Arch Virol (2010) 155:1261–1271
DOI 10.1007/s00705-010-0715-z

ORIGINAL ARTICLE

Comparative study of ranavirus isolates from cod (*Gadus morhua*) and turbot (*Psetta maxima*) with reference to other ranaviruses

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Received: 24 February 2010 / Accepted: 25 May 2010 / Published online: 15 June 2010

Abstract Two iridovirus isolates recovered from cod (*Gadus morhua)* and turbot (*Psetta maxima*) in Denmark were examined in parallel with a panel of other ranaviruses including frog virus 3 (FV3), the reference strain for the genus *Ranavirus*. The isolates were assessed according to their reactivity in immunofluoresent antibody tests (IFAT) using both homologous and heterologous antisera and their amplification in PCR using primers targeting five genomic regions. The corresponding PCR fragments were sequenced, and the sequences obtained were used in phylogenetic analysis. In addition, the pathogenicity to rainbow trout under experimental challenge conditions was investigated. The viruses were serologically and genetically closely related to highly pathogenic ranaviruses such as European catfish iridovirus (ECV), European sheatfish iridovirus (ESV) and epizootic haematopoietic necrosis virus (EHNV). The challenge trials indicate that rainbow trout fry cultured at 15°C are not target species for the virus isolates in the present panel. We suggest that the two isolates belong in the genus *Ranavirus* and propose the name Ranavirus maxima (Rmax) for the turbot isolate.

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Introduction

Ranavirus is a genus in the family *Iridoviridae* [1]. It possesses a large dsDNA genome, which is replicated in two stages, the main replication occurring in the nucleus, and the second phase of replication in the cytoplasm of the host cell [2]. Virions exit the host cell either by budding, whereby they obtain a host-derived envelope, or by cell lysis [1]. Ranaviruses cause disease in both amphibians [3–6] and reptiles [7–12] and have progressively become prominent pathogens of fish on a global scale. Some isolates are highly pathogenic and cause systemic infection in both cultured and free-living fish. Epizootic haematopoietic necrosis virus (EHNV) was isolated from redfin perch (*Perca fluviatilis*) in Australia during an epizootic outbreak [13, 14] and later from rainbow trout (*Oncorhyncus mykiss*) [15]. In 1989, Ahne et al. [16] reported an incidence of high mortality in sheatfish (*Silurus glanis*) associated with a ranavirus, and in 1992 and 1993, a ranavirus was found to cause high mortality in catfish (*Ameiurus melas*) in France and Italy [17, 18]. Repeated epizootics caused by ranaviruses in northern Italy have all but put an end to the *Ameiurus melas* industry in that area. Other ranavirus isolates such as pike perch iridovirus (PPIV) and short-finned eel ranavirus (SERV) appear to be have been isolated by chance from symptomless fish from both freshwater and marine environments [19, 20].

Frog virus 3 (FV3) is the type species of the genus *Ranavirus*, and was isolated from a frog with a tumour [21]. Bohle iridovirus (BIV) was isolated from moribund tadpoles [22] and caused mortality in other species of frogs under experimental conditions, especially in the metamorphosis stage [23]. It was also found to be highly pathogenic to certain fish, showing an ability to infect hosts from different classes [24, 25]. Rana esculenta virus 282/I02 (REV

282/I02) was isolated from moribund tadpoles [26]. Many reports of ranaviruses in amphibians have been published and linked to the worldwide decline in frogs [27–29].

The two Danish isolates that are the focus of this study were isolated from apparently healthy fish. Cod iridovirus (CodV) was identified during an investigation of the cause of ulcus syndrome in free-living populations of cod (*Gadus morhus*) in Danish waters, and the infected fish were caught in Lillebælt, the narrow strait between Jutland and Fynen [30]. The ranavirus isolated from cod was one of several suspected etiological agents, none of which was ever confirmed as the cause of the ulcers [30]. An iridovirus was visualized by EM in several organs in turbot (*Psetta maxima*) fry with general disease symptoms and 70% mortality [31]. It was only in 1999 that a virus was isolated during a general health certification of apparently healthy turbot fry from the same aquaculture facility. There are recent reports of iridoviruses in farmed turbot from China and Korea [32, 33]. They both appear to belong to the proposed genus *Megalocytivirus* within the family *Iridoviridae*.

