

Isolation and characterization of *Scophthalmus maximus* rhabdovirus

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ABSTRACT: A rhabdovirus associated with a lethal hemorrhagic disease in cultured turbot *Scophthalmus maximus* Linnaeus was isolated. The virus induced typical cytopathogenic effects (CPE) in 9 of 15 fish cell lines examined and was then propagated and isolated from infected carp leucocyte cells (CLC). Electron microscopy observations revealed that the negatively stained virions had a typical bullet-shaped morphology with one rounded end and one flat base end. The bullet-shaped morphology was more obvious and clear in ultrathin sections of infected cells. Experimental infections also indicated that the *S. maximus* rhabdovirus (SMRV) was not only a viral pathogen for cultured turbot, but also had the ability to infect other fish species, such as freshwater grass carp. A partial nucleotide sequence of the SMRV polymerase gene was determined by RT-PCR using 2 pairs of degenerate primers designed according to the conserved sequences of rhabdovirus polymerase genes. Homology analysis, amino acid sequence alignment, and phylogenetic relationship analysis of the partial SMRV polymerase sequence indicated that SMRV was genetically distinct from other rhabdoviruses. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified SMRV revealed 5 major structural proteins, and their molecular masses were estimated to be about 250, 58, 47, 42, and 28 kDa. Significant serological reactivity differences were also observed between SMRV and its nearest neighbor, spring viremia of carp virus (SVCV). The data suggest that SMRV is likely a novel fish rhabdovirus, although it is closely related to rhabdoviruses in the genus *Vesiculovirus*.

KEY WORDS: Rhabdovirus · *Scophthalmus maximus* · Fish viral disease · Rhabdovirus polymerase

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INTRODUCTION

Turbot *Scophthalmus maximus* Linnaeus, which originated in Europe, has become an important aquaculture fish in northern China. Unfortunately, a disease with typical hemorrhagic syndrome has affected cultured turbot. In the past, several lethal viruses, such as grass carp hemorrhage virus (GCHV), mandarin fish *Siniperca chuatsi* spherical virus (SCSV), rhabdovirus (SCRV), and baculovirus (SCBV), Chinese sucker rhabdovirus (CSRV), and flounder lymphocystis virus (LCDV-C), have been identified and isolated from diseased grass carp *Ctenopharyngodon idellus* (Chen & Jiang 1984), from diseased mandarin fish (Zhang & Li 1999a,b), from diseased Chinese sucker *Myxocyprinus asiaticus* (Zhang et al. 2000), and from diseased floun-

der *Paralichthys olivaceus* (Zhang et al. 2003a, 2004). These viruses are associated with severe hemorrhagic disease and lymphocystis disease (Zhang 2002). Recently, we prepared the infectious agent from diseased turbot and used it to infect a carp leucocyte (CLC) cell line. Subsequently, a rhabdovirus termed *Scophthalmus maximus* rhabdovirus (SMRV) was observed from the diseased turbot, and some typical characteristics of apoptosis were revealed in CLC cells infected by the rhabdovirus (Du et al. 2004). The aims of the present study were to isolate and characterize the rhabdovirus and to determine the partial polymerase sequence of the virus. Moreover, the rhabdovirus was compared to some previously characterized rhabdoviruses based on the biochemical and molecular data.

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MATERIALS AND METHODS

Original viral isolate preparation. Diseased turbot showing hemorrhagic syndrome on the skin were sampled from a fish farm in Shandong Province of northern China, and the original viral isolate was prepared from the diseased tissues of liver, spleen, and kidney as described previously (Zhang et al. 2001). Briefly, a tissue sample was ground in a mortar and diluted 1:10 in phosphate buffered saline (PBS) containing antibiotics (100 IU penicillin ml⁻¹, 100 µg streptomycin ml⁻¹). The extract was stored overnight at -20°C. The supernatant was then collected by centrifugation at 2000 × *g* and filtered through a 300 to 450 nm filter membrane. The filtered supernatant was used as the original viral isolate for infecting cell lines and was stored at -80 or -20°C.

Cell culture and virus infection. A total of 15 cell lines, including *Carassius auratus* blastula embryos (CAB), *C. auratus* rear-fin (CAR), channel catfish ovary (CCO), chinook salmon embryo (CHSE), *Ctenopharyngodon idellus* kidney (CIK), carp leucocytes (CLC) *Epithelioma papulosum cyprini* (EPC), fathead minnow (FHM), grass carp fins (GCF), grass carp heart (GCH), grass carp ovary (GCO), grass carp vertebral column bone cell (GCVB), pike gonad (PG), rainbow trout gonad (RTG-2), and silver carp embryonic cells (SCE), were used for viral sensitivity tests (Zhang 1997). The cells were grown in TC 199 medium with 5% fetal bovine serum and antibiotics (100 IU penicillin ml⁻¹; 100 µg streptomycin ml⁻¹), and the growth temperature was 20°C unless otherwise stated.

After the stored original viral isolate was thawed at room temperature (RT), it was serially diluted from 10⁻¹ to 10⁻⁸ and inoculated onto the culturing cells in 96-well plates as described previously (Zhang et al. 1996, 1999). The virus titration was measured on the basis of the tissue culture infectious dose 50 (TCID₅₀) ml⁻¹ as described by Reed & Muench (1938). Stock SMRV extract was prepared by propagating the original viral isolate in CLC cells using 199 medium supplemented with 5% fetal bovine serum for cell growth at 20°C.

Experimental infection of fish. A total of 20 healthy grass carp, with mean weight of about 20 g, were collected from a fish farm with no outbreak disease history. They were split into an experimental group and a control group, each containing 10 fish. In addition, 4 healthy turbot, with mean weight of about 80 g, were divided into one infected group of 2 fish and another control group of 2 fish. The fish were transferred to 0.25 m³ tanks with cycling water and kept at a temperature of about 20°C. The fish in the experimental groups were challenged by an intraperitoneal injection of 10⁴ TCID₅₀ ml⁻¹ of the stock SMRV extract prepared from CLC cell culture, using 0.1 ml per grass carp

and 0.4 ml per turbot. The fish in the control groups were intraperitoneally injected with physiologic salt solution.

