Isolation and identification of a lethal rhabdovirus from farmed rice field eels *Monopterus albus*

Tong Ou, Ruo-Lin Zhu, Zhong-Yuan Chen, Qi-Ya Zhang*

State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Graduate University of Chinese Academy of Sciences, Wuhan 430072, PR China

ABSTRACT: We provide the first description of a virus responsible for a systemic hemorrhagic disease causing high mortality in farmed rice field eels *Monopterus albus* in China. Typical signs exhibited by the diseased fish were extensive hemorrhages in the skin and viscera and some neurological signs, such as loss of equilibrium and disorganized swimming. Histopathological examination revealed various degrees of necrosis within the spleen and liver. Virus isolation was attempted from visceral tissues of diseased fish by inoculation on 6 fish cell lines. Typical cytopathic effects (CPE) were produced in bluegill fry (BF2) cells, so this cell line was chosen for further isolation and propagation of the virus. Electron microscopy observation showed that the negative stained viral particles had the characteristic bullet shape of rhabdoviruses and an estimated size of 60 × 120 nm. We therefore tentatively refer to this virus as *Monopterus albus* rhabdovirus (MoARV). Molecular characterization of MoARV, including sequence analysis of the nucleoprotein (N), phosphoprotein (P), and glycoprotein (G) genes, revealed 94.5 to 97.3% amino acid similarity to that of *Siniperca chuatsi* rhabdovirus. Phylogenetic analysis based on the amino acid sequences of N and G proteins indicated that MoARV should be a member of the genus *Vesiculovirus*. Koch's postulates were fulfilled by infecting healthy rice field eels with MoARV, which produced an acute infection. RT-PCR analysis demonstrated that MoARV RNA could be detected in both naturally and experimentally infected fish. The data suggest that MoARV was the causative pathogen of the disease.

KEY WORDS: Fish viral disease · Rice field eel · *Monopterus albus* rhabdovirus · MoARV · Histopathology · Electron microscopy · Phylogenetic analysis

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INTRODUCTION

The rice field eel *Monopterus albus* is an economically important freshwater fish characterized by the ability to breathe air and by the natural occurrence of sex reversal (Zhou et al. 2011). The species belongs to the teleost family Synbranchidae of the order Synbranchiformes (Neoteleostei, Teleostei, Vertebrata), and is distributed mainly in Asia (Jang et al. 2006). With its high culinary value both in taste and nutrients, and its medicinal properties, this fish is a regionally popular food in the Asian diet, and its farming in China has developed rapidly in recent years (Weng et al. 2011). Some lethal dis-

eases causing mass mortalities of rice field eels have occurred in farms (Shen et al. 2001). In the spring of 2011, a previously undescribed infectious and lethal disease was observed in farmed rice field eels in Hubei Province. Typical clinical signs exhibited by the diseased fish were extensive red foci in the skin and neurological signs, such as loss of equilibrium and disorganized swimming. Examination of the diseased fish failed to reveal pathogenic bacteria or parasitic agents, which suggested that the systemic hemorrhagic disease may have a viral etiology.

Several lethal viruses, such as *Siniperca chuatsi* rhabdovirus (SCRV), Chinese sucker rhabdovirus (CSRV), and *Scophthalmus maximus* rhabdovirus

*Corresponding author. Email: zhangqy@ihb.ac.cn

(SMRV), have been identified and isolated from diseased mandarin fish *Siniperca chuatsi* (Zhang & Li 1999), Chinese sucker *Myxocyprinus asiaticus* (Zhang et al. 2000), and turbot *Scophthalmus maximus* (Zhang et al. 2007), respectively. These viruses have been reported to be associated with high morbidity and mortality in a number of aquaculture species and have tentatively been assigned to the family *Rhabdoviridae*. Rhabdoviruses have a serious socio-economic impact on aquaculture, particularly infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), and spring viremia of carp virus (SVCV), which are all responsible for major disease epizootics in aquaculture and generally result in an acute hemorrhagic syndrome (Betts et al. 2003, Crane & Hyatt 2011).