Several comparative studies have been carried out on ranaviruses [34–37], with the findings that BIV, EHNV, ECV and ESV are distinct isolates that are similar to each other and to FV3 in terms of morphology, size, number and weight of structural proteins, and appear to share antigens that cause cross-reactivity in serology.

Infection with ranavirus is listed by the Office International des Epizooties (OIE) as a notifiable disease for amphibians; for fish, EHNV is the only notifiable ranavirus. This paper reports on a study comparing two Danish ranavirus-like isolates to notifiable and other ranaviruses with respect to their reactivity in IFAT with polyclonal antisera, sequencing of the major capsid protein (MCP), DNA polymerase (DNApol), RNase III, ribonucleoside diphosphate reductase alpha (RNR-α) and beta (RNR-β) subunit genes, and the pathogenicity of the isolates in experimental challenge of rainbow trout (*Onchorhyncus mykiss*) fry at 15°C.

Materials and methods

Viruses and cell culture

Ranavirus (DK-9995205) was isolated from turbot fry with no clinical signs in the spring of 1999 as part of health certification for export. The fish were investigated for the presence of viruses by cell culture and nodavirus by standard histological and immunohistochemical examination. The ranavirus was isolated from tissue homogenate of fry diluted 1:10 in cell culture medium. The suspension was centrifuged at 4000×*g* and the resultant supernatant was inoculated onto bluegill fry (BF-2) cells [38] and striped snakehead (SSN-1) cells [39] at two dilutions (10^{-1} and 10^{-2}). In addition to CodV and Rmax, nine other isolates were included in the investigation: BIV, EHNV, ECV (French and Italian isolates), ESV, FV3, PPIV, REV282/I02 and SERV (Table 1). The isolates were propagated and titrated in BF-2 cells prior to use in the different laboratory investigations and the experimental challenge trials. The cells were grown at 20°C in Eagle's MEM supplemented with 10% foetal bovine serum, 1% glutamine, 100 IU/ml penicillin and 40 g/ml streptomycin. After inoculation with virus, the flasks were transferred to a 15°C incubator. The cells were grown in either 25- or 175-cm^2 tissue culture flasks (Nunc A/S; Roskilde, Denmark). At full cytopathic effect (CPE), the suspensions underwent three cycles of freeze-thawing, after which the cell debris were separated from the viral suspension by centrifugation at 4,000×*g*. The virus was stored at −80°C. For titration of infectious virus, six replicates of tenfold dilutions of virus were inoculated into subconfluent BF-2 cells in 96-well plates (Nunc A/S; Roskilde, Denmark), and the titre was calculated as 50% tissue culture infective dose ($TCID_{50}$) according to Reed and Muench [40] prior to use in laboratory testing and in experimental challenge.

Virus purification was carried out according to Hedrick et al. [34], with slight modifications: Virus was pelleted from the supernatant prepared as above by a 60-min ultracentrifugation in a Beckman SW 28 rotor at 20,000 rpm at +4°C. The pellet was resuspended in 1 ml of TN buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl), layered on top of a 20–60% w/v continuous sucrose gradient and ultracentrifuged in a Beckman L8-60 M SW 41 rotor at 34,000 rpm for 60 min at +4°C. The resulting opalescent viral bands were collected by aspiration, diluted in TN buffer and pelleted in a Beckman SW 28 rotor at 20,000 rpm for 60 min at +4°C. The final virus pellet was resuspended in 200 µl of TN buffer, aliquoted and frozen at −80°C.

Polyclonal antisera

Two New Zealand white rabbits, F61 and F62, were immunized by intraperitoneal and subcutaneous injection according to the protocols described by Olesen et al. [41].