Biophysical and biochemical property detection. The optimal temperature for the virus propagation was detected by infecting monolayers of CLC cell cultures with 10-fold dilutions of the tissue isolate at 15, 18, 20, and 25°C. Heat lability of the virus was measured by incubating the virus extract at 56°C for 1 h, and samples were removed at selected intervals and the titer determined. Stability to pH was tested by incubating the virus extract at 18°C in TC 199 medium adjusted to pH 2.0, 3.0, 4.0, 9.0, and 10.0. After 1 h of incubation, the samples were titrated. Chloroform sensitivity and resistance to 5-iododeoxyuridine (IUdR) were determined as described previously (Zhang et al. 2000).

Virus isolation and purification. SMRV was isolated and purified from the infected CLC cells by the original viral isolate as described previously (Zhang et al. 2000). Briefly, following complete development of cytopathogenic effects (CPE), cell culture fluids were harvested and cell debris removed by centrifugation at 7000 × *g* for 20 min. The supernatant was then ultracentrifuged at 38 000 rpm (100 000 × *g*) (Beckman rotor SW41) for 40 min. The virus pellet was resuspended in 1 ml PBS and further purified by using discontinuous sucrose (20, 30, 40, and 50%, w/w) gradient centrifugation at 38 000 rpm (100 000 × *g*) for 40 min. The virus particle band was collected, and sucrose was removed by centrifugation at 38 000 rpm (100 000 × *g*) for 30 min in PBS. The resulting pellet was resuspended in 0.5 ml TE (1 mM EDTA, 10 mM Tris-Cl, pH 8.0) buffer, and this purified virus was stored at -20°C until use.

Fluorescent staining and microscope observation of virus-infected cells. The CLC cells, grown on glass microscope slides, were infected with the virus. The infected cells were fixed at different times with 3.7% formaldehyde (20°C, 15 min), dried in acetone, and stained with the DNA-binding fluorescent dye 4',6'-diamidino-2-phenylindole (DAPI, Sigma) at a final concentration of 4 µg ml⁻¹ in PBS (pH 7.5) for 20 min at RT. The samples were examined by fluorescence microscopy (Leica; Du et al. 2004).

Electron microscopy. The infected CLC cells were collected after appearance of CPE and then fixed with 2% glutaraldehyde, post-fixed in aqueous 1% osmium tetroxide (OsO₄), dehydrated, and embedded. Ultrathin sections were prepared with an ultramicrotome (Leica Ultracut microtome) and stained with 2% uranylacetate and lead citrate. Purified virus particles were negatively stained with 2% (w/v) uranylacetate. All sample grids were then examined with transmission electron microscopy (JEM-1230).

Virus RNA extraction and RT-PCR assays. The purified viruses were mixed with 10% sodium dodecyl

sulfate (SDS) and incubated for about 1 h after adding Proteinase K. Proteins were removed with phenol:chloroform (1:1), subsequently centrifuging at $12\,000 \times g$ for 10 min at 4°C. The upper aqueous phase (containing the viral RNA) was extracted again and transferred to a fresh tube. The RNA was precipitated by mixing with 1/10 volume of 3 M NaAc (pH 4.6) and adding 2.5× volume of cold 100% ethanol. The solution was stored at -20°C overnight before centrifugation at $12\,000 \times g$ for 30 min. The RNA pellets were washed 2 times in 70% ethanol, dried for 10 min, dissolved in 15 µl Tris-ethylenediaminetetraacetic (TE) buffer, and stored at -80°C until use.

SMRV genomic RNA isolated from purified viral particles was used as template for RT-PCR amplification. Two pairs of degenerate primers were designed and synthesized according to the conserved nucleotide sequences of polymerase genes in some rhabdoviruses. One pair of primers was targeted to the positions of 1106-1128 nucleotides (nt) (5'-TCDDT-YAGRCATTGGGGWCATCC-3') and 1863-1841 nt (5'-TARTCAATGTGRGCWATGCA-3') in the spring viremia of carp virus (SVCV) polymerase gene (Hoffmann et al. 2002), and the other was located at the positions of 1425-1405 nt (5'-ATCSRTRATWACTC-WGACAA-3') and 2166-2151 nt (5'-TTRTCHCCYT-GHGCA-3') in the SVCV polymerase gene (Hoffmann et al. 2002). RT-PCR was performed as described by Zhang et al. (2003b). Briefly, the reaction mix was subjected to 5 temperature cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C with the annealing temperature decreasing by 1°C per cycle, followed by 30 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C, and completed by a final extension step of 10 min at 72°C. Aliquots (10 µl) of the PCR reaction mixture were electrophoresed on 1% agarose (1× Tris-acetate-EDTA [TAE, 40 mM Tris-acetate, 5 mM EDTA]) gel containing ethidium bromide and scanned under UV transillumination.

Sequencing and alignment analysis. PCR products were purified using a DNA Extraction Kit according to the manufacturer's protocol (Fermentas) and cloned into pMD-18-T vector (Takara) following the manufacturer's protocol. DNA fragment sequencing was performed with a BigDye Terminator reagent kit in an ABI 377 apparatus (Perkin-Elmer) according to the manufacturer's specifications. Phylogenetic analysis was performed on the partial polymerase sequence using the DNASTar software package, and the partial polymerase sequences were obtained from the databases in GenBank. A BLAST search was used to compare homologous regions of SMRV and 12 other rhabdovirus sequences. A phylogenetic tree was constructed using the MegAlign ClustalW program in the DNASTar software.

Electrophoresis of SMRV and SVCV structure polypeptides. In order to reveal the structure polypeptide profile, SVCV (Ahne et al. 2002), a known rhabdovirus related to SMRV, was also propagated and purified from the infected EPC cells. The purified SMRV and SVCV particles were respectively dissolved in loading buffer (50 mM Tris-Cl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled at 100°C for 5 min. Polypeptides were separated by SDS 12% polyacrylamide gel electrophoresis (SDS-PAGE) on a Mini-Protein II electrophoresis system (Bio-Rad) described previously (Gui et al. 1994). The bands were visualized using Coomassie brilliant blue.