In this study, we collected diseased fish showing typical signs and attempted to isolate a virus. Based on a series of observations and analysis, a rhabdovirus was identified as the causative pathogen responsible for the systemic hemorrhagic disease. This is the first disease caused by a virus to be recorded in rice field eels.

MATERIALS AND METHODS

Sample collection and histopathology study

In April 2011, a total of 15 diseased rice field eels (mean weight = 180 g) showing systemic hemorrhagic syndrome were collected from a farm located in Wuhan, Hubei Province, China. Data were collected on overall diseased fish condition including size, observable lesions, and organ coloration. Visceral organs (pools of spleen, liver, and kidney) were excised and homogenized in phosphate-buffered saline (PBS) containing antibiotics (penicillin, 100 IU ml⁻¹; streptomycin, 100 µg ml⁻¹). For each pool, the tissue homogenate was stored overnight at -20°C, thawed, and then clarified by centrifugation at 4000 × g (15 min at 4°C). The supernatant was filtered (0.45 µm pore membrane, Millipore) and stored frozen at -80°C until assayed for virus.

Spleens and livers were dissected from all individuals and processed for histology using standard histological techniques. Each sample was fixed in 10% phosphate-buffered formalin and embedded in paraffin wax. Paraffin-embedded tissues were serially sectioned (5 µm), and stained with Mayer's hematoxylin and eosin (H&E). Sections were examined by light microscopy for pathological examination.

Cell cultures and virus isolation

In total, we used 8 cell lines: bluegill fry (BF2; American Type Culture Collection [ATCC] catalog no. CCL 91), *Ctenopharyngodon idellus* kidney (CIK; Zuo et al. 1986), carp leucocytes (CLC; Faisal & Ahne 1990), epithelioma papulosum cyprini (EPC; ATCC catalog no. CRL-2872), grass carp fins (GCF; Lu et al. 1990), grass carp ovary (GCO; Zhang et al. 2000), chinook salmon embryo (CHSE-214; ATCC catalog no. CRL 1681), and fathead minnow (FHM; ATCC catalog no. CCL 42). The cells were grown at 25°C in TC 199 medium with 10% fetal bovine serum (FBS).

Primary viral isolation was conducted in BF2, CIK, CLC, EPC, GCF, and GCO cells in 24-well plates with TC 199 medium containing 5% FBS and 20 mmol l⁻¹ Hepes (*N*-2-hydroxyethylpiperazine-*N*-ethane-sulfonic acid). After the stored filtered supernatant was thawed at room temperature, 3 consecutive 10-fold dilutions of the tissue sample supernatant were inoculated onto cultured cells in 24-well plates. The original viral isolate was adapted to cell culture through 3 successive blind passages on these cell lines. In order to obtain a cloned infectious agent, plaque formation was performed to further isolate individual viruses as described previously (Tao et al. 2007, He et al. 2012). Briefly, BF2 cells were infected with the original viral isolate and harvested at 72 h post infection. The harvested virus suspension was serially diluted and inoculated onto the BF2 cell monolayer grown on a 24-well plate. After adsorption for 1.5 h, the virus suspension was removed and the infected cells were cultured in TC 199 medium containing 0.7% melted soft agar. When the plaques appeared and developed to 4–5 mm in diameter, a single plaque was selected to infect fresh cells. A new round of viral infection and plaque isolation was conducted as above until homogeneous plaques appeared. The plaque-isolated virus was designated as *Monopterus albus* rhabdovirus (MoARV).

Susceptibilities of cell lines

The susceptibilities of different fish cell lines to MoARV were tested using BF2, CHSE, EPC, and FHM. The viral titers of MoARV in these cell lines were measured by the tissue culture infectious dose 50 (TCID₅₀) ml⁻¹ method as previously described (Zhang et al. 2007). The cytopathic effect (CPE) induced by MoARV was observed under the light microscope.