Staining of cells for immunofluorescence

A 1:1,000 dilution of each of the isolates in the panel was added to cover-glass cultures of epithelioma papulosum cyprini (EPC) cells (Fijan et al. 1983). The cultures were incubated for 72 h at 15°C. They were then rinsed and fixed in 80% acetone prior to immunofluorescence staining as described by Jørgensen et al. [42]. Primary antibodies (rabbit sera) produced against specific isolates were tested

Table 1 Ranavirus isolates used in this investigation, with reference to original host, first publication and supplier

Virus	Acronym	Host	Reference	Isolate obtained from	Infection trials	IFAT	Molecular studies
Bohle iridovirus	BIV	Burrowing frog (*Limnodynastes ornatus*)	Speare and Smith [22]	L.Owens[a]	X	X	ND
Bohle iridovirus	BIV	Burrowing frog (*Limnodynastes ornatus*)	Speare and Smith [22]	A. Hyatt[b]	ND	X	X
Cod ranavirus	CodV	Cod (*Gadus morhua*)	Jensen et al. [30]	J.L. Larsen[c]	X	X	X
Epizootic haematopoietic necrosis virus	EHNV	Red-fin perch (*Perca fluviatilis*)	Langdon et al. [13]	R. Whittington[d]	X	X	X
European catfish virus (France)	ECV (Fr)	European catfish (*Ameiurus melas*)	Pozet et al. [17]	G. Bovo[e]	X	X	ND
European catfish virus (Italy)	ECV (It)	European catfish (*Ameiurus melas*)	Bovo et al. [18]	G. Bovo[e]	X	X	X
European sheatfish virus	ESV	European sheatfish (*Silurus glanis*)	Ahne et al. [16]	W. Ahne[f]	X	X	X
Frog virus 3	FV3	Leopard frog (*Rana pipiens*)	Granoff et al. [21]	W. Ahne[f]	X	X	X
Pike-perch iridovirus	PPIV	Pike-perch (*Stizostedion lucioperca*)	Tapiovaara et al. [19]		X	X	X
Rana esculenta virus 282/I02	REV 282/I02	Edible frog (*Pelophylax esculentus*)	G. Bovo pers. comm.; Holopainen et al. [26]	G. Bovo[e]	ND	X	X
Ranavirus maxima	Rmax DK 9995205	Turbot (*Psetta maxima*)			X	X	X
Short-finned eel ranavirus	SERV	Short-finned eel (*Anguilla australis*)	Bang Jensen et al. [20]	G. Bovo[e]	ND	X	X

X included, *ND* not done

[a] James Cook University

[b] Australian Animal Health Laboratory, Australia

[c] University of Copenhagen

[d] University of Sydney, Australia

[e] Instituto Zooprofilattico delle Venezie, Italy

[f] University of Munich, Germany

for reactivity to homologous and heterologous isolates at 1:800 dilution and incubated for 30 min at 37°C. Additionally, a monoclonal antibody against red sea bream iridovirus (RSIV) was tested with the panel at a 1:10 dilution, as were antisera against infectious pancreatic necrosis virus (IPNV) serotypes Sp and Ab [43], using the rabbit sera F48 + F51 batch 18.11.1998, and lake trout rhabdovirus (LTR) [44], with rabbit serum K 2705-02.04.90 [45] at a dilution of 1:1,000. The antisera were kindly made available by P. de Kinkelin (anti-EHNV, -ECV (Fr), -ESIV), G. Bovo (anti-ECV(It)) and K. Nakajima (anti-RSIV), or produced at the National Veterinary Institute, Denmark, and National Veterinary and Food Research Institute, Finland (anti-BIV, -CodV, -PPIV, -Rmax, -IPN, -LTR). Rhodamine- or fluorescein-conjugated swine antibodies to rabbit immunoglobulin (R015 and F0205, respectively, Dako, Copenhagen, Denmark) at a dilution of 1:100 and 1:40, respectively, were applied as secondary antisera for 30 min at 37°C.

DNA extraction, PCR amplification, DNA sequencing and sequence analysis

CodV DNA was extracted from concentrated virus preparations by ultracentrifugation. The DNA of Rmax was purified directly from growth medium collected from virus-infected cell culture after freezing and thawing three times. Twenty microlitre of purified virus suspension or 200 µl growth medium was used for DNA extraction. The preparations were lysed with 200 µl of DNA extraction buffer containing 10 mM Tris, pH 7.5, 100 mM NaCl, 5 mM EDTA and 2% SDS. Proteinase K (final concentration

200 μg/ml) was added, and the lysates were incubated overnight at 37°C. The lysates were extracted twice with phenol–chloroform, and DNA was precipitated overnight at −20°C with ethanol containing 0.3 M sodium acetate. The dried DNA was dissolved in 10–20 μl of sterile water.