Anti-serum preparation and Western blot analysis. The purified SMRV was mixed 1:1 with Freund's adjuvant (Sigma), and used to immunize an 8 wk old BALB/c mouse by intraperitoneal injection with 0.5 ml of the SMRV mixture 4 times at intervals of 5 wk. Five wk after the first immunization, a serum sample was taken from the immunized mouse and used for Western blot analysis as described previously (Zhang et al. 2006). Briefly, polypeptides of the purified SMRV and SVCV were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane using a Bio-Rad transfer apparatus for 45 min at 70 V. The membrane was rinsed with distilled water and then blocked overnight in 5% skim milk in TBST (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 0.05% Tween-20). The diluted (1:1000) anti-SMRV serum was then applied for 1 h at RT. After rinsing 3 times for 15 min in TBST, the membranes were incubated with goat anti-mouse IgG alkaline phosphatase conjugate (Vector) diluted 1:1000 in 5% skim milk for 1 h at RT. After rinsing in TBST, the membranes were developed with an NBT-BCIP substrate system for 20 min in the dark. The reaction was stopped with water.

RESULTS

Infectivity and CPE in different fish cell lines

The original viral isolate prepared from the diseased turbot with hemorrhagic syndrome was demonstrated to have strong infectivity to the CLC cell line, and the bullet-shaped virus particles SMRV, were observed in the infected CLC cells (Du et al. 2004). To further reveal the basic characteristics, 15 fish cell lines were used to examine SMRV infectivity and CPE. The SMRV isolate induced typical CPE in 9 cell lines, and obvious differences in cytopathogenic appearance were observed under the same conditions. In CHSE, CLC, EPC, GCF, GCO, PG, and RTG-2 cell lines, CPE was observed in cell monolayers after 1 to 2 d of incu-

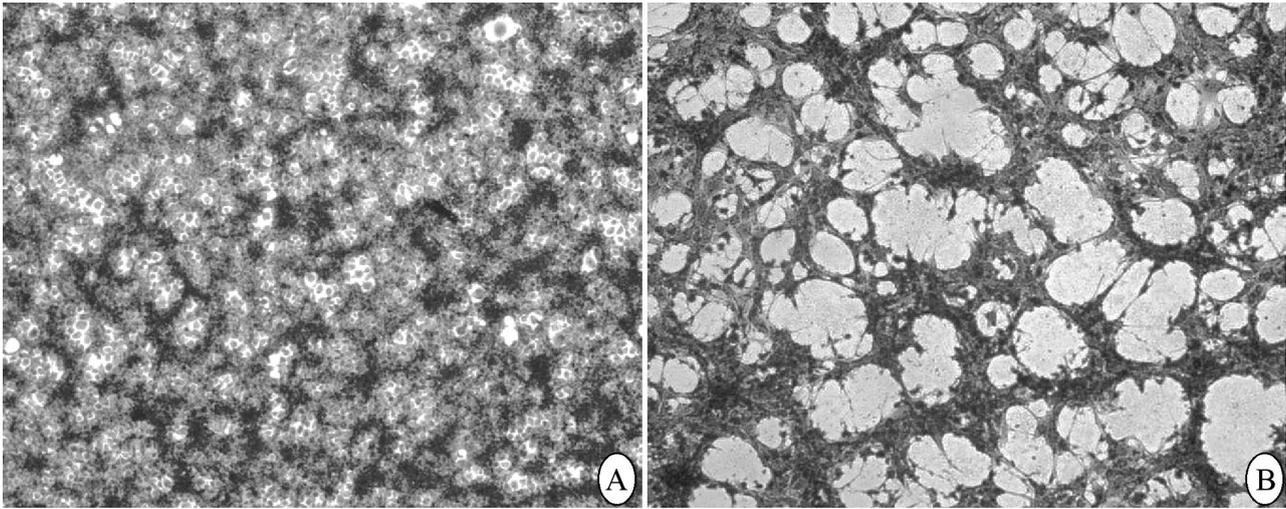


Fig. 1. Cytopathogenic effects (CPE) produced by the original viral isolate in the infected carp leucocyte cell (CLC) monolayer. (A) Uninfected control CLC cell monolayer. (B) Infected CLC cell monolayer with CPE at 1 d ($\times 50$)

bation at 20°C, and the entire cell sheets were almost completely lysed after 3 to 4 d. In CCO and FHM cell lines, the SMRV isolate showed moderate infectivity, and CPE was observed in the cell monolayers until 4 to 5 d of incubation at 20°C. No obvious CPE was observed after 1 wk of incubation in the other 6 cell lines, i.e. CAR, SCE, CAB, CIK, GCH, and GCVB. In comparison with other cell lines, the CLC cell line was the most susceptible to SMRV infection. As shown in Fig. 1, when CLC cells were infected by the original viral isolate for only 1 d, the plaques that resulted from CPE had almost extended across the entire cell sheets. Therefore, the CLC cell line was used for the subsequent investigation.

Clinical signs of infected fish

Healthy turbot and grass carp were infected with virus isolate prepared from diseased turbot with typical hemorrhage syndrome, and the infected fish began to show some clinical signs within 5 to 7 d. At the early stage, some hemorrhage spots were observed on the skin of turbot (Fig. 2A). As infection time increased, the hemorrhage spots resulted in skin erosion and ulceration, and the fins became swollen and reddened because of extensive and severe hemorrhaging (Fig. 2B). The infected turbot died 10 d post-infection. Anatomical observation revealed numerous hemorrhage spots on the liver and other tissues (Fig. 2C). The disease symptoms of the infected grass carp were basically similar to the turbot, and some bleeding speckles were observed on the skin (Fig. 2D). All diseased grass carp died at about 10 d after infection.

Biophysical and biochemical characteristics

Monolayers of CLC cell cultures were infected with 10-fold dilutions of the original viral isolate at 15, 18, 20, and 25°C. The temperature range of virus proliferation extended from 15 to 20°C, and the maximum virus infectivity was obtained with $TCID_{50} 10^7$ at 18°C. The virus lost its infectivity at 56°C, and thus was unstable at high temperatures. The infectivity was basically stable between pH 4.0 and 9.0, but caused significant reduction below pH 3.0 and above pH 10.0.

The deoxyuridine analogs IuDR and chloroform were used to treat the virus isolate. We found that IuDR did not affect infectivity of the virus, whereas chloroform treatment reduced its infectivity. These results indicate that the virus possesses a possible RNA genome and a lipid-containing envelope.

