinophilic inclusions (arrow) of the hepatocytes. HE. Bar = 25 μm. (B) The focal necrosis and hemorrhage with the inflammatory cell infiltration of the lung. HE. Bar = 12.5 μm. (C) A focal necrosis area of the spleen, with lymphocytolysis and lymphoid depletion. HE. Bar = 12.5 μm. (D) Vacuolar degeneration and eosinophilic inclusions (arrows) of the renal tubular epithelial cells. HE. Bar = 25 μm.

4. Discussion

It was clear that members of the genus *Ranavirus* are closely related to mass mortality of amphibians (Chinchar, 2002; Gray et al., 2009). The Chinese giant salamander (*Andreas davidianus*) as the world's largest amphibian species is important for research and extensive breeding in China. The first *Ranavirus* infection in China was reported in a mass die-off of the pig frog, *R. grylio*, during 2001 (Zhang et al., 2001). Since then, the Chinese giant salamander has frequently been reported to have infections and mass mortality due to *Ranavirus* (Dong et al., 2011; Geng et al., 2011). In this study, we collected diseased Chinese giant salamanders from Sichuan province that had typical clinical pathological and histopathological symptoms, specifically the ulceration of skin and necrosis of the limbs,

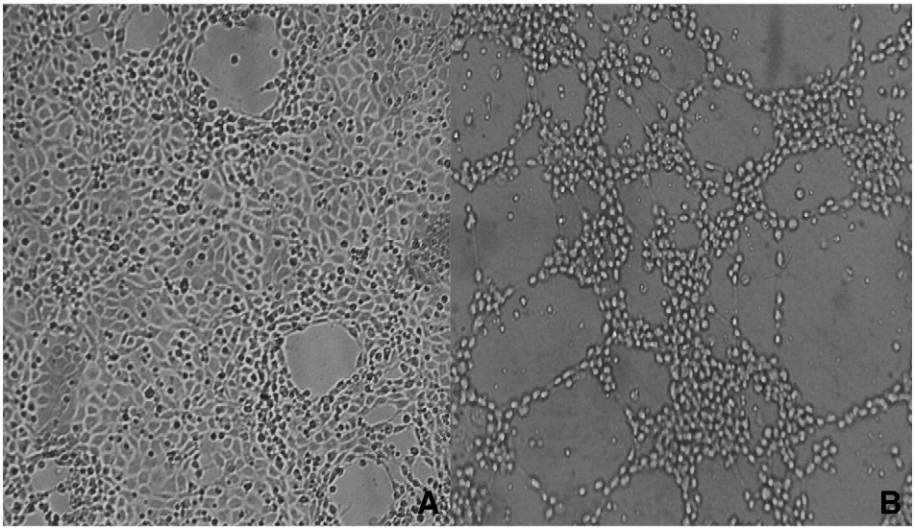

Fig. 4. CPE in the CGSV-L infected EPC cell. (A) Small foci CPE with some cell rounding and floating apparent in monolayer EPC cells on the 3rd day after infection. (B) Numerous plaques in monolayer EPC cells on the 7th day after infection.

Table 2
Sensitivity of virus to physicochemical treatments.

Physicochemical treatments	$TCID_{50}$/0.1 ml	
	Before treatment	After treatment
Chloroform	$10^{4.8}$	0
pH 3	$10^{5.0}$	$10^{1.5}$
pH 10	$10^{5.0}$	$10^{1.5}$
0.5% trypsin solution	$10^{5.3}$	$10^{2.6}$
56 °C, 30 min	$10^{5.0}$	0
BrdU	$10^{5.4}$	$10^{1.0}$

eosinophilic inclusions of hepatocyte and renal tubular epithelial cell, those were almost the same as those observed in other reported *Ranavirus* infected amphibians (Converse and Green, 2005; Cunningham et al., 1996; Miller et al., 2007), and other reported Chinese giant salamanders in Shanxi province, China (Dong et al., 2011; Geng et al., 2011). In addition, compared with the negative control, the infected salamanders showed the typical clinical signs in the pathogenicity tests. Molecular techniques can be useful for diagnosis, but the preliminary diagnosis of amphibian ranaviral disease requires an understanding of clinical, gross and histopathological signs. The gross and histopathological pathology of ranaviral disease in Chinese giant salamanders should be summarized and used as a basis for its clinical diagnosis.

