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Isolation and characterization of a novel reovirus from white bream *Blicca bjoerkna*

Oskar Schachner[1], Hatem Soliman[1,2], Michael Straif[3], Franz Schilcher[4], Mansour El-Matbouli[1,*]

[1]Clinical Division of Fish Medicine, University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria
[2]Fish Medicine and Managements, Faculty of Veterinary Medicine, Assiut University, 71515 Assiut, Egypt
[3]Institute of Hydrobiology and Aquatic Ecosystem Management, Department of Water – Atmosphere – Environment, University of Natural Resources and Applied Life Sciences, 1180 Vienna, Austria
[4]Institute of Pathology and Forensic Veterinary Medicine, Department of Pathobiology, University of Veterinary Medicine, 1210 Vienna, Austria

ABSTRACT: During a fish health inspection in the Viennese waterway 'Old Danube', a virus was isolated exclusively from white bream *Blicca bjoerkna* (L.) (formerly *Abramis bjoerkna* L.), one of the most abundant cyprinids present and not known as a host species for this virus. The virus preferentially replicated in cultures of the epithelioma papulosum cyprini cell line where focal plaques of infection developed slowly. Examination of infected cell cultures by electron microscopy revealed non-enveloped 60 to 70 nm icosahedral virions that had characteristic multiple segregated protrusions of their outer capsid. A partial RNA-dependent RNA polymerase gene sequence was obtained and a BLAST search indicated 76% identity to golden shiner reovirus and grass carp reovirus. These results suggested that the virus belonged to the genus *Aquareovirus* (Family *Reoviridae*). Phylogenetic analysis placed the isolated virus within a clade of the species *Aquareovirus C* species. Accordingly, the virus was tentatively designated as white bream reovirus (WBRV) strain A-127/06 within the species *Aquareovirus C*.

KEY WORDS: *Aquareovirus* · White bream · *Blicca bjoerkna* · Cell line susceptibility · Gill histopathology · Electron microscopy · Phylogenetic analysis

INTRODUCTION

Reoviridae is the largest family of double-stranded RNA viruses, and these infect a diverse host range (Mertens 2004, Day 2009). The genus *Aquareovirus* comprises reoviruses isolated from fish, shellfish and crustaceans (Attoui et al. 2006, Guo et al. 2008, Attoui et al. 2011) which are non-enveloped, double-stranded RNA viruses containing 11 segments (Reinisch 2002, Mertens 2004, Samal et al. 2005, Attoui et al. 2006, Wellehan et al. 2009). In fish cell lines, aquareoviruses characteristically produce large syncytia (Attoui et al. 2002, Mohd Jaafar et al. 2008). The International Committee for the Taxonomy of Viruses (ICTV) recognizes 7 aquareovirus species (*Aquareovirus A* to *G*) and several tentative species (Seng et al. 2004, Samal et al. 2005, Mohd Jaafar et al. 2008, Attoui et al. 2011).

Aquareoviruses pose a significant threat to aquaculture, depending on the viral strain and host species (DeWitte-Orr & Bols 2007). Some aquareovirus species can cause hemorrhagic disease, hepatitis and pancreatitis in fish (Amend et al. 1984, Chen & Jiang 1984, Marshall et al. 1990). Piscine aquareoviruses have been isolated from a wide host range including salmonids, ictalurids and cyprinids (Winton et al.

*Corresponding author: mansour.el-matbouli@vetmeduni.ac.at

1987, Wolf 1988) and have a broad geographic range. The first aquareovirus was isolated from golden shiner *Notemigonus crysoleucas* in North America (Plumb et al. 1979). Subsequently, aquareoviruses have been described from multiple cyprinid species including grass carp *Ctenopharyngodon idella* L. in China (Chen & Jiang 1984) and Europe (Ahne & Koelbl 1987), chub *Leuciscus cephalus* L. and tench *Tinca tinca* L. in Europe (Ahne & Koelbl 1989) and common carp *Cyprinus carpio* L. in China (Jiang & Ahne 1989). Prior to the present study, white bream (*Abramis bjoerkna* L. or *Blicca bjoerkna* L.) was not known as a host for reoviruses. Studies on hydrobiology and fish population dynamics in the 'Old Danube', remnant floodplains within Vienna, Austria were accompanied by a fish health inspection. The inspection resulted in isolation of a novel reovirus from white bream. The isolation and identification of this virus is described herein.