Isolation and morphology of SMRV

The virus was isolated and purified by sucrose gradient centrifugation from CLC cells infected by the original viral isolate. Electron microscopy observations revealed that the negatively stained virions had a typical bullet-shaped morphology with one rounded end and one flat base end (Fig. 3A) and were about $80\text{--}100 \times 40\text{--}60$ nm in size. The bullet-shaped morphology was more obvious and clear in the ultrathin sections of infected cells (Fig. 3B). The bullet-shaped nucleocapsids were measured and ranged in size from 110 to 150 nm in length and 40 to 60 nm in diameter. The spikes (about 10 nm long) dispersed evenly over the entire surface of the virus (Fig. 3C). One central

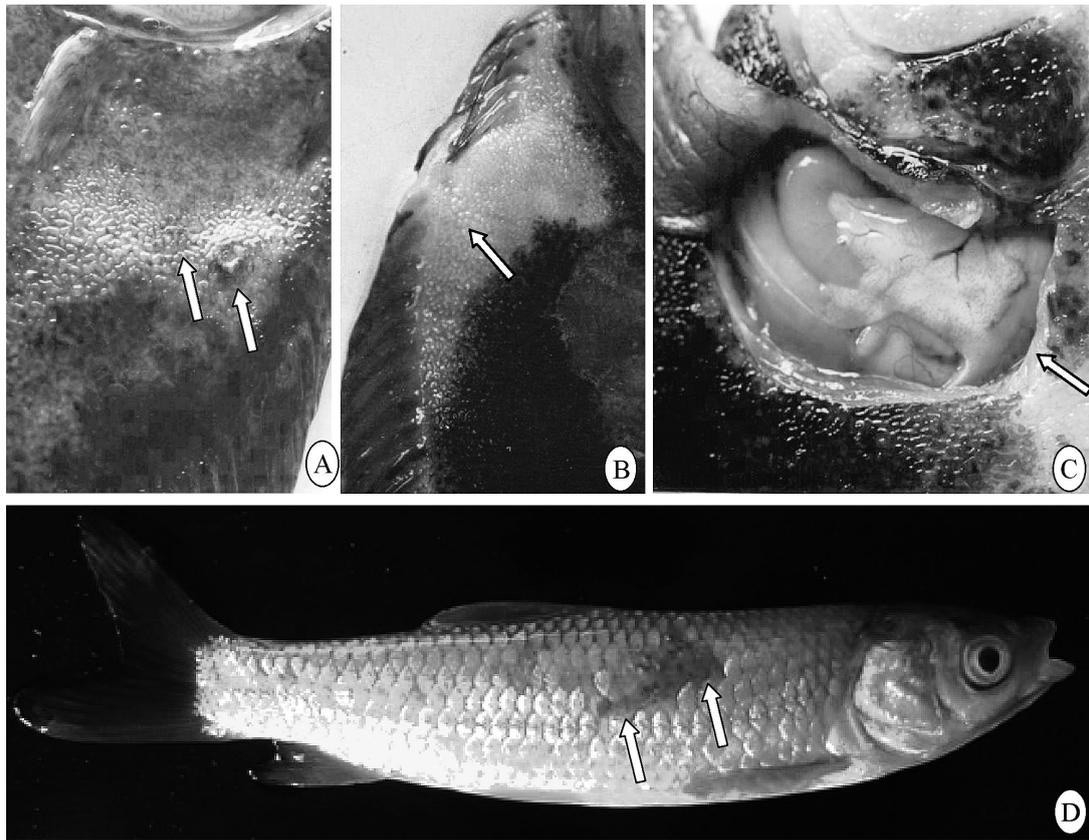


Fig. 2. *Scophthalmus maximus* and *Ctenopharyngodon idellus*. Clinical signs of infected fish. (A) Hemorrhage spots (arrows) on the skin of infected turbot. (B) Swollen and reddened basal fin resulting from extensive and severe hemorrhaging (arrow) in infected turbot. (C) Liver of infected turbot with mottled brown color (arrow). (D) Bleeding spots on the skin of infected grass carp (arrows)

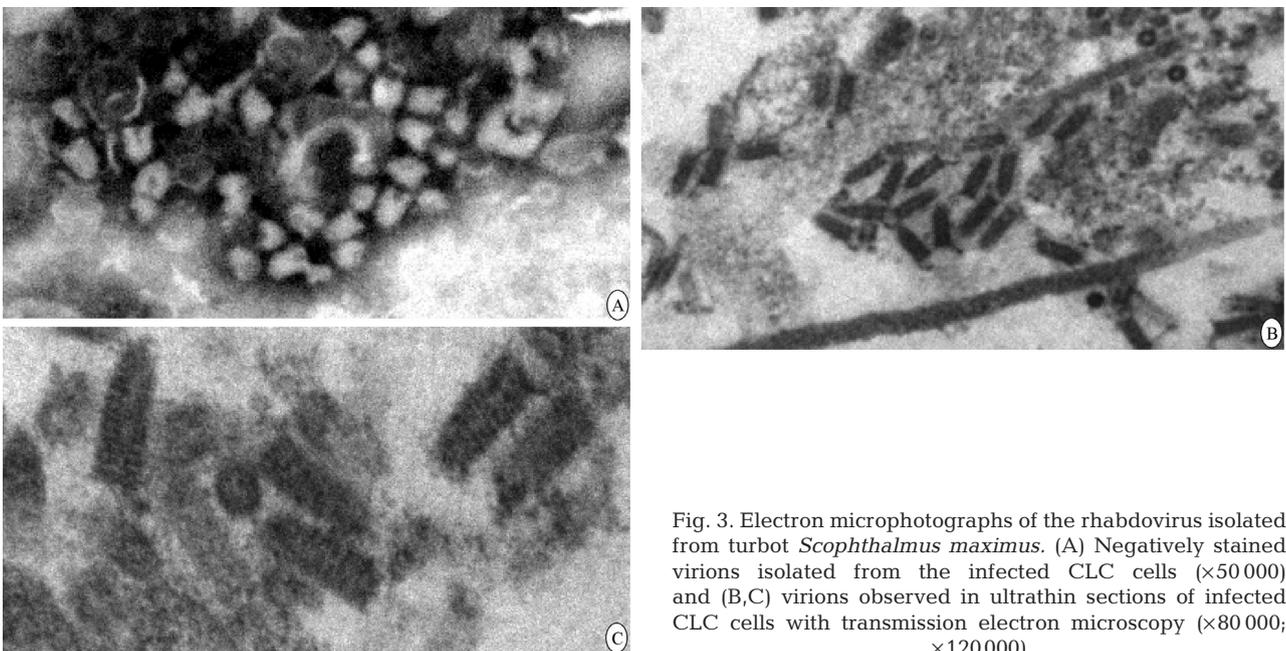


Fig. 3. Electron microphotographs of the rhabdovirus isolated from turbot *Scophthalmus maximus*. (A) Negatively stained virions isolated from the infected CLC cells ($\times 50\,000$) and (B,C) virions observed in ultrathin sections of infected CLC cells with transmission electron microscopy ($\times 80\,000$; $\times 120\,000$)