Viruses in the genus of *Ranavirus* are emerging killers with a wide host range, including fish, amphibians, and reptiles (Ariel et al., 2009a; Green et al., 2002; Muths et al., 2006; Williams et al., 2005). Numerous fish cell lines have been used to isolate ranavirus at most temperatures (Ariel et al., 2009b), and the EPC cell line is a common fish cell line that was used in this study to multiply the virus at 25 °C (Ariel et al., 2009a; Bollinger et al., 1999; Zhang et al., 2001). The isolated virus could replicate in cells to produce a high titer $TCID_{50}$ with obvious CPE (Fraser et al., 1993; Jancovich et al., 1997). Tests of the physicochemical properties indicated that the virus possessed an envelope and a DNA the genome. Electron microscopy studies of the morphogenesis of viruses and their cellular interactions have also been conducted. Based on the morphogenesis, pathogenesis, and taxonomic relationships of *Ranavirus* (Qin et al., 2001; Watson et al., 1998; Zhang et al., 1999), as well as the morphology of the isolated virus in the infected EPC cell line in this study, it was apparent that the Chinese giant salamander virus-L (CGSV-L) particles showed the typical iridovirus morphology. Studies of the morphogenesis and morphological interactions between viruses and cells are a prerequisite for understanding and characterizing the life cycles of pathogenic viruses (Watson et al., 1998; Zhang et al., 2001). Furthermore, the morphogenesis and cellular interactions of CGSV-L should be investigated to discover differences of the pathological changes in host cells and to study the pathogenic mechanisms by distinguishing different types of cell damage.

The three gene regions (MCP, DNA polymerase, and NF-H1) obtained from Chinese giant salamander virus-L (CGSV-L) were analyzed using NCBI-BLAST and MEGA tools. MCP is a highly conserved protein among *Ranavirus* (Gray et al., 2009) and it widely used to detect and identify possible ranavirus pathogens (Geng et al., 2011; Gray et al., 2009). The 500 bp region at the 5′ end of the MCP gene has frequently been analyzed (Mao et al., 1999; Webby and Kalmakoff, 1998), but this fragment is not evident in viral isolates of particular *Ranavirus* species (Gray et al., 2009; Zhang et al., 2006). The DNA polymerase gene was commonly used to characterize large DNA viruses (Balseiro et al., 2009; Hanson et al., 2006). In this study, based on the cloned and analyzed complete MCP and the partial DNA polymerase gene, CGSV-L showed the most DNA sequence identity with FV3 and soft-shelled turtle iridovirus, while the phylogenetic analysis showed that it was particularly close to *Hynobiusnebulosus* virus and FV3. In addition, the NF-H1 gene is considered to exhibit higher variation than DNA polymerase and MCP genes in *Ranavirus* where it is used to distinguish closely related viral isolates (Holopainen et al., 2009; Kim et al., 2011). After our analysis of the NF-H1 gene, CGSV-L was shown to be closely related to *Ranavirus* KRV-1, soft-shelled turtle iridovirus, and *R. grylio* iridovirus, and it formed a cluster with FV3 in the phylogenetic analysis. Because of the genetic similarity of ranaviruses, members of the genus *Ranavirus* have been classified based on their different hosts and geographical ranges (Chinchar, 2002; Williams et al., 2005).