Virus purification and electron microscope observation

Cell cultures were grown to confluent monolayer, infected with MoARV, and incubated at 25°C until CPE was complete. Cell cultures were harvested, and cell debris was removed by centrifugation at $8000 \times g$ (45 min). Virus particles were pelleted by centrifugation at $110\,000 \times g$ (Beckman SW 41; 1.5 h at 4°C). The virus pellet was resuspended in 50 μ l TE buffer (10 mM Tris-cl, 1 mM EDTA, pH 7.5). The concentrated virus particles were negatively stained with 2% (w/v) uranyl acetate and examined with a JEM-1230 transmission electron microscope.

RNA extraction

MoARV-infected cell cultures were harvested after the appearance of CPE as follows: the culture medium was removed and the adherent cells were digested with 0.25% trypsin solution prior to centrifugation ($800 \times g$, 15 min), and subsequent total RNA was extracted from the harvested cell cultures using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The resulting RNA was resuspended in DEPC-treated water and stored at -80°C until use.

RT-PCR amplification and sequencing

The extracted RNA was used as template for RT-PCR amplification of MoARV genes, and total RNA extracted from mock-infected cells was used as the negative control. Firstly, one pair of degenerate primers, designated as MoARV-Fn and MoARV-Rn (Table 1), was designed and synthesized according to the conserved nucleoprotein (N) gene sequences of fish rhabdoviruses (Tao et al. 2007). The cDNA was synthesized in a 25 μ l reaction volume containing 100 pmol random primer, 200 U M-MLV reverse transcriptase (Promega), 1 $\times$ M-MLV RT reaction buffer, 50 μ M dNTPs, 40 U of RNase inhibitor, and 5 μ l RNA extracted above. The following PCR was performed in a volume of 25 μ l, containing 0.5 U of *Taq* polymerase (TransGen), 1 $\times$ *Taq* buffer, 0.2 μ M of each primer, 0.2 mM dNTPs, and 1 μ l cDNA. A PCR program was carried out as follows: pre-denaturation at

Table 1. Primers used for RT-PCR. MoARV: *Monopterus albus* rhabdovirus

Primer name	Sequence (5'-3')	Usage
MoARV-Fn	GAA GAT CAG GYC RAN TAY CC	Degenerate primers for N gene
MoARV-Rn	GTD GCB AGT GCW GCA CAR TCT	
N-F	ATT ATT GGA TTT CTT TCT CTC G	Nucleoprotein (N) gene cloning
N-R	CCA GCT TCT CAA GAT CAT AAG	
P-F	TTA GGG TCA ATA AGG TGA TGG G	Phosphoprotein (P) gene cloning
P-R	GAG TAT GGC GGA GGT GCT TG	
G-F	ATC AAT GGT GTT GGT GGT G	Glycoprotein (G) gene cloning
G-R	GCA AAT GGA TCA TCG TAA TC	
Det-F	CCC TCG TGA TCC TGT TAT TGG	Detection of MoARV infection
Det-R	GCA TCT CTC TTT TTG GGC ATT C	
β -actin-F	CAC TGT GCC CAT CTA CGA G	Internal control gene cloning
β -actin-R	CCA TCT CCT GCT CGA AGT C	

94°C for 5 min; 32 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1.5 min; followed by a final extension step of 72°C for 10 min. The PCR products were analyzed by electrophoresis in 1% agarose gel and visualized under a gel imaging analysis system (Syngene G: Box) after staining with ethidium bromide. The target fragment was purified using a Silica Bead DNA Gel Extraction Kit according to the manufacturer's protocol (Fermentas), cloned into pMD-18T vector (TaKaRa), and sequenced in both directions using an automated ABI sequencer (Sangon).