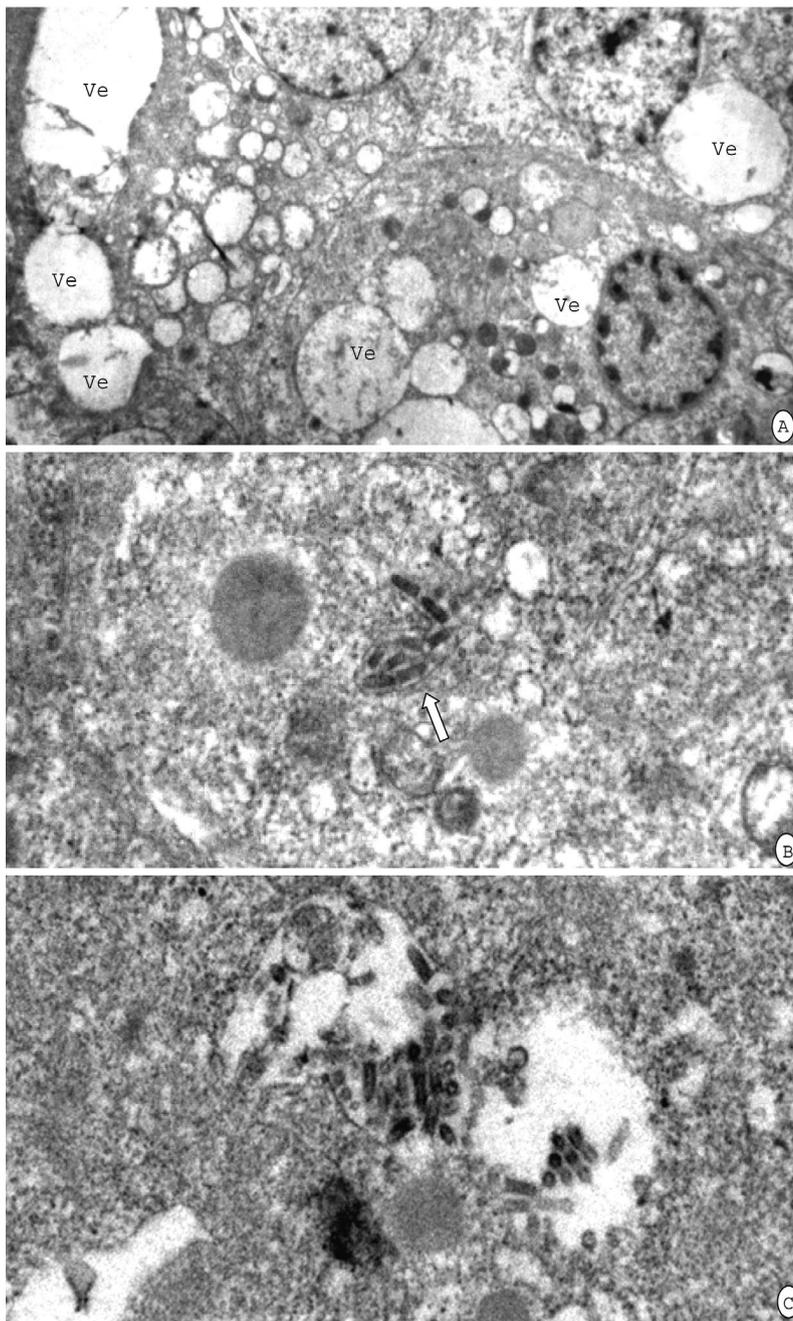


Fig. 4. *Scophthalmus maximus* rhabdovirus (SMRV) morphogenesis. (A) Large number of intracytoplasmic vesicles (Ve) in SMRV-infected CLC cells ($\times 40\,000$). (B) Early small vesicle containing virus particles and viromatrix-like area (arrow) in SMRV-infected CLC cells ($\times 30\,000$). (C) Joined large vesicle full of numerous virus particles in SMRV-infected CLC cells ($\times 6\,000$)

electron-lucent hole of 10 to 20 nm in diameter was observed in the cross sections of nucleocapsids (Fig. 3B). The isolated virus is likely a member of the family *Rhabdoviridae*, and was therefore called *Scophthalmus maximus* rhabdovirus (SMRV).

Ultrastructural characterization of SMRV morphogenesis

One striking characteristic of SMRV morphogenesis was the strong proliferation of intracytoplasmic vesicles in the SMRV-infected CLC cells (Fig. 4A). Some viral components in different stages of morphogenesis were found only within the cytoplasm, and most of them were observed in these intracytoplasmic vesicles. At the early stages of virus infection, the majority of the intracytoplasmic vesicles were empty (Fig. 4A). As the infection time increased, some virus particles at different stages of assembly appeared in the vesicles (Fig. 4B). As the infection process extended further, the vesicles enlarged, or 2 or more vesicles joined together, and numerous virus particles were crowded into the vesicles (Fig. 4C). The early small vesicles (Fig. 4B), similar to viromatrix, may be the central area for the SMRV assembly in cytoplasm. The late large vesicles (Fig. 4C), which were full of virus particles, may be virus inclusion bodies that can be observed under light microscopy by staining with hematoxylin and eosin (data not shown).