The nucleotide sequence of the complete MCP gene region and partial sequences of DNApol, RNase III, RNR-α and RNR-*β* subunit genes were analysed in this study. Several PCR primer pairs (Table 2) were used in the amplification of the different genomic regions. The entire open reading frame (ORF) of the MCP gene was amplified in three overlapping fragments. One primer pair for each gene was used to obtain partial gene sequences of DNApol, RNase III, RNR-α and RNR-*β* genes.

The PCR conditions for primer pairs MCP-AF & MCP-AR and MCP-BF & MCP-BR were as follows: 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. For primer pairs RNase III-F & RNase III-R, RNR-AF & RNR-AR and RNR-BF & RNR-BR, the cycling conditions were 25 cycles of 95°C for 1 min, 50°C for 2 min, and 72°C for 2 min. The PCR reaction mixture contained 0.5 μM of each primer, 160 μM of each nucleotide (dATP, dTTP, dGTP, dCTP), 10× PCR buffer (150 mM Tris–HCl, 500 mM KCl, pH 8.3), 1 mM $MgCl_2$ and 2 units of *Taq* polymerase (AmpliTaq Gold, Applied Biosystems). The sequences of primer pairs MCP-5 & MCP-6R and DNApol-F & DNApol-R, as wells as with the suitable PCR conditions, were published by Hyatt et al. [37] and Holopainen et al. [26], respectively. At least two independent amplification events were performed for each gene region to eliminate errors introduced by the polymerase.

Amplified PCR fragments were purified using MinElute PCR purification colums (Qiagen, Valencia, CA, USA) and sequenced. The sequencing was carried out using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Unincorporated dye terminators were removed using a DyeEx 2.0 Spin Kit (Qiagen), and the reactions were run on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Each PCR product was sequenced at least twice in both directions using the forward and reverse PCR primers.

Sequence data were analyzed using Sequencing Analysis Software 5.1 (Applied Biosystems) and SeqScape v.2.1.1 (Applied Biosystems). The individual gene sequences of each virus isolate were assembled into one continuous sequence using SeqMan Pro v.8.0.2 from the DNASTAR Lasergene 8 application package (DNASTAR Inc.). Multiple sequence alignments of the continuous sequences were done with ClustalX 1.81 [46] and edited with GeneDoc version 2.6.02 [47]. Sequence pair percent identity values were calculated using the MegAlign program from the DNASTAR Lasergene 8 application package. Maximum parsimony phylogenetic analysis was performed with Mega 4.1 software [48]. The reliability of the phylogeny was assessed by bootstrapping.

Table 2 Primers used to amplify different ranavirus genes

Primer	Target	Primer position	Nucleotide Sequence (5′-3′)	Amplicon size	Reference
*MCP-AF	MCP	97279–97299	CCTCCAAAGAGAGCGATATGC	626	U36913[a]
*MCP-AR		97904–97887	AAGAATGGGAGGGGAAGA		
*MCP-BF	MCP	97813–97830	ACCAGCGATCTCATCAAC	548	U36913[a]
*MCP-BR		98360–98341	AGCGCTGGCTCCAGGACCGT		
MCP-5	MCP	98244–98263	CGCAGTCAAGGCCTTGATGT	585	Hyatt et al. [37]
MCP-6R		98828–98807	AAAGACCCGTTTTGCAGCAAAC		
DNApol-F	DNApol	67188–67208	GTGTAYCAGTGGTTTTGCGAC	560	Holopainen et al. [26]
DNApol-R		67747–67728	TCGTCTCCGGGYCTGTCTTT		
*RNase III-F	RNase III	88858–88836	GAGGCKCTGGAGATYGTGGGSGA	717	AY548484[b], AY666015[c], AF389451[d]
*RNase III-R		88142–88159	CCCRCTRCCCTCVACRAC		
*RNR-AF	RNR-α	43729–43748	CTGCCCATCTCKTGCTTTCT	806	AY548484[b], AY666015[c]
*RNR-AR		44534–44513	CTGGCCCASCCCATKGCGCCCA		
*RNR-BF	RNR-*β*	78029–78012	AGGTGTRCCRGGGYCGTA	646	AY548484[b], AY666015[c]
*RNR-BR		77384–77403	GACGCTCCAYTCGACCACTT		