Sequence comparison and phylogenetic analysis

The nucleotide and deduced amino acid sequences were analyzed using the EditSeq program (Laser-gene). Full length N, phosphoprotein (P), and glycoprotein (G) protein sequences were selected from 13 other completely sequenced rhabdoviruses in GenBank (see legend of Table 2 for GenBank accession numbers). Protein sequences were aligned with Clustal X 1.83 and the homology percentages of N, P, and G proteins of MoARV with the corresponding proteins of other rhabdoviruses were calculated using the Clustal W method in the MegAlign program (Thompson et al. 1994). Phylogenetic trees were constructed using a neighbor-joining (NJ) method as implemented in MEGA version 4 (Tamura et al. 2007).

Experimental challenge and RT-PCR detection

A total of 30 healthy rice field eels, with a mean weight of approximately 8 g, were randomly split

into an experimental group and a control group, each containing 15 fish. The fish were acclimated in 0.25 m³ tanks with flow-through aerated water at a temperature of about 25°C prior to experimental infection. The fish in the experimental group were challenged by an intraperitoneal injection with 0.05 ml culture medium containing 10⁶ TCID₅₀ of MoARV prepared from BF2 cells. In the control group, the fish were mock-infected in the same way using uninfected BF2 cell culture medium. The development of clinical signs and mortality was monitored daily for 2 wk, and inoculated or control fish were randomly sampled and tested for the presence of MoARV by RT-PCR. Spleen and liver tissues were also collected for histopathological examination and processed as described above.

The livers of all sampled fish, which were randomly selected from the naturally-infected diseased fish and experimental fish, were dissected from each individual, and tissue samples were examined for the presence of MoARV by RT-PCR as described above. A β -actin PCR was used as an internal control.

RESULTS

Clinical signs, gross pathology, and histopathology

Lethargy and anorexia were the first signs of the disease. In contrast to healthy fish, the sick fish gasped at the surface of the water and their movements became agitated. Prior to death, abnormal clinical signs included loss of equilibrium, disorganized swimming, accumulation of skin mucus, and

regular/irregular red spots (approximately 1 mm in diameter) on the skin of the head, back, abdomen, and caudal trunk. In moribund fish, numerous reddened areas became more obvious, and the anus was red and swollen (Fig. 1A,B). Upon necropsy, the abdominal cavity usually contained clear or bloody ascitic fluid, and the alimentary tract was almost free of food. The spleen was swollen, the gallbladder reddened, and the liver discolored with multiple red foci (2–3 mm; Fig. 1C).

Histopathological changes were investigated in the spleen and liver from diseased fish and compared with the corresponding organs from healthy individuals. The results revealed various degrees of damage and necrosis within the diseased fish spleen and liver. Spleen tissue sections from diseased fish exhibited widespread multifocal necrosis and various-sized cavities (Fig. 2A). Liver damage in diseased fish ranged from focal to locally extensive nuclear pyknosis and cytoplasmic degeneration to generalized hemorrhagic hepatocellular necrosis (Fig. 2B). No pathological changes were observed in tissue sections from healthy fish (data not shown).

Isolation, morphology, and cell infection of MoARV

Primary viral isolation was conducted in BF2, CIK, CLC, EPC, GCF, and GCO cells. After 3 successive blind passages on these cell lines, the BF2 cell line was found to be the most susceptible to the original viral isolate, and typical CPE was observed in the cell monolayer at 72 h post infection. In the GCO and

Fig. 1. *Monopterus albus* infected with *Monopterus albus* rhabdovirus (MoARV). (A) Gross external appearances of an infected fish with red swollen anus (arrowhead) and regular/irregular red foci (arrows) in the skin. (B) Detail of red foci in the abdomen. (C) Gross appearance of the spleen (S), gallbladder (GB), and liver (L) in MoARV-infected (Inf) and normal uninfected (Con) fish. The internal organs of MoARV-infected fish displayed signs including swollen spleen, reddened gallbladder, and discolored liver with irregular red foci (arrowhead)