Partial sequence analysis of SMRV polymerase

Two fragments of the polymerase gene were amplified by RT-PCR using 2 pairs of the designed primers from SMRV genomic RNA isolated from infected CLC cells. The genes were determined by sequencing to be 758 and 761 bp long, respectively, and to have a 435 bp overlap with each other. Thus, a 1084 bp nucleotide sequence of the SMRV polymerase gene was obtained, and the partial sequence corresponded to nucleotide positions ranging from 1106 to 2166 bp of the SVCV polymerase gene (Hoffmann et al. 2002). After the primer sequences were truncated from the 2 terminals, the SMRV polymerase gene portion encoded 334 amino acids (aa). Homology analysis (Table 1) showed that the 334 aa portion had 68.3% identity to that of SVCV (Hoffmann et al. 2002), 67.9% identity to that of the Adelaide River virus

Table 1. Identity percentage of the partial amino acid sequences of *Scophthalmus maximus* rhabdovirus (SMRV) polymerase with corresponding regions of 18 other rhabdoviruses. SMRV: (bankit678369_); SVCV: spring viremia of carp virus (CAC51337); ADRV: Adelaide River virus (AAG10421); CHPV: Chandipura virus (AJ810083); VSIV-MS: vesicular stomatitis Indiana virus Mudd-Summers strain (K02378); ISFV: Isfahan virus (AJ810084); VSV-OG: vesicular stomatitis New Jersey virus Ogden strain (AAL73076); VSNJV-HZ: vesicular stomatitis New Jersey virus Hazelhurst strain (P13615); SFRV: starry flounder rhabdovirus (AAS02285); BEFV: bovine ephemeral fever virus (NP-065409); TRV: tupaia rhabdovirus (YP-238534); FLV: Flanders virus (AAN73288); ABLV: Australian bat lyssavirus (NP-478343); RABV: rabies virus (NP-056797); MV: Mokola virus (YP-142354); HIRRV: hirame rhabdovirus (NP-919035); IHNV: infectious hematopoietic necrosis virus (CAA61500); SHRV: snakehead rhabdovirus (NP-050585); VHSV: viral hemorrhagic septicemia virus (NP-049550)

	SMRV	SVCV	ADRV	CHPV	VSIV-MS	ISFV	VSV-OG	VSNJV-HZ	SFRV	BEFV	TRV	FLV	ABLV	RABV	MV	HIRRV	IHNV	SHRV
SVCV	68.3																	
ADRV	67.9	68.3																
CHPV	67.4	70.7	62.2															
VSIV-MS	67.4	73.7	66.1	78.4														
ISFV	67.1	71.3	63.8	83.5	77.2													
VSV-OG	66.8	77.2	66.5	75.4	77.5	74.6												
VSNJV-HZ	66.5	76.6	66.1	74.9	77.5	74.3	97.6											
SFRV	66.5	71.2	66.8	71.2	72.7	72.4	70.6	70.6										
BEFV	62.6	60.8	74.1	62.6	62.0	62.0	61.4	61.4	62.2									
TRV	62.5	63.7	60.3	63.4	63.1	62.5	64.0	63.4	62.2	55.6								
FLV	59.8	60.4	56.2	61.0	61.6	61.9	62.2	60.7	59.8	62.8	58.9							
ABLV	49.4	50.9	50.9	48.5	50.9	50.6	52.1	51.8	50.2	49.1	49.8	48.0						
RABV	49.4	51.8	51.3	49.4	51.2	50.9	52.4	52.1	50.8	50.0	49.5	48.3	93.4					
MV	48.5	51.2	47.8	49.4	50.9	51.5	51.8	51.5	49.5	48.2	51.1	47.1	87.1	86.6				
HIRRV	21.3	24.0	23.7	21.9	22.2	18.6	22.8	19.5	17.7	21.9	23.4	17.7	21.9	23.0	23.3			
IHNV	21.0	23.1	23.2	20.7	21.6	17.1	21.3	21.3	20.1	21.9	22.8	15.6	21.0	23.3	23.0	90.0		
SHRV	19.5	24.9	24.6	24.0	23.7	19.5	20.1	20.1	21.6	22.8	23.4	17.4	22.5	20.3	24.8	74.6	73.9	
VHSV	18.3	23.1	24.6	19.8	19.8	17.1	20.7	20.1	16.5	19.2	23.1	21.0	19.2	22.1	23.3	73.1	71.0	77.2

(ADRV; Dhillon et al. 2000), 67.4% identities to that of Chandipura virus (CHPV; GenBank accession no. AJ810083) and vesicular stomatitis Indiana virus (VSIV-MS; Schubert et al. 1984), 67.1% identity to that of Isfahan virus (ISFV; GenBank accession no. AJ810084), 66.8% identity to that of vesicular stomatitis virus (VSV-OG; GenBank accession no. AAL73075), 66.5% identity to that of vesicular stomatitis New Jersey virus (VSNJV-HZ; Barik et al. 1990), and 65.5% identity to that of starry flounder rhabdovirus (SFRV; Mork et al. 2004). Very low identities were observed with rhabdoviruses of the genus *Novirhabdovirus*. For example, there was only 18.3% identity (Table 1) to that of viral hemorrhagic septicemia virus (VHSV), the type species of *Novirhabdovirus* (Schütze et al. 1999).

Amino acid sequence alignment (Fig. 5) indicated that the partial SMRV polymerase sequence corresponds to the regions ranging from 343 to 676 aa of SVCV polymerase, from 356 to 689 aa of SFRV polymerase, from 356 to 689 aa of FLV polymerase, and from 354 to 687 aa of VSIV-MS, VSV-OG, and VSNJV-HZ polymerases. As shown in Fig. 5, the partial sequence of SMRV polymerase is homologous to the corresponding parts of the known rhabdovirus polymerases, and some strongly conserved motif regions contain invariant amino acids, although a difference obviously exists between SMRV and other rhabdoviruses.

Phylogenetic analysis

Based on the homology analysis and multiple alignments, the partial sequence of SMRV polymerase was used to construct phylogenetic relationships with 18 other rhabdoviruses. As shown in Fig. 6, the 19 rhabdoviruses were divided into 3 large groups. SMRV was clustered into the largest group with VSIV-MS, CHPV, ISFV, SVCV, VSV-OG, VSNJV-HZ, and SFRV of the genus *Vesiculovirus*, bovine ephemeral fever virus (BEFV) and ADRV of the genus *Ephemerovirus*, and unclassified tupaia rhabdovirus (TRV) and Flanders virus (FLV), but it appeared to locate between *Vesiculovirus* and *Ephemerovirus*. Thus, SMRV was genetically distinct from other rhabdoviruses.