The position of the primer is presented relative to FV3 genome AY548484. New primers used in this study are marked with an asterisk. The reference for each primer is either the original publication or the GenBank accession number for previously published sequences based on which the primers were designed

Y = C/T, K = G/T, S = C/G, R = A/G, V = A/C/G

[a] Mao et al. [54], [b]Tan et al. [51], [c]Tsai et al. [58], [d]He et al. [56]

Experimental challenge of rainbow trout

The pathogenicity of the panel of ranavirus isolates was tested by experimental challenge of rainbow trout fry. The fry (average weight 1.5 g) were obtained from a certified virus-free hatchery, Rønhøjgård in Denmark, and were screened for viral, parasitic and bacterial infections prior to challenge. The challenges were carried out at the challenge facilities of DTU in Århus. Fish were kept in 10–l tanks with 50 animals per tank in 15°C water with constant aeration and a flow-through system. Three challenge treatments were tested: Intraperitoneal inoculation of 50 µl viral suspension (10^4 $TCID_{50}$) per fish, bath exposure at a high dose (10^4 $TCID_{50}$/ml) and at a lower dose (10^3 $TCID_{50}$/ml) for 2 h. The two bath treatments were tested in duplicate. Nine ranavirus isolates in the panel described above were tested in the three treatments. A negative and positive control, consisting of cell culture medium and the viral haemorrhagic septicaemia (VHS) virus strain DK-3592B [49], respectively, were included in each treatment. Fish were tranquilised with benzocaine prior to i.p. inoculation. For bath exposure, the water levels were lowered, and circulation was stopped for 2 h whilst aeration was maintained. Fish were monitored and fed twice daily. Mortality was recorded daily, and moribund or dead fish were collected and frozen at 20°C for virological examination by standard virological procedures [50] at the end of each trial. Five fish were collected from each tank on day seven post-challenge and examined individually. On day 28, a pool of five fish from each tank was likewise examined.

Results

The ranavirus isolate from turbot was obtained from 3 of 10 samples of pooled organ material of apparently healthy turbot fry originating from a Danish turbot aquaculture facility. No other pathogens were detected. The material was inoculated onto BF-2 and SSN-1 cells and incubated at both 15 and 21°C. Cytopathic effect (CPE) was observed in both cell lines after 7 days and was most prominent at 21°C. Cytopathic effect was characterised by small foci of rounded cells in a cobweb-like matrix. The CPE developed into plaques with rounded cells along the edges. At total CPE, the cells had all detached from the culture surface. The isolate tested negative for IPN, VHS and infectious haematopoietic necrosis virus (IHNV) in ELISA. Infected cultures of EPC cells grown on coverslips stained positive in IFAT using rabbit antisera against the ranaviruses, CodV, BIV and EHNV. There was no cross-reaction with antisera against RSIV, IPNV or LTR (Table 3).

Rabbit F61 reacted strongly in IFAT with homologous and heterologous ranaviruses at dilution 1:800–1:1,000, while the staining with F62 was less pronounced (not shown). Differentiation of the isolates in the panel was not possible in IFAT due to complete cross-reaction with all of the ranavirus antisera tested (Table 3). Positive staining was observed for all combinations, although some reacted more strongly than others. The monoclonal antisera produced against RSIV did not produce any staining when tested against the isolates in the panel, and neither did the antisera against IPNV or LTR. The staining of the isolates with antibodies against EHNV is shown in Fig. 1. Positive

Table 3 Immunofluorescent staining of a panel of 11 iridoviruses with rabbit antisera produced against 8 different iridovirus isolates. A monoclonal antibody against RSIV and rabbit antisera against IPNV and lake trout rhabdovirus were tested as well