Fig. 2. *Monopterus albus* infected with *Monopterus albus* rhabdovirus. Photomicrographs of hematoxylin and eosin-stained tissue sections illustrating lesions. (A) Widespread multifocal necrosis (arrows) and various size cavities in the diseased fish spleen. Inset shows spleen necrosis at higher magnification. (B) Locally extensive necrosis (arrows) with erythrocyte infiltration in the diseased fish liver. Inset shows liver necrosis with erythrocyte infiltration (arrowheads) at higher magnification. Scale bars = 100 µm

GCF cell lines, the original viral isolate showed moderate infectivity. No obvious CPE was produced in the other 3 cell lines within 1 wk post incubation. Therefore, the BF2 cell line was used to further isolate the original viruses by plaque formation. After 3 rounds of propagation and selection in BF2 cells, homogeneous plaques were formed with a diameter of about 6 mm (Fig. 3B), instead of the varied plaque sizes formed by the original viral isolate (Fig. 3A). This suggests that the plaque-isolated virus represents a cloned infectious agent.

Electron microscopy observations showed that the negative stained virions had the characteristic bullet shape of rhabdoviruses and an estimated size of 60×120 nm (Fig. 4). Based upon the morphological features, the plaque-isolated virus is likely to be a member of the family *Rhabdoviridae*, and was provisionally named *Monopterus albus* rhabdovirus (MoARV).

The susceptibilities of different fish cell lines to MoARV were tested using

Fig. 3. Plaque formation and cytopathic effects (CPE) in bluegill fry cell culture. (A) Various plaques with diameters ranging from 2 (S) to 8 (L) mm formed by original viral isolate. (B) Homogeneous plaques with a diameter of about 6 mm formed by plaque-isolated virus. (C) Uninfected control cultures. (D) Typical CPE induced by *Monopterus albus* rhabdovirus. Scale bars = 500 µm

Fig. 4. Transmission electron micrograph of *Monopterus albus* rhabdovirus particles isolated from the infected bluegill fry cell culture. The negatively stained virions display the characteristic bullet shape of rhabdoviruses

BF2, CHSE, EPC, and FHM, and the resultant viral titers were $10^{7.7}$, $10^{7.7}$, $10^{4.0}$, and 0 TCID₅₀ ml⁻¹, respectively. Significant CPE was observed in BF2 cells at 48 h post infection with MoARV at a multiplicity of infection of approximately 0.1 (Fig. 3D), while no CPE was observed in the mock-infected cells (Fig. 3C).

Nucleotide and deduced amino acid sequences of MoARV N, P, and G genes

A homology search for the resulting sequence was conducted using the BLAST program from NCBI, which showed high similarity to the SCR V N gene (Tao et al. 2008). Therefore, 3 pairs of primers (Table 1)

were designed and synthesized according to the complete genome sequence of SCR V, and used to clone MoARV N, P, and G genes. The RT-PCR amplification and cloning results are shown in Fig. 5. Non-specific DNA fragments in the control were confirmed by sequencing. The 3 fragments of MoARV N, P, and G genes were amplified and cloned by RT-PCR from MoARV-infected BF2 cell cultures, and the corresponding sequences have been deposited in GenBank under accession nos. KF184325, KF184326, and KF184324, respectively. The N gene is 1456 nt long from the consensus mRNA start sequence AACAG to the transcription stop signal ATG AAA AAA A. The open reading frame (ORF) of the N gene (1290 nt) is predicted to encode a 429 amino acid protein. The P gene is composed of 971 nt, and comprises an 864 nt ORF encoding a 287 amino acid protein. The G gene comprises 1658 nt and includes a 1527 nt ORF, leading to a 508 amino acid protein.