MATERIALS AND METHODS

Fish specimens

During a study on the hydrobiology and fish population dynamics in the 'Old Danube', Vienna, Austria, random samples of various fish species were inspected (Table 1). The sampled fishes were caught by electrofishing. The fish were overdosed with 0.05% (w/v) MS-222 (Sigma Chemical) before sampling. Fish health was assessed by inspection of gross anatomy, by gill histopathology, for the presence of external and internal parasites as well as viruses, and by virus isolation using fish cell lines.

Table 1. Number and size of fish species collected from Old Danube for investigations

Fish species	No. of samples	Length (cm)
Pike *Esox lucius* L.	2	16–18.5
Perch *Perca fluviatilis* L.	2	7.5–15.5
Bluegill *Lepomis macrochirus* L.	4	4–11
Chub *Leuciscus cephalus* L.	1	48
Rudd (redfin) *Scardinius erythrophtahlmus* L.	12	13–19
White bream *Blicca bjoerkna* L.	12	13–24

Light microscopy

Gill material was fixed in 10% formalin, dehydrated in ascending ethanol concentrations and embedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin (H&E), followed by Alcian blue for the demonstration of acid mucopolysaccharide-containing goblet cells.

Virus isolation in cell culture

Tissue samples consisting of pools of spleen, head kidney and heart from 1 to 3 fish, and of individual organs (hematopoietic organs [spleen and kidney], skin, gill and brain samples) from single fish were homogenized in MEM-Glutamax (Gibco) containing 2% foetal bovine serum (FBS) and 0.1% Gentamycin. Following 15 min centrifugation at 2000 × *g*, the supernatants were filter-sterilized using 0.45 µm syringe filters (Sartorius), inoculated at final dilutions of 1:100 and 1:1000 on to confluent 24-well plate cultures of epithelioma papulosum cyprini (EPC), Chinook salmon embryo (CHSE-214), bluegill fry (BF-2) and rainbow trout gonad (RTG-2) cell lines seeded 12 to 24 h previously. The cultures were incubated at 20°C for 2 to 3 wk, with passage intervals of 7 d. Cell culture infectivity was determined in 96-well plates (Greiner) using the same cell lines as above and 10-fold serial dilutions of the virus according to Reed & Muench (1938).

Electron microscopy

Cells from infected EPC cell monolayers that showed advanced stages of viral cytopathic effect (CPE) were centrifuged (2000 × *g* for 10 min), the cell pellet fixed in 5% glutaraldehyde and post-fixed in 1% osmium tetroxide (OsO_4). After dehydration of cells in an ascending acetone series, the cell pellet was embedded in glycidether 100 (Serva). Ultra-thin sections of 70 to 80 nm were stained with uranyl acetate and lead citrate and examined using a transmission electron microscope EM900 (Zeiss-Leo). For negative contrast staining, 50 µl of bovine serum albumin solution (1% in phosphate buffered saline) together with 50 µl of resuspended pellet were applied to a Formvar-coated and carbonized copper grid, stained with 4% phosphotungstic acid and examined as above.