Major structural proteins of SMRV and comparison to those of SVCV

SDS-PAGE analysis of the purified SMRV and SVCV revealed significant protein profile differences. As shown in Fig. 7A, their protein profiles were composed of 5 major structure polypeptides, most of which were obviously different from each other in size. Similar to a previous report (Walker et al. 2000), the 5 major structure proteins of SVCV were estimated to be about 238, 57, 45, 36, and 26 kDa, and are polymerase (L), glyco-

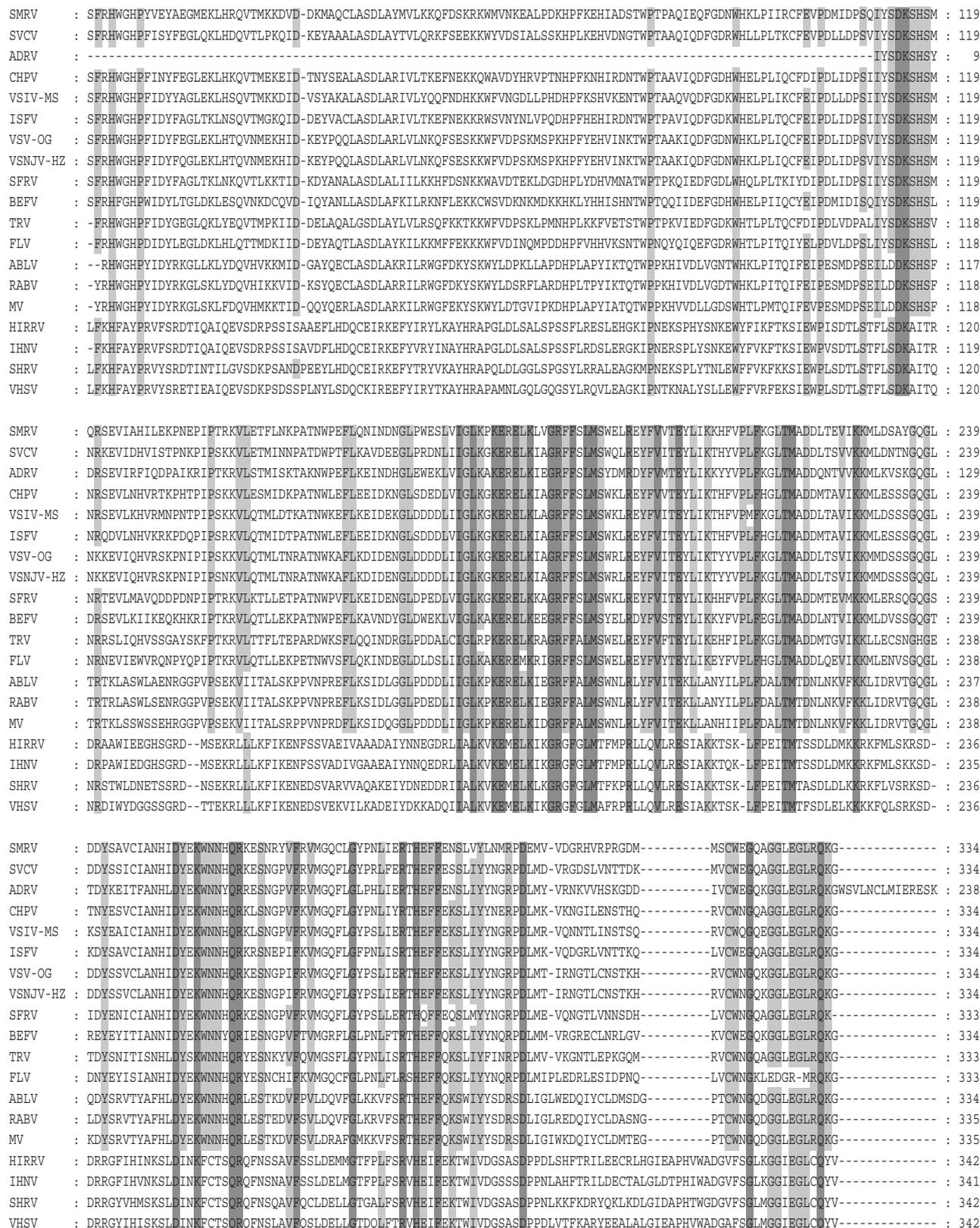


Fig. 5. Multiple amino acid sequence alignment. The sequences were aligned with Clustal X 1.83 software, and the amino acid identities were calculated with the GeneDoc program. Dark grey bars: 100% conservation; light grey bars: <100% but >80% conservation. Abbreviations as in Table 1

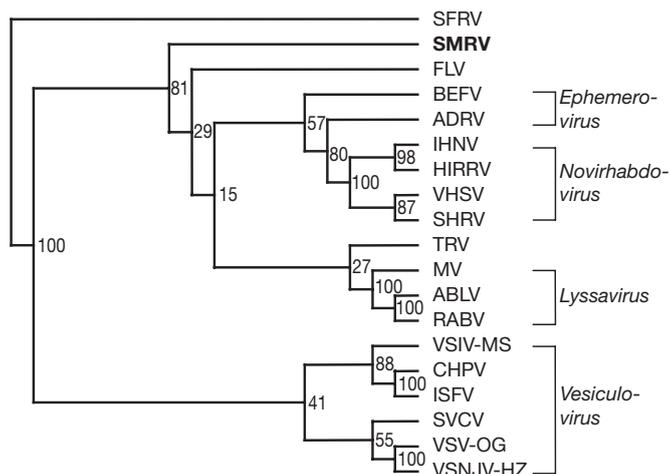


Fig. 6. Phylogenetic tree constructed from the alignment of the deduced partial amino acid sequences of L proteins of 19 rhabdoviruses by the neighbor-joining method in the PHYLIP 3.63 software. Values at the nodes of the branches indicate the statistical reliability obtained by bootstrap analysis (100 replications). Viruses are mainly divided into 4 established genera: *Ephemero-virus*, *Novirhabdo-virus*, *Lyssavirus*, and *Vesiculo-virus*. Abbreviations as in Table 1

protein (G), nucleoprotein (N), phosphoprotein (P), and matrix protein (M), respectively. The 5 major structure proteins of SMRV were estimated to be about 250, 58, 47, 42, and 28 kDa by comparison with the relative mobility of the known protein molecular masses, and the 47 kDa proteins appeared to be most abundant. Obviously, the major structure proteins of SMRV are larger than the corresponding products of SVCV.