Homology analysis (Table 2) indicated that the G protein is the most conserved (19.4–97.3% similarity), followed by the N protein (13.3–96.5% similarity) and the P protein (11.9–94.5% similarity). The N, P, and G protein sequences of SCR V all showed the highest amino acid sequence similarity to those of MoARV (96.5, 94.5, and 97.3%, respectively), followed by eel virus European X (EVEX; 43.9, 22.5, and 40.0%, respectively). The analysis also revealed that MoARV showed higher similarity to vesiculoviruses than to novirhabdoviruses.

Phylogenetic analysis

To investigate the phylogenetic relationships of MoARV within the family *Rhabdoviridae*, the complete sequences of MoARV N and G proteins were used to construct phylogenetic trees with 13 other rhabdoviruses based on the homology analysis and

Fig. 5. RT-PCR cloning of nucleoprotein (N), phosphoprotein (P), and glycoprotein (G) genes of *Monopterus albus* rhabdovirus (MoARV) from MoARV-infected bluegill fry cell culture. M: DL2000 DNA marker; Actin: internal control gene. The fragments with the approximate sizes of 1600, 1100, and 1800 bp are the target fragments for N, P, and G genes, respectively

Table 2. Comparison of the amino acid sequences of *Mono-pterius albus* rhabdovirus (MoARV) and other rhabdoviruses: SCR (V) (*Siniperca chuatsi* rhabdovirus; GenBank accession no. NC008514), EVE (X) (eel virus European X; FN557213), PFR (V) (pike fry rhabdovirus; FJ872827), SVC (V) (spring viremia of carp virus; NC002803), VSN (V) (vesicular stomatitis New Jersey virus; P04881, P04877, and P04882), HIR (V) (hirame rhabdovirus; NC005093), IHN (V) (infectious hematopoietic necrosis virus; NC001652), SHR (V) (snakehead rhabdovirus; NC000903), VHS (V) (viral hemorrhagic septicemia virus; NC000855), ADR (V) (Adelaide River virus; JN935380), BEF (V) (bovine ephemeral fever virus; NC002526), ABL (V) (Australian bat lyssavirus; NC003243), RAB (V) (rabies virus; NC001542). The highest values of similarity with MoARV are shown in **bold**. N: nucleoprotein; P: phosphoprotein; G: glycoprotein

Genus Virus	Protein name		
	Similarity (%) of MoARV proteins to:		
	N	P	G
<i>Vesiculovirus</i>			
SCR	96.5	94.5	97.3
EVE	43.9	22.5	40.0
PFR	39.1	17.7	39.5
SVC	38.2	18.9	39.3
VSN	35.4	18.4	35.4
<i>Novirhabdovirus</i>			
HIR	13.9	18.5	28.1
IHN	15.2	18.7	28.0
SHR	13.6	17.4	28.0
VHS	13.3	19.4	29.2
<i>Ephemerovirus</i>			
ADR	32.2	11.9	19.4
BEF	31.5	17.0	21.2
<i>Lyssavirus</i>			
ABL	22.7	17.0	30.9
RAB	21.8	18.2	30.8

multiple alignments. As shown in Fig. 6A, based on N protein analysis, the 14 rhabdoviruses were divided into 4 groups. MoARV and SCR clustered together, and further grouped with 4 other rhabdoviruses in the genus *Vesiculovirus*, including EVE, pike fry rhabdovirus (PFR), SVC, and vesicular stomatitis New Jersey virus (VSN). Other rhabdoviruses clustered within the tree were in agreement with the established genera. The tree derived from analysis of G protein sequences showed similar results (Fig. 6B). The phylogenetic analysis revealed that MoARV should belong to the genus *Vesiculovirus* in the family *Rhabdoviridae*.