Table 2. Oligonucleotide primers used in this study. M: A or C; R: A or G; W: A or T; S: G or C; Y: C or T; V: A, G or C; H: A, C or T; D: A, G or T; B: G, C or T; N: A, G, C or T

Primer name	Length (bp)	Sequence (5′–3′)	Reference
1607 F	26	CAR MGN CGN SCH MGH TCH ATH ATG CC	Wellehan et al. (2009)
2608 R	26	TAV AYR AAV GWC CAS MHN GGR TAY TG	Wellehan et al. (2009)
2090 F	26	GGB TCM ACN GCY ACY TCB ACY GAG CA	Wellehan et al. (2009)
2334 R	22	CDA TGT CRT AHW YCC ANC CRA A	Wellehan et al. (2009)
GSP2	25	TCC TCT CTC AAG CGT ATC CTG TCT G	This study
NGSP2	27	ATA TGT CTA TCC AAC GCA ACT ATG TTT	This study

RNA extraction

Viral genomic RNA was extracted from 140 µl of infected EPC cell culture supernatant using QIAamp viral RNA kit (Qiagen) according to the manufacturer's instructions. Genomic RNA of non-infected EPC cells served as a control for any cell culture contamination. A negative extraction control (sterile water) was included to exclude any contaminations in the extraction reagents or procedures. RNA was eluted in 60 µl elution buffer, and its purity and concentration assessed by measuring the optical density at 260 and 280 nm. RNA samples were aliquoted and stored at −80°C.

PCR amplifications

A nested PCR utilizing degenerate primers was used for amplification of a conserved region on the reovirus RNA-dependent RNA polymerase gene (VP2) according to Wellehan et al. (2009). The 1-step RT-PCR was performed using OneStep RT-PCR Kit (Qiagen). For 50 µl reaction volume, 1 µg RNA was mixed with 5× QIAGEN OneStep RT-PCR Buffer, dNTPs mix, 15 pmol of each 1607F and 2608R degenerate primers (Table 2), RNase inhibitor, 2 µl QIAGEN OneStep RT-PCR Enzyme Mix, and RNase-free water. The reaction conditions for the 1-step procedure comprised incubation at 50°C for 30 min, then heating to 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 1 min. There was a final extension step of 72°C for 10 min.

Following the 1-step RT-PCR, the nested PCR was carried out in 50 µl reaction volume utilizing 2× ready mix PCR Master mix (Thermo Scientific), which contained 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween-20, 0.2 mM each nucleotide triphosphate, 1.25 U Thermoprime Plus DNA polymerase, and red dye for electrophoresis, 3 µl of the 1-step RT-PCR product and 15 pmol each of 2090F and 2334R degenerate nested primers (Table 2). The cycling profile was 95°C for 3 min, then 45 PCR cycles of 95°C for 1 min, 47°C for 1 min and 72°C for 30 s, with a terminal extension step of 72°C for 10 min.

Detection of the PCR product

PCR products were analysed by electrophoresis on 2% agarose gels in Tris-acetate-EDTA buffer (TAE, 0.04 M Tris-acetate, 1 mM EDTA), Superladder-low 100 bp DNA molecular weight ladder (ABgene) was used to determine the molecular size of the PCR amplicons. Gels were stained with ethidium bromide and visualized on an UV transilluminator.

Cloning and sequencing of the PCR products

The 274 bp PCR amplicon was purified using a MinElute gel extraction kit (Qiagen), then cloned into the pDrive Cloning Vector using a QIAGEN PCR cloning plus kit according to the manufacturer's instructions. Recombinant plasmids were purified from *Escherichia coli* using a QIAprep Miniprep kit (Qiagen) following the manufacturer's instructions. Cloned PCR products were sequenced in a commercial sequencing laboratory (AGOWA), then subjected to BLASTn search for sequence similarity in the GenBank database according to Altschul et al. (1997).

Rapid amplification of 3′ cDNA ends (RACE)

Two gene-specific primers, GSP2 and NGSP2 (Table 2), were designed based on the 274 bp amplicon to be used in a RACE assay; 3′ RACE was carried out using a SMARTer RACE cDNA amplification kit

(Clontech) as per the manufacturer's instructions. To facilitate sequencing, purified 3′ RACE product was cloned into pDrive Cloning Vector using a QIAGEN PCR cloning kit according to the manufacturer's instructions. Recombinant plasmids were purified using a QIAprep Spin Miniprep kit (Qiagen), as per manufacturer's instructions and sequenced (AGOWA). A BLASTn search was conducted for sequence similarity against the GenBank database according to Altschul et al. (1997).