Virus	Polyclonal antiserum against										MAb
	BIV	CodV	ECV It	ECV Fr	EHNV	ESV	PPIV	Rmax	*IPNV*	*L.trout*	*RSIV*
BIV	+	+	+	+	+	+	+	+	*ND*	*ND*	*Neg*
CodV	+	+	+	+	+	+	+	+	*ND*	*ND*	*Neg*
ECV It	+	+	+	+	+	+	+	+	*ND*	*ND*	*Neg*
ECV Fr	+	+	+	+	+	+	+	+	*ND*	*ND*	*Neg*
EHNV	+	+	+	+	+	+	+	+	*ND*	*ND*	*Neg*
ESV	+	+	+	+	+	+	+	+	*ND*	*ND*	*Neg*
FV3	+	+	+	+	+	+	+	+	*ND*	*ND*	*Neg*
PPIV	+	+	+	+	+	+	+	+	*ND*	*ND*	*Neg*
REV 282/I02	ND	ND	ND	ND	+	ND	ND	ND	*ND*	*ND*	*Neg*
Rmax	+	+	+	+	+	+	+	+	*ND*	*ND*	*Neg*
SERV	ND	ND	ND	ND	+	ND	ND	ND	*ND*	*ND*	*Neg*
IPNV	*Neg*	*Neg*	*Neg*	*Neg*	*Neg*	*Neg*	*Neg*	*Neg*	+	*Neg*	*Neg*
L. Trout	*Neg*	*Neg*	*Neg*	*Neg*	*Neg*	*Neg*	*Neg*	*Neg*	*Neg*	+	*Neg*

Negative controls are italisized. *Neg* no staining, *ND* not done, + indicates positive reaction

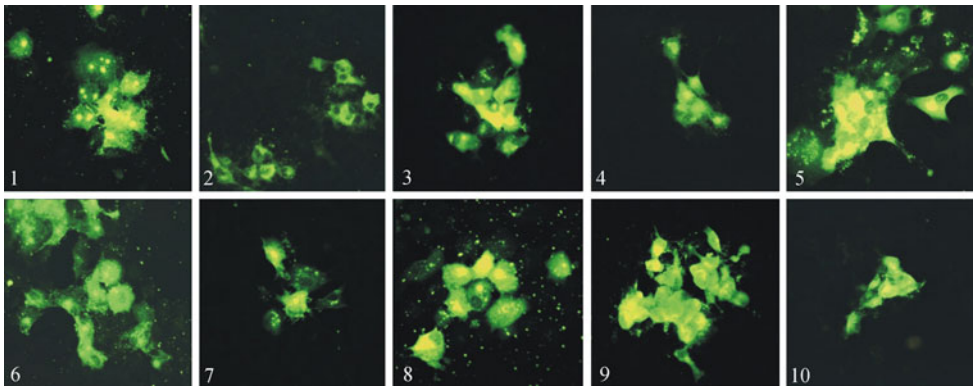

Fig. 1 IFAT staining of ranavirus infected EPC cells with antibodies against EHNV. Cytoplasms of the infected cells are stained with fluorescein (*green*). Virus isolates used in the infection: *1* BIV, *2* CodV, *3* ECV, *4* EHNV, *5* ESV, *6* FV3, *7* PPIV, *8* REV 282/I02, *9* Rmax, *10* SERV. Pictures were taken with 400× magnification