Experimental challenge and RT-PCR detection

To confirm pathogenicity, an artificially induced infection was performed on healthy rice field eels. BF2-propagated MoARV was used to infect 15 fish by

intraperitoneal injection. In general, the disease in experimental fish was indicative of an acute infection. First signs of disease in experimentally infected fish occurred at about 3 d post infection (dpi), and mortality began at 5 dpi. The majority of infected fish died within the first 8 dpi, and the final death occurred at 10 dpi, resulting in 86.7% cumulative mortality (Table 3). Moribund fish displayed similar signs and histopathology as described above. In the control group, no mortality or pathological changes were observed in any of the rice field eels. The results indicate that the MoARV is the causative pathogen of the disease.

To detect MoARV infection in rice field eels, a pair of specific primers (Det-F and Det-R; Table 1) derived from the MoARV N gene was used to amplify a target sequence with the predicted length of 743 bp by RT-PCR. MoARV was detected by RT-PCR in liver tissue samples of rice field eels which were randomly selected from the original diseased and experimental fish (Fig. 7). A fragment of approximately 750 bp was amplified from naturally infected fish and experimentally MoARV-infected fish, and no corresponding band was detected in the experimental mock-infected fish and those fish collected before injection. As an internal control, β -actin was amplified from all of the detected fish samples. Therefore, the experimental rice field eels were negative for MoARV before infection and suitable for experimental challenge, and MoARV was detected in both naturally and experimentally infected fish.

DISCUSSION

In this study, we present data on the pathology, isolation, and characterization of a lethal rhabdovirus responsible for a systemic hemorrhagic disease causing high mortality in farmed rice field eels. The diagnosis of rhabdovirus infection was based on a combination of characteristic pathological changes, virus isolation in fish cell lines, cell infection, electron microscopy observation, molecular characterization, experimental challenge, and RT-PCR detection. This is the first record of a viral pathogen associated with significant morbidity and mortality in farmed rice field eels.

Rhabdoviruses are significant viral pathogens in aquaculture, and usually result in an outbreak of acute disease characterized by systemic hemorrhages or necrotic ulcerations (Johnson et al. 1999, Ahne et al. 2002, Elston & Meyers 2009, Toplak et al. 2010, Zhang & Gui 2012). More than 10 species of

Fig. 6. Phylogenetic relationships of *Monopterus albus* rhabdovirus (MoARV) to representative rhabdoviruses. The alignment of whole amino acid sequences of (A) nucleoprotein and (B) glycoprotein was used to construct the neighbor-joining trees using the MEGA package. Bootstrap values (%) for 1000 tree replicates are shown at each branch node. MoARV is shown in bold. Viruses from the same genera are indicated

Fig. 7. RT-PCR detecting the infection of *Monopterus albus* rhabdovirus (MoARV) in *Monopterus albus*. M: DL2000 DNA marker; Lane 1: the original diseased fish used for isolating virus; Lanes 2–4: random samples of experimental fish collected before infection; Lanes 5–7: random samples from 15 fish in the infected group collected within 5 to 10 d after infection; Lanes 8–10: random samples from 15 fish in the control group collected 2 wk after mock infection. Every fish liver was examined by RT-PCR using primers for the nucleoprotein gene (Det-F and Det-R, listed in Table 1), and the β -actin gene was used as an internal control

Table 3. *Monopterus albus*. Cumulative mortality of rice field eels after experimental infection with *Monopterus albus* rhabdovirus; each group contained 15 specimens

Groups	Cumulative mortality								Final %
	Time (d)								
	4	5	6	7	8	9	10	14	
Infected	0	1	2	6	11	12	13	13	86.7
Control	0	0	0	0	0	0	0	0	0