Phylogenetic analysis

The resultant viral sequence was aligned with the *Aquareovirus* VP2 gene sequences available in GenBank using the multiple sequence alignment tools such as Clustal-X, T-Coffee, MUSCLE and DNAstar (Thompson et al. 1997, Notredame et al. 2000, Edgar 2004). A phylogenetic tree was constructed using the neighbour-joining method according to Saitou & Nei (1987).

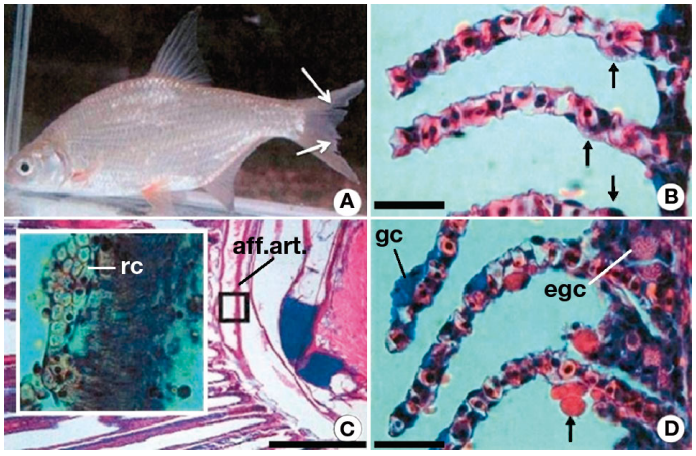

Fig. 1. White bream showing (A) slight erosion at the caudal fin. (B) Normal white bream gill lamellae: respiratory epithelial cells (arrows) are attached to the capillaries and do not contain acid Alcian blue stained mucus; scale bar = 50 µm. (C) White bream gill tissue with epitheloid rodlet cell (rc) accumulation in the endothelium of the afferent artery running between the base of filaments and gill arch supportive tissue; scale bar = 20 µm. (D) Gill histopathology of white bream: eosinophilic extravasations through the damaged capillary wall and respiratory epithelium (arrow), replacement of respiratory epithelial cells by mucus-containing goblet cells (gc), eosinophilic granular cells (egc) infiltrating the basal interlamellar epithelium; scale bar = 50 µm

RESULTS

Macroscopic and light microscopic findings

Unlike other sampled species, white bream of different sizes exhibited skin and gill damage to varying degrees including scale loss, fin erosion (Fig. 1A), increase in mucus production, epidermal swelling as well as haemorrhages mainly along fin and gill rays.

Gill histology (Fig. 1B–D) was characterized by an extremely delicate respiratory epithelium tending to either detach from capillaries by oedematous swelling or be replaced by mucus-secreting goblet cells. The interlamellar epithelium exhibited some infiltration. The most marked and unique finding in individual white bream gill tissue was extravasation of an eosinophilic substance resembling agglutinated erythrocytes extruding from destroyed lamellar capillaries and respiratory epithelium. There was accumulation of eosinophilic granular cells at the base of the lamellae and of rodlet cells lining the endothelium of afferent arteries next to the gill arch supportive tissue. While proliferation of goblet and granular cells was found in other cyprinid species, these were different from the extravasation observed in white bream.