Table 4 GenBank accession numbers of the sequences used in the analyses

Virus	MCP	DNApol	RNase III	RNR-α	RNR-β
BIV	FJ358613[a] (58.9%)	FJ374280[a] (62%)	GU391273* (58.1%)	GU391286* (60.6%)	GU391264* (55.8%)
CodV	GU391284* (59.3%)	GU391282* (62%)	GU391274* (58.3%)	GU391287* (61.5%)	GU391265* (55.9%)
ECV	FJ358608[a] (60.3%)	FJ374277[a] (62.6%)	GU391275* (59.2%)	GU391288* (61.5%)	GU391266* (55.9%)
EHNV	AY187045[b] (60.0%)	FJ374274[a] (62.2%)	GU391276* (59.8%)	GU391289* (61.1%)	GU391267* (56.3%)
ESV	FJ358609[a] (60.3%)	FJ374278[a] (62.2%)	GU391277* (59.3%)	GU391290* (61.5%)	GU391268* (55.9%)
FV3	FJ459783[a] (59.1%)	AY548484[c] (62.0%)	AY548484[c] (58.3%)	AY548484[c] (60.6%)	AY548484[c] (55.8%)
PPIV	FJ358610[a] (59.7%)	FJ374276[a] (62.0%)	GU391278* (58.6%)	GU391292* (61.1%)	GU391269* (55.8%)
REV282/I02	FJ358611[a] (59.5%)	FJ374275[a] (61.5%)	GU391280* (58.4%)	GU391293* (60.9%)	GU391271* (55.8%)
Rmax	GU391285* (59.3%)	GU391283* (61.8%)	GU391279* (58.3%)	GU391291* (61.3%)	GU391270* (55.6%)
SERV	FJ358612[a] (60.5%)	FJ374279[a] (63.2%)	GU391281* (60.4%)	GU391294* (61.8%)	GU391272* (55.9%)
TFV	AF389451[d] (59.1%)	AF389451[d] (62.4%)	AF389451[d] (57.5%)	AF389451[d] (61.1%)	AF389451[d] (55.6%)
SGIV	NC_006549[e] (50.6%)	NC_006549[e] (48.7%)	NC_006549[e] (49.2%)	NC_006549[e] (52.7%)	NC_006549[e] (48.6%)
GIV	AY666015[f] (50.6%)	AY666015[f] (49.7%)	AY666015[f] (49.1%)	AY666015[f] (52.9%)	AY666015[f] (48.8%)

Sequences marked with an asterisk (*) were obtained in this study. GC contents of the sequences are given in brackets

[a] Holopainen et al. [26], [b]Marsh et al. [53], [c]Tan et al. [51], [d]He et al. [56], [e]Song et al. [57], [f]Tsai et al. [58]

staining was detected in the cytoplasm of infected EPC cells with all of the virus isolates studied.

The complete MCP gene and partial DNApol, RNase III, RNR-α and RNR-β genes were successfully amplified from both CodV and Rmax. Based on sequence analysis, the lengths of the amplified gene fragments corresponded to the predicted amplicon sizes estimated from the FV3 genome [51] (GenBank AY548484). The MCP gene sequences of CodV and Rmax were obtained in three overlapping fragments, and the length of the open reading frame (ORF), 1,392 bp, was identical with the other ranaviruses studied. In addition, the partial RNase III, RNR-α and RNR-β genes of BIV, EHNV, ECV (It), ESV, FV3, PPIV, REV282/I02 and SERV were amplified and sequenced. The complete genome of FV3 has been published earlier [51] and the RNase III, RNR-α and RNR-β sequences of FV3 obtained in this study matched the published data. For BIV, EHNV, ECV (It), ESV, PPIV, REV282/I02 and SERV, there were no previously published sequence data for the RNase III, RNR-α and RNR-β genes. The novel MCP, DNApol, RNase III, RNR-α and RNR-β gene sequences obtained from the isolates studied were deposited into the GenBank database (Table 4).

In order to study the phylogenetic relationships of the virus isolates, the individual gene sequences of each virus isolate were assembled into one continuous sequence. The order of the gene sequences was arranged to match the gene order of the FV3 genome [51] (AY548484): RNR-α, DNApol, RNR-β, RNase III and MCP. The sequences of different gene regions were concatenated according to the ORFs of each gene; missing nucleotides in the junction of gene fragments were coded into the sequence in order to maintain the correct reading frame. Previously published sequences of the virus isolates studied were used in the sequence analysis (Table 4). The length of the concatenated sequence was 3,959 bp in all 10 virus isolates studied. Previously published sequences of three other ranaviruses, tiger frog virus (TFV), Singapore grouper iridovirus (SGIV) and grouper iridovirus (GIV), were also included into the maximum parsimony phylogenetic

Table 5 Ranavirus sequence pair percent identity values based on the concatenated nucleotide (3,959 bp) and amino acid (1,317 aa) sequences of the RNR-α, DNApol, RNR-β, RNase III and MCP genes

	BIV	CodV	ECV	EHNV	ESV	FV3	PPIV	REV 282/I02	Rmax	SERV	TFV	GIV	SGIV
BIV		97.6	97.7	98.2	97.7	98.8	98.6	98.4	97.5	95.1	98.5	65.3	64.8
CodV	96.9		98.3	98.6	98.3	97.6	98.2	98.0	99.7	95.4	97.6	65.2	64.8
ECV	97.4	97.3		99.0	100	97.8	98.4	98.2	98.3	96.2	97.7	65.5	65.0
EHNV	98.1	97.7	98.4		99.1	98.2	98.8	98.6	98.6	96.1	98.1	65.1	64.8
ESV	97.4	97.3	100	98.4		97.8	98.4	98.2	98.3	96.2	97.7	65.4	65.0
FV3	99.2	96.8	97.3	97.9	97.3		98.6	98.4	97.5	95.1	98.5	65.3	64.8
PPIV	98.9	97.6	98.0	98.7	98.0	98.6		99.2	98.1	95.5	98.5	65.4	65.0
REV282/I02	98.4	97.3	97.7	98.4	97.7	98.3	99.0		97.9	95.3	98.2	65.3	64.9
Rmax	97.1	99.5	97.4	97.8	97.4	97.0	97.7	97.4		95.4	97.5	65.3	64.9
SERV	95.9	95.7	96.8	96.5	96.8	95.9	96.3	96.0	96.0		94.9	65.6	65.2
TFV	98.6	96.6	97.0	97.7	97.0	98.5	98.3	97.9	96.8	95.6		65.2	64.8
GIV	67.8	67.5	67.8	67.3	67.8	67.5	67.7	67.5	67.6	67.5	67.5		98.2
SGIV	67.8	67.5	67.6	67.2	67.6	67.6	67.8	67.5	67.6	67.5	67.5	98.9	

Nucleotide sequence identity values are presented in the upper diagonal, and amino acid identity values in the lower diagonal. The GenBank accession numbers of the sequences used in the analyses are presented in Table 4

analysis as well as into the nucleotide (nt) and amino acid (aa) sequence identity comparisons. The GC content of all of the sequences analysed varied between 48.6% in RNR-β of SGIV and 63.2% in DNApol of SERV.

The nt and aa sequence pair percent identity values of CodV and Rmax compared with other ranavirus sequences are presented in Table 5. Based on the sequences obtained for the RNR-α, DNApol, RNR-β, RNase III and MCP genes, the overall nt identity among the 10 ranavirus isolates studied was between 95.1 and 100%. ECV and ESV had identical aa sequences and only one nt difference in the entire concatenated sequence (in RNR-β). SERV was the most divergent of the isolates studied. CodV and Rmax had 13 nt and 6 aa differences in the entire concatenated sequence: 2 nt differences and 1 aa difference in RNR-α, 5 nt and 3 aa in DNApol, 2 nt and 1 aa in RNR-β, 2 nt and 1 aa in RNase III and 2 nt and no aa differences in MCP. The overall nt sequence identity between CodV and Rmax was 99.7%; the aa sequence identity was 99.5%. The closest relatives to Rmax and CodV were EHNV, with 98.6%, and ECV and ESV, with 98.3% nt sequence identity. The same affinity can be seen in the results of the maximum parsimony phylogenetic analysis; Rmax and CodV cluster closely with EHNV (Fig. 2).

Mortality in experimental tanks was low (max 2/50 fish per tank) and did not exceed that recorded in the placebo treatments. The positive-control tanks experienced 70% accumulated mortality by day 28, and VHS was isolated from dead fish. Of the fish collected at day 7 from the different treatments, one isolation was made from an individual fish in the bath treatment with EHNV, and two other isolations were made from individual fish inoculated with FV3 and ESV, respectively. At day 28, no virus was isolated from the pooled samples collected from each treatment. From a pool of the dead fish from the third week of the experiment, virus was isolated from fish that had been inoculated with ESV and EHNV. Virus was not isolated from fish that died during weeks 2 and 4. All viruses isolated from challenged fish were confirmed to be ranaviruses in IFAT using anti-EHNV antisera. Histopathological examination of 