rhabdoviruses have been isolated from cultured fish, including some important economic species from both fresh and marine water (Meyers et al. 1999, Lumsden et al. 2007, Zhang et al. 2007, Gui & Zhu 2012). Rice field eels infected with MoARV also exhibited an acute disease characterized by extensive hemorrhages in the skin and viscera. In order to obtain more detail on pathological changes, histopathological examination of the spleen and liver was performed. Focal to extensive necrosis with hemorrhage was observed in these tissues, and similar histopathological changes have been reported associated with other fish viral diseases (Sano 1976, Negele 1977, Chen et al. 2008, Zhang & Gui 2012). In addition, the virus could replicate well in some cell lines derived from other fish species, such as BF2 and CHSE-214, and produce typical CPE in these cells within as little as 2 d. These data suggest that MoARV is a highly virulent virus causing acute systemic infections and multiple organ damage. Early diagnosis of MoARV is required in order to avoid large disease outbreaks in rice field eel farming. In this study, a pair of specific primers based on the MoARV N gene was designed and used for RT-PCR detection of MoARV in rice field eels. The N gene, as the most highly expressed gene in members of the family *Rhabdoviridae*, is usually considered an ideal target for PCR-based diagnostic methods (Talbi et al. 2011). The results indicated that MoARV RNA could be detected in naturally and experimentally infected fish, and RT-PCR detection with diagnostic primers is an effective method for the diagnosis of MoARV infection in the farmed rice field eels and other fish.

Rhabdoviruses have nonsegmented and single-stranded RNA genomes encoding 5 major functional proteins, namely the N, P, matrix protein (M), G, and RNA-dependent RNA polymerase (L) in the order 3' N-P-M-G-L 5' (Zhu et al. 2011). Significantly, MoARV N, P, and G proteins all have the highest amino acid similarities (94.5–97.3%) to those of SCR. For the MoARV N and G proteins, the highest similarities (35.4–97.3%) are to members of the genus *Vesiculo-*

virus. The family *Rhabdoviridae* currently includes 6 genera: *Vesiculovirus*, *Lyssavirus*, *Ephemerovirus*, *Novirhabdovirus*, *Cytorhabdovirus*, and *Nucleorhabdovirus* (Tordo et al. 2005), and to date all reported rhabdoviruses infecting fish have been classified in the genera *Vesiculovirus* or *Novirhabdovirus* (Galinier et al. 2012). Phylogenetic analysis also revealed SCR as the closest relative of MoARV, and they were further grouped with the recently characterized EVEX. Moreover, these viruses formed a genetic cluster distinct from the one represented by SVCV and PFRV, supporting the potential existence of a new fish vesiculovirus clade as previously reported (Galinier et al. 2012, Stone et al. 2013). Rice field eels belong to the Synbranchiformes, whose members are taxonomically closely related to those of the Perciformes (Johnson & Patterson 1993). SCR was originally isolated from mandarin fish (also called Chinese perch) (Zhang & Gui 2012). Rhabdoviruses infecting mandarin fish and rice field eels are of particular interest, since their impact on percid farming is increasing in Europe after the first record of a rhabdovirus pathogenic for perch in France in 1980 (Dorson et al. 1984). The phylogenetic study performed by Talbi et al. (2011) showed that all rhabdoviruses from perch, as well as SCR and EVEX, were clustered together. Stone et al. (2013) proposed the new genus *Perhabdovirus* for the fish rhabdoviruses and created 3 further species, viz. *Perch rhabdovirus*, *Anguillid rhabdovirus*, and *Sea trout rhabdovirus*, within the new genus. Although SCR was not included in the genus *Perhabdovirus*, it was closely related to the proposed members of this genus. EVEX was considered a member of the species *Anguillid rhabdovirus* (Stone et al. 2013). After EVEX was isolated in a shipment of European elvers *Anguilla anguilla* from France to Tokyo, another rhabdovirus appearing to be serologically similar to EVEX was reported in Japan (Kobayashi & Miyazaki 1996, Galinier et al. 2012). This information suggests that rhabdoviruses also have a noticeable impact on percid farming in Asia and may evolve to infect taxonomically related host species. Isolation and characterization of a rhabdovirus from farmed rice field eels in China may help us to better understand the evolution and circulation of these rhabdoviruses between regions and hosts.

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