Virus isolation in cell culture

Virus-associated CPE developed slowly (during the second week of incubation) in EPC cell cultures inoculated with hematopoietic tissue pools of 3 white bream. Infected cells became pycnotic but remained in contact with the intact monolayer up to 7 d. After 5 to 8 d (first passage), several areas of focal degeneration appeared as streams of plaques, all arranged in one direction (Fig. 2A) with only a few exhibiting a central hole where dead cells had lost adherence to the monolayer and the substrate (Fig. 2B). Single hyperplastic cells contained inclusion bodies (Fig. 2C). Development of CPE proceeded slowly, resulting in complete destruction of the monolayer during the third week of incubation. Passage of CHSE-214, BF-2 and RTG-2 supernatants onto new homologous monolayers did not yield infectious virus. In EPC cell cultures, however, the infectivity slowly increased with the number of passages. When highly infective doses of EPC supernatant were transferred onto cultures of CHSE-214, BF-2 and RTG-2 cell lines, the virus propagated well, rapidly inducing characteristic syncytia and inclusion bodies (Fig. 2D–H). Complete destruction of the monolayer occurred at 11 d incubation. EPC remained the most susceptible cell

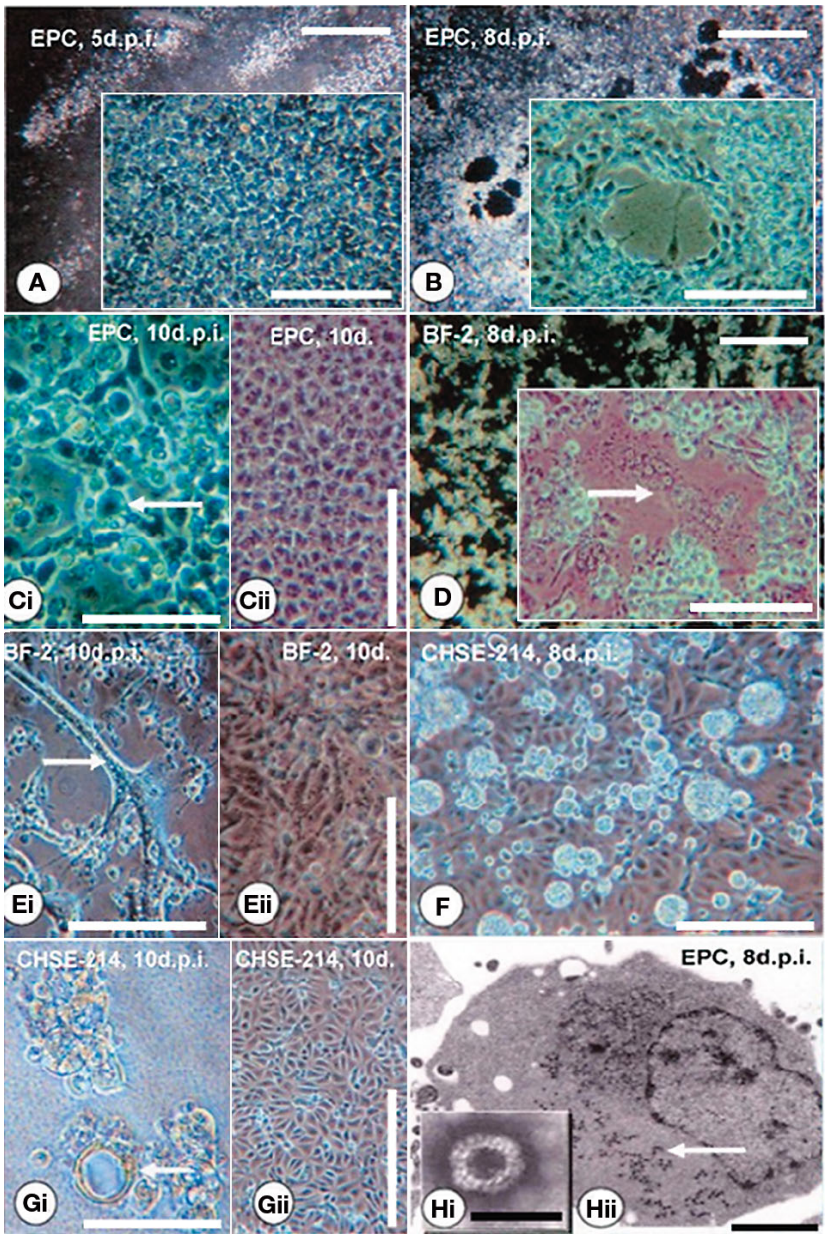

Fig. 2. (A) Epithelioma papulosum cyprini (EPC) monolayer, 5 d post-infection (dpi) with 10^{-3} 50% tissue culture infective dose ($TCID_{50}$) white bream reovirus (WBRV) A-127/06: bright plaques of infected cells; dark field illumination, scale bar = 50 µm. Insert: the same monolayer showing single pyknotic cells still adherent; bright field, scale bar = 20 µm. (B) EPC monolayer, 8 dpi with 10^{-3} TCID WBRV A-127/06: dark holes in the monolayer surrounded by pyknotic still adherent cells; dark field illumination, scale bar = 50 µm. Insert: one of the holes within the diffusely infected monolayer; bright field, scale bar = 20 µm. (Ci) EPC cells, 10 dpi showing still mainly attached