Moreover, the structure proteins of SMRV and SVCV were subjected to Western blot analysis. As shown in Fig. 7B, 3 SMRV structure proteins, i.e. 58 kDa G protein, 47 kDa N protein, and 28 kDa M protein, were

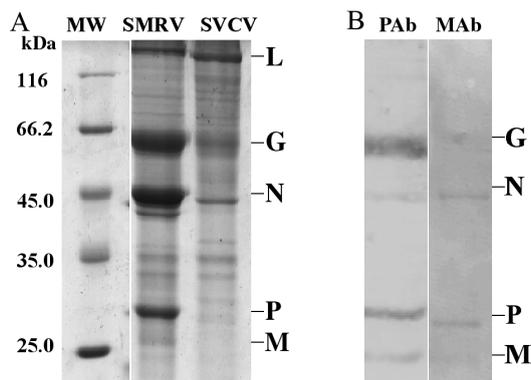


Fig. 7. (A) SDS-PAGE and (B) Western blot analysis of the *Scophthalmus maximus* rhabdovirus (SMRV) proteins. Five protein bands were similar to spring viremia of carp virus (SVCV), a strain of rhabdovirus. N, P, and M proteins were observed in the corresponding positions on the Western blot. MW: molecular weight; PAb: polyclonal antibody; MAb: monoclonal antibody

recognized by the anti-SMRV serum, whereas only one 45 kDa N protein was detected in the SVCV structure proteins. The data further indicated that significant differences existed between SMRV and SVCV.

DISCUSSION

The above studies reveal the presence of a viral pathogen in cultured turbot *Scophthalmus maximus* Linnaeus, and indicate that the virus is likely a member of the family *Rhabdoviridae* (van Regenmortel et al. 2000, Tordo et al. 2004) according to its morphology and ultrastructural characterization. For example, the virus particles exhibit typical bullet-shaped morphology, and the formation of intracytoplasmic vesicles and inclusion bodies in infected cells is similar to that of another rhabdovirus described previously (Zhang et al. 2000). Moreover, most of the characteristics of these virus particles, including their size, extensive virus infectivity to fish cell lines, strong CPE, stability at high temperatures, a few major structural proteins, and a possible RNA genome, very closely resemble those of other rhabdoviruses (Wolf 1988, Kasornchandra et al. 1992, Lilley & Frerichs 1994, Zhang et al. 2000, Isshiki et al. 2001).

Rhabdoviruses are significant viral pathogens in aquaculture, and more than 10 species of rhabdoviruses have been isolated from cultured fish. The infected fish include some important freshwater and marine species, including rainbow trout, common carp, and Japanese flounder, and these rhabdoviruses have caused significant economic losses (Ahne et al. 2002, Dopaz et al. 2002, Iwanowicz & Goodwin 2002, Johansson et al. 2002, Betts et al. 2003, Mork et al. 2004). Rhabdoviruses all have bullet or bacilliform morphology and have been reassigned to 6 genera: *Vesiculovirus*, *Lyssavirus*, *Ephemero-virus*, *Cytorhabdovirus*, *Nucleorhabdovirus* and *Novirhabdovirus*. Some fish rhabdovirus isolates, e.g. infectious hemorrhagic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV), were previously placed in the genus *Lyssavirus* based on the electrophoretic profile of their structural proteins (Kasornchandra et al. 1992), but they have more recently been assigned to the genus *Novirhabdovirus* (Walker et al. 2000). The most significant results of this study were the isolation and characterization of the SMRV on a molecular level. Obviously, the SMRV partial polymerase sequence differs from any corresponding sequences of other known rhabdoviruses, because the highest amino acid identity was only 68.3% of that of SVCV in the genus *Vesiculovirus* (Hoffmann et al. 2002). Homology analysis, amino acid sequence alignment, and phylogenetic relationship analysis of the partial SMRV polymerase

sequence indicated that SMRV was genetically distinct from other rhabdoviruses, and its relatedness was closest to rhabdoviruses in the genus *Vesiculovirus*, such as SVCV, SFRV, ISFV, VSIV-MS, VSNJV-OG, and VSNJV-HZ. SMRV was less related to rhabdoviruses in the genera *Novirhabdovirus*, *Cytorhabdovirus*, and *Nucleorhabdovirus*, because lower identities were obtained between SMRV and representatives of these genera (Table 1). Before this study, we had tried to amplify gene fragments using primers that were designed according to conserved sequences in *Novirhabdovirus*; however, no products were obtained from the SMRV. As a result, we alternatively designed the primers according to the conserved sequences of polymerase genes in the genus *Vesiculovirus* and obtained the essential data. In this study, the partial polymerase sequence was obtained with the proliferated SMRV in CLC cells; thus some nucleotide mutations may have occurred. However, the significant sequence difference with other known rhabdoviruses may suggest that the SMRV is related to rhabdoviruses in the genus *Vesiculovirus* and may be a novel rhabdovirus. Further evidence to support the suggestion of a novel rhabdovirus should be obtained by sequencing the whole virus genome, especially from naturally infected fish.

In the present study, 9 fish cell lines, including marine and freshwater fish, were susceptible to the isolated SMRV, and the susceptibility was unrelated to particular clades of fish and particular types of tissue because the susceptible cell lines originated from different fish in various taxonomical groups and from different tissues. The temperature range of virus infection and proliferation was examined only in the CLC cell line, and the optimum temperature of SMRV replication in CLC cells was revealed to be 18°C. Experimental infections also indicated that SMRV was not only a viral pathogen for cultured turbot, but also had the ability to infect other fish species, such as freshwater grass carp. The extensive host ranges and lower temperature pathogenicity of SMRV are basically similar to some rhabdoviruses, such as SFRV (Mork et al. 2004).

Most fish rhabdoviruses have been analyzed and compared in terms of their morphology, cytopathogenicity, serological relatedness, and structural polypeptide composition (Zhang et al. 2000, Ahne et al. 2002, Dopaz et al. 2002, Iwanowicz & Goodwin 2002, Johansson et al. 2002). The SMRV antibody has been prepared in our laboratory, and significant serological reactivity differences have been observed between SMRV and its nearest neighbor SVCV. Complete genomic sequences of 5 rhabdoviruses, i.e. IHNV, VHSV, SVCV, TRV, and ISFV, have been determined by Schütze et al. (1995, 1999), Hoffmann et al. (2002),

Springfield et al. (2005), and Marriott (2005), respectively. Genetic relatedness among Japanese, American, and European isolates of VHSV has been analyzed based on partial G and P genes (Nishizawa et al. 2002). Rhabdoviruses have a wide host range, and numerous rhabdoviruses have been discovered from fish and other aquatic organisms. Therefore, further comparison and detailed molecular anatomy will be needed to reveal the similarities and differences between SMRV and other rhabdoviruses. For this purpose, we are performing a complete genome sequence analysis of SMRV.

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