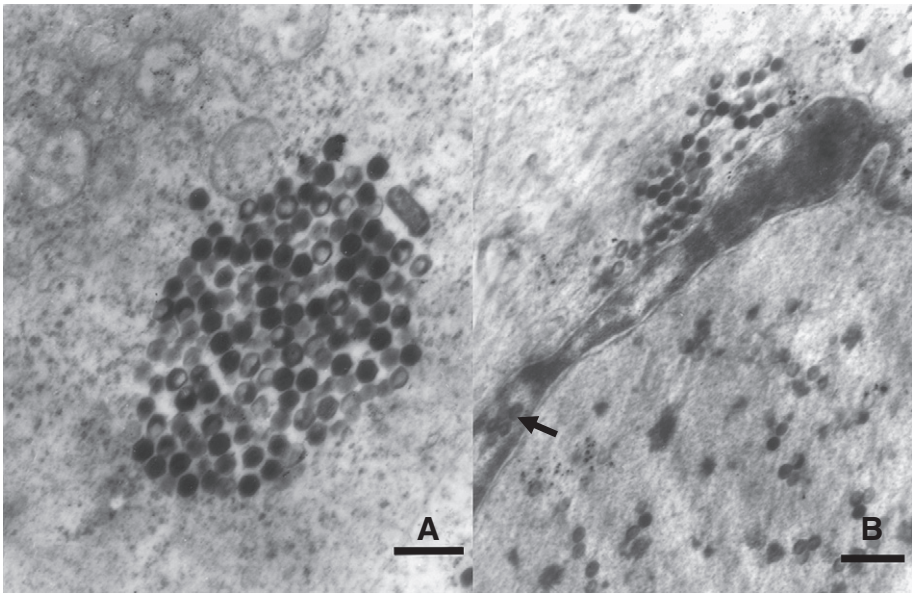

Fig. 5. Morphological study by electron microscopy. (A) The mature virus and nucleocapsid aggregated in pseudocrystalline arrays. Bar = 400 nm. (B) Viral particles in the cytoplasm and nuclei (arrow) of infected EPC cells. Bar = 600 nm.

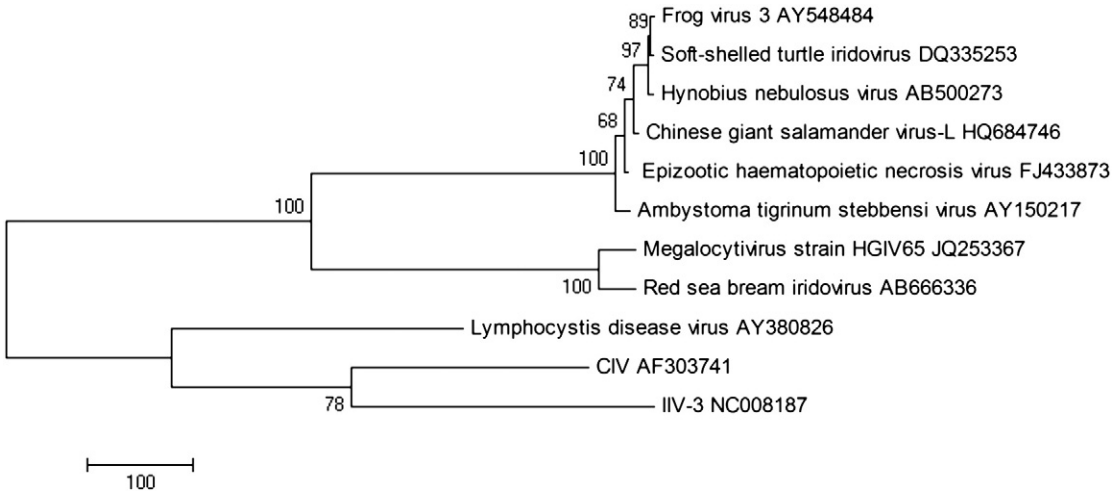

Fig. 6. The phylogenetic tree generated based on the complete MCP gene sequences using the Maximum Parsimony method, where the numbers at the nodes indicate the bootstrap values after 1000 replicates. The bootstrap values (%) are shown by the clades, while the accession numbers are stated by the names of the viruses, and the scale bars represent distance values.

The analysis of various characteristics revealed genomic differences, but the clinical pathological changes rarely changed, nor did the physicochemical properties and morphogenesis of CGSV-L, these all clearly demonstrated that CGSV-L was a member of the genus *Ranavirus*. Some ranaviruses have a broad host range and can infect fish and amphibians (Moody and Owens, 1994). Thus, ranaviruses are killers of lower vertebrates, which are spread in amphibians or fish in different parts of the world. Studies have shown that virions shed into the aquatic environment can successfully infect new amphibian hosts, the infection is most likely to occur on the epithelial surfaces leading to damaged skin, or the ingested ranavirus infects animal tissue (Holopainen et al., 2009). The Chinese giant salamanders may be fed on living frogs or fish that were infected with ranavirus in its environment. The possibility of the existence of the virus in the environment means it is a need to differentiate specific ranaviruses. The method of CGSV transmission, virus maintenance in the environment, and the environmental conditions of accompany mortality events are all important to understand these local Chinese giant salamander mortality events and to determine how they contribute to more widespread amphibian decline. In this study, the physicochemical properties, morphogenesis, and molecular analysis as the main steps to characterize CGSV-L, are all significant for identifying the virus and ultimately controlling the viral disease.

Acknowledgments

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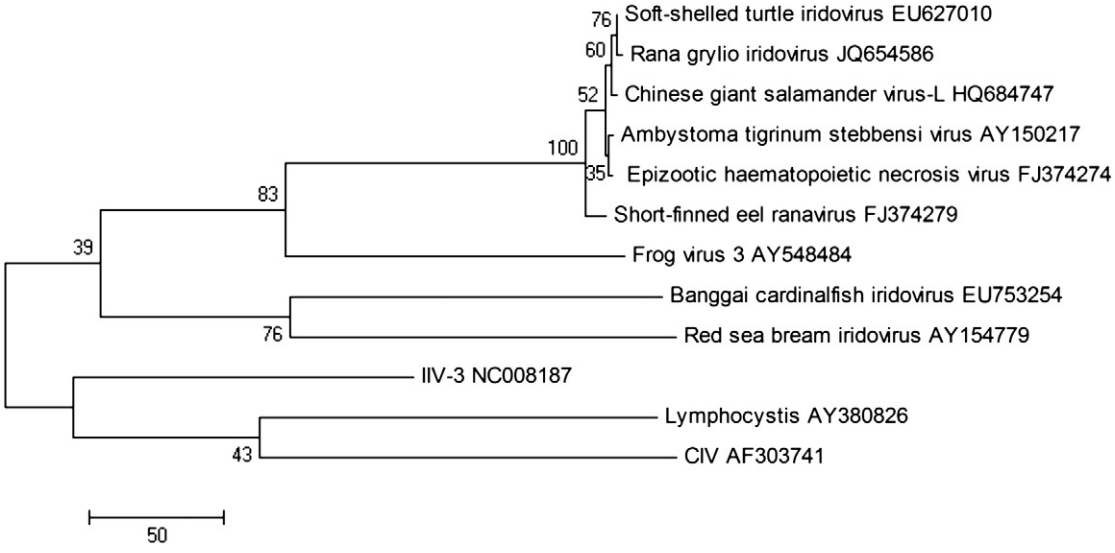

Fig. 7. The phylogenetic tree generated based on the DNA polymerase gene sequences using the Maximum Parsimony method, where the numbers at the nodes of the tree indicate the bootstrap values after 1000 replicates. The bootstrap values (%) are shown by the clades, while the accession numbers are stated by the names of the viruses, and the scale bars represent distance values.

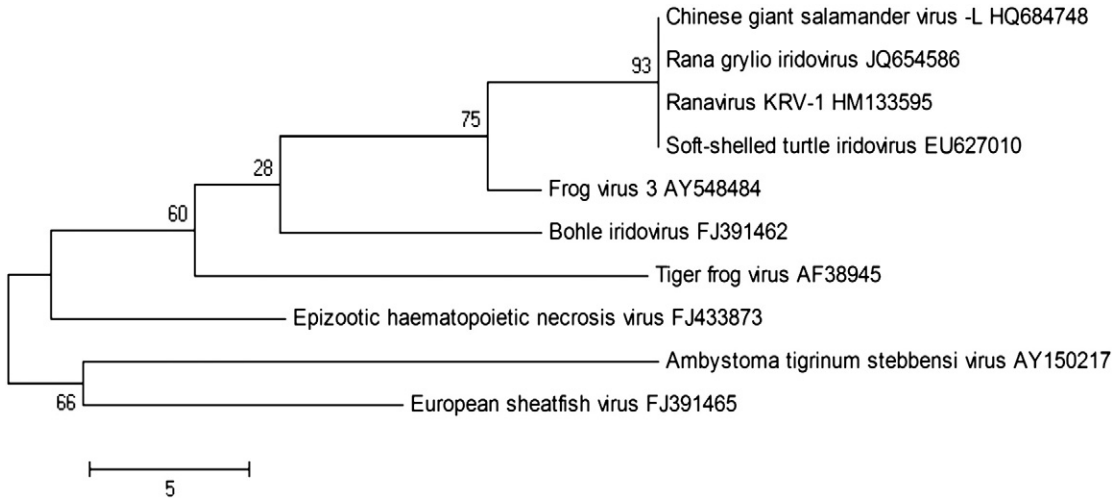

Fig. 8. The phylogenetic tree generated based on the NH-F1 gene sequences using the Maximum Parsimony method, where the numbers at the nodes of the tree indicate the bootstrap values after 1000 replicates. The bootstrap values (%) are shown by the clades, while the accession numbers are stated by the names of the viruses, and the scale bars represent distance values.

References

Ariel, E., Nicolajsen, N., Christophersen, M.B., Holopainen, R., Tapiovaara, H., Bang Jensen, B., 2009a. Propagation and isolation of ranaviruses in cell culture. Aquaculture 294, 159–164.

Ariel, E., Kielgast, J., Svart, H.E., Larsen, K., Tapiovaara, H., Bang Jensen, B., Holopainen, R., 2009b. Ranavirus in wild edible frogs *Pelophylax kl. esculentus* in Denmark. Diseases of Aquatic Organisms 85.

Balseiro, A., Dalton, K.P., Del Cerro, A., Marquez, I., Cunningham, A.A., Parra, F., Prieto, J.M., Casais, R., 2009. Pathology, isolation and molecular characterisation of a ranavirus from the common midwife toad *Alytes obstetricans* on the Iberian Peninsula. Diseases of Aquatic Organisms 84, 95.

Bollinger, T.K., Mao, J., Schock, D., Brigham, R.M., Chinchar, V.G., 1999. Pathology, isolation, and preliminary molecular characterization of a novel iridovirus from tiger salamanders in Saskatchewan. Journal of Wildlife Diseases 35, 413–429.

Chinchar, V., 2002. Ranaviruses (family Iridoviridae): emerging cold-blooded killers. Archives of Virology 147, 447–470.

Converse, K., Green, D., 2005. Diseases of salamanders. In: Majumdar, S.K., Huffman, J.E., Brenner, F.J., Panah, A.I. (Eds.), Wildlife Diseases: Landscape Epidemiology, Spatial Distribution and Utilization of Remote Sensing Technology. PAS, pp. 118–130.

Cunningham, A., Langton, T., Bennett, P., Lewin, J., Drury, S., Gough, R., Macgregor, S., 1996. Pathological and microbiological findings from incidents of unusual mortality of the common frog (*Rana temporaria*). Biological Sciences 1539–1557.

Docherty, D.E., Meteyer, C.U., Wang, J., Mao, J., Case, S.T., Chinchar, V.G., 2003. Diagnostic and molecular evaluation of three iridovirus-associated salamander mortality events. Journal of Wildlife Diseases 39, 556–566.

Dong, W., Zhang, X., Yang, C., An, J., Qin, J., Song, F., Zeng, W., 2011. Iridovirus infection in Chinese giant salamanders, China, 2010. Emerging Infectious Diseases 17, 2388.

Duffus, A.L.J., Cunningham, A.A., 2010. Major disease threats to European amphibians. Herpetological Journal 20, 117–127.

Feldman, H.A., Wang, S.S., 1961. Sensitivity of Various Viruses to Chloroform. RSM, pp. 736–738.

Fraser, W., Keefe, T., Bolon, B., 1993. Isolation of an iridovirus from farm-raised gouramis (*Trichogaster trichopterus*) with fatal disease. Journal of Veterinary Diagnostic Investigation 5, 250.

Geng, Y., Wang, K., Zhou, Z., Li, C., Wang, J., He, M., Yin, Z., Lai, W., 2011. First report of a ranavirus associated with morbidity and mortality in farmed Chinese giant salamanders (*Andrias davidianus*). Journal of Comparative Pathology 145, 95–102.

Gray, M.J., Miller, D.L., Hoverman, J.T., 2009. Ecology and pathology of amphibian ranaviruses. Diseases of Aquatic Organisms 87, 243–266.

Green, D., CoNVerSe, K.A., SCHrADer, A.K., 2002. Epizootiology of sixty-four amphibian morbidity and mortality events in the USA, 1996–2001. Annals of the New York Academy of Sciences 969, 323–339.

Hanson, L.A., Rudis, M.R., Vasquez-Lee, M., Montgomery, R.D., 2006. A broadly applicable method to characterize large DNA viruses and adenoviruses based on the DNA polymerase gene. Virology Journal 3, 28.

Holopainen, R., Ohlemeyer, S., Schütze, H., Bergmann, S., Tapiovaara, H., 2009. Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes. Diseases of Aquatic Organisms 85, 81–91.

Jancovich, J.K., Davidson, E.W., Morado, J.F., Jacobs, B.L., Collins, J.P., 1997. Isolation of a lethal virus from the endangered tiger salamander *Ambystoma tigrinum stebbinsi*. Diseases of Aquatic Organisms 31, 161–167.

Kim, Y.R., Hikima, J., Jang, H.B., Nho, S.W., Park, S.B., Cha, I.S., Ohtani, M., Eom, A.H., Aoki, T., Jung, T.S., 2011. Identification and determination of antigenic proteins of Korean ranavirus-1 (KRV-1) using MALDI-TOF/TOF MS analysis. Comparative Immunology, Microbiology and Infectious Diseases 34, 237–245.

Mao, J., Wang, J., Chinchar, G.D., Chinchar, V.G., 1999. Molecular characterization of a ranavirus isolated from largemouth bass *Micropterus salmoides*. Diseases of Aquatic Organisms 37, 107–114.

Miller, D.L., Rajeev, S., Gray, M.J., Baldwin, C.A., 2007. Frog virus 3 infection, cultured American bullfrogs. Emerging Infectious Diseases 13, 342.

Moody, N., Owens, L., 1994. Experimental demonstration of the pathogenicity of a frog virus, Bohle iridovirus, for a fish species, barramundi *Lates calcarifer*. Diseases of Aquatic Organisms 18, 95–102.

Muths, E., Gallant, A.L., Campbell Grant, E.H., Battaglin, W.A., Green, D.E., Staiger, J.S., Walls, S.C., Gunzburger, M.S., Kearney, R.F., 2006. The Amphibian Research and Monitoring Initiative (ARMI): 5-year report. U. S. Geological Survey, 77, pp. 2006–5224.

Qin, Q., Lam, T., Sin, Y., Shen, H., Chang, S., Ngoh, G., Chen, C., 2001. Electron microscopic observations of a marine fish iridovirus isolated from brown-spotted grouper, *Epinephelus tauvina*. Journal of Virological Methods 98, 17–24.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. American Journal of Epidemiology 27, 493–497.

Schoegel, L.M., Daszak, P., Cunningham, A.A., Speare, R., Hill, B., 2010. Two amphibian diseases, chytridiomycosis and ranaviral disease, are now globally notifiable to the World Organization for Animal Health (OIE): an assessment. Diseases of Aquatic Organisms 92, 101–108.

Watson, L., Groff, J., Hedrick, R., 1998. Replication and pathogenesis of white sturgeon iridovirus (WSIV) in experimentally infected white sturgeon *Acipenser transmontanus* juveniles and sturgeon cell lines. Diseases of Aquatic Organisms 32, 173.

Webby, R., Kalmakoff, J., 1998. Sequence comparison of the major capsid protein gene from 18 diverse iridoviruses. Archives of Virology 143, 1949–1966.

Whittington, R.J., Becker, J.A., Dennis, M.M., 2010. Iridovirus infections in finfish — critical review with emphasis on ranaviruses. Journal of Fish Diseases 33, 95–122.

Williams, T., Barbosa-Solomieu, V., Chinchar, V.G., 2005. A decade of advances in iridovirus research. Advances in Virus Research 65, 173–248.

Zhang, Q.Y., Li, Z.Q., Gui, J.F., 1999. Studies on morphogenesis and cellular interactions of *Rana grylio* virus in an infected fish cell line. Aquaculture 175, 185–197.

Zhang, Q.Y., Xiao, F., Li, Z.Q., Gui, J.F., Mao, J., Chinchar, V.G., 2001. Characterization of an iridovirus from the cultured pig frog *Rana grylio* with lethal syndrome. Diseases of Aquatic Organisms 48, 27–36.

Zhang, K.J., Wang, X.M., Wu, W., Wang, Z.H., Huang, S., 2002. Advances in conservation biology of Chinese giant salamander. Biodiversity Science 10, 291–297.

Zhang, Q.Y., Zhao, Z., Xiao, F., Li, Z.Q., Gui, J.F., 2006. Molecular characterization of three *Rana grylio* virus (RGV) isolates and *Paralichthys olivaceus* lymphocystis disease virus (LCDV-C) in iridoviruses. Aquaculture 251, 1–10.