pyknotic and normal cells, one enlarged but still polygonal cell (arrow) contains bright inclusions, presumably compartments of virus replication; bright field, scale bar = 10 µm. (Cii) Normal EPC monolayer, 10 d after seeding; bright field, scale bar = 10 µm. (D) Bluegill fry (BF-2) monolayer, 8 dpi with 10^{-3} $TCID_{50}$ WBRV A-127/06: about half of the cells are detached from the substrate; dark field, scale bar = 50 µm. Insert: islet of still adherent pyknotic and normal cells surround large hyperplastic syncytia (arrow) prior to detachment; bright field, scale bar = 20 µm. (Ei) BF-2 cell syncytium (arrow); 10 dpi bright field, scale bar = 10 µm. (Eii) Normal BF-2 monolayer, 10 d after seeding; bright field, scale bar = 10 µm. (F) Chinook salmon embryo (CHSE-214) monolayer showing a characteristic CPE 8 dpi: bright, spherical syncytia of different size are extruded from the monolayer; bright field, scale bar = 10 µm. (Gi) CHSE-214 cells, 10 dpi, almost completely destroyed monolayer, the arrow showing a firm, spherical, valve-shaped remnant of a syncytium; bright field, scale bar = 10 µm. (Gii) Normal CHSE-214 monolayer, 10 d after seeding; bright field, scale bar = 10 µm. (Hi) Virus particle from EPC cell supernatant, 8 dpi; negative contrast showing a dark nearly hexagonal core and a bright capsid zone, consisting of about 20 capsomeres, scale bar = 250 nm. (Hii) TEM-section of an infected EPC cell, 8 dpi: cytoplasmic compartment containing numerous virions (arrow); scale bar = 50 nm

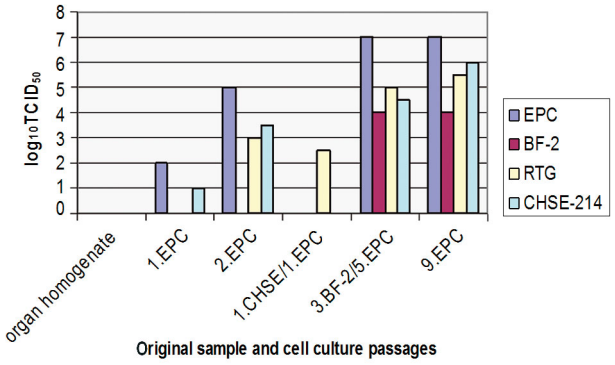

Fig. 3. $TCID_{50}$ of various passages of white bream reovirus (WBRV) A-127/06 in different cell lines. Highest titers are recorded in epithelioma papulosum cyprini (EPC) cells

line for any of the isolates obtained from either the homologous or the heterologous cultures. The final titres yielded on EPC cultures were 1000-fold higher than titres on BF-2 cultures, but only 10-fold higher than those using CHSE-214 cultures (Fig. 3).

Electron microscopy

Transmission electron microscopy of the infected EPC cells revealed cytoplasmic aggregates of non-enveloped ca. 60 to 70 nm virions that consisted of an electron-dense icosahedral core of about 40 nm in diameter, and a thick translucent capsid. Aggregates appeared within separate compartments of cytoplasm. Negative-stained virus particles exhibited numerous segregated protrusions of the outer capsid, making up the typical wheel-like shape of reoviruses (Fig. 2Hi, Hii).

Molecular characterization

The nested PCR amplified the expected 274 bp segment of reovirus RNA-dependent RNA polymerase gene. No amplification products were detected from either uninfected cell culture or no-template controls. The sequence obtained from the 3′ RACE reaction (1002 bp) was deposited in GenBank (accession number HQ448931). The BLASTn search revealed a 76% identity with both grass carp reovirus (GCRV) and golden shiner reovirus (GSRV); a 71% identity to golden ide reovirus (GIRV) and American grass carp reovirus (AGCRV); 57% to Atlantic salmon reovirus (TSRV) and chum salmon reovirus (CSRV). The BLASTx search of the deduced amino acid sequence revealed an 87 to 89% identity to GCRV, 79% to GIRV, 73% to AGCRV, 62% to striped bass reovirus (SBRV), and 59% to TSRV. The phylogenetic analysis indicated that the virus isolated from white bream was affiliated with both GSRV and GCRV within a clade of *Aquareovirus C* species (Fig. 4). The combined results obtained from cell culture, electron microscopy and molecular analysis support identification of the white bream isolate as a novel reovirus, which we have tentatively designated as white bream reovirus (WBRV) strain A-127/06.

DISCUSSION

A novel virus, designated WBRV A-127/06, was isolated from white bream and propagated on different fish cell lines. Independent of infective dose, the development of CPE proceeded slowly in cultures of the EPC cell line to reach titres up to 1000-fold higher

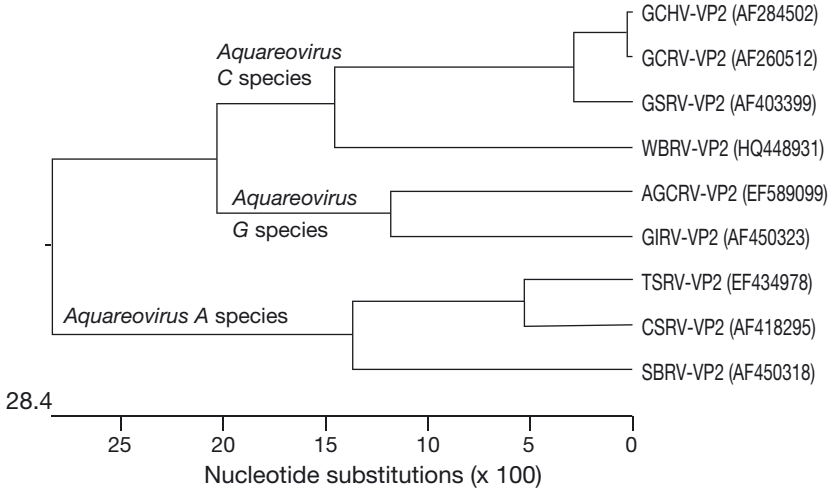

Fig. 4. Phylogenetic tree constructed using DNAstar software based on the nucleotide sequence of the available *Aquareovirus* VP2 genes in GenBank and the novel white bream reovirus (WBRV) isolate. GCHV: grass carp hemorrhagic virus; GCRV: grass carp reovirus; GSRV: golden shiner reovirus; AGCRV: American grass carp reovirus; GIRV: golden ide reovirus; TSRV: Atlantic salmon reovirus; CSRV: chum salmon reovirus; SBRV: striped bass reovirus. GenBank accession number of each virus is present on the tree

than in cultures of the BF-2 cell line. This finding is similar to that of Ahne & Koelbl (1987), who reported that chub and tench reoviruses did not replicate well on BF-2 cells. WBRV produced characteristically different types of CPE in different cell lines. CPE common to the cell lines used included formation of large syncytia and inclusion bodies, both of which are typical for aquareoviruses (Ahne & Koelbl 1987, Winton et al. 1987). WBRV also produced a response similar to other aquareoviruses in BF-2, RTG-2 and CHSE-214 cell lines. The CPE that developed in EPC cultures was characterized by pyknosis, leaving the cells adherent for several days. In contrast, BF-2 cells needed higher concentrations of WBRV for infection and presented completely different CPEs, perhaps due to the fibroblastic nature of this cell line. GCRV, the highly pathogenic agent of grass carp haemorrhagic disease (Chen & Jiang 1984) does not induce any CPE in any of the cell lines used but is able to produce a species-specific viral inhibitory interferon-like substance that inhibited viral CPE in cyprinid cell lines only (Jiang & Ahne 1989). It is possible that a similar substance is responsible for the viral resistance exhibited by BF-2 cells and for the slow development of WBRV-induced CPE in EPC cells. Further investigation is needed to prove this suggestion.

Transmission electron microscopy revealed the presence of non-enveloped, icosahedral virus particles 60 to 70 nm in size, strongly indicating that this virus belongs to the family *Reoviridae* and is similar to members of the genus *Aquareovirus* (Winton et al. 1981, Joklik 1983, Amend et al. 1984, Ahne & Koelbl 1987, Samal et al. 1990).

Preliminary RT-PCRs failed to amplify any segment from the novel WBRV-RNA using published primers of different fish aquareoviruses (data not shown). Conserved genes that are of significant basic function for organisms are the best choice for phylogenetic analysis (Attoui et al. 2002, González et al. 2003). The reoviral RNA-dependent RNA polymerase gene (VP2 gene) is one of these conserved genes as it is important for viral function (Wellehan et al. 2009). Therefore, a consensus nested PCR was used to obtain initial sequence data from this novel virus utilizing degenerative primers that target the reovirus' VP2 gene according to Wellehan et al. (2009). Additional sequence data from the WBRV VP2 gene were obtained by RACE assay and used in BLAST search and phylogenetic analysis. The obtained sequence was deposited in GenBank (accession number HQ448931). Results of BLAST search analysis of the obtained sequence displayed varying percentages of similarity to different species of the genus *Aquareovirus* at both the nucleotide sequence and amino acid sequence levels. Previous studies have indicated that most reoviruses from a single genus will have amino acid identity >30% in the polymerase gene (Attoui et al. 2002, 2005). The WBRV displayed 59 to 89% amino acid identity to the genus *Aquareovirus*, thus it is considered a species of the genus *Aquareovirus*. The phylogenetic tree based on VP2 nucleotide sequences (Fig. 4) placed Atlantic salmon reovirus (TSRV), chum salmon reovirus (CSRV) and striped bass reovirus (SBRV) in one group (*Aquareovirus A*; Seng et al. 2004); American grass carp reovirus (AGCRV) and golden ide reovirus (GIRD) in one group (*Aquareovirus G*; Mohd Jaafar et al. 2008); and golden shiner reovirus (GSRV), grass carp reovirus (GCRV) and white bream reovirus (WBRV) in one group (*Aquareovirus C*). Phylogenetic analysis placed WBRV within the *Aquareovirus C* group although it has 76% nucleotide sequence identity with GSRV and GCRV. This result is comparable with the presence of SBRV, TSRV and CSRV in one species group (*Aquareovirus A*) although the sequence identity between them is 77% (data not shown).

In conclusion, a novel virus, tentatively designated WBRV strain A-127/06, was isolated from white bream. Based on sequence analyses, virion size, cell line susceptibility and CPE characteristics, this cyprinid virus aligns with viruses of the genus *Aquareovirus* within the family *Reoviridae*. Phylogenetic analysis placed it within a clade of the species *Aquareovirus C*. Complete sequencing of the virus genome and pathogenicity of the virus to other fish species will be investigated in future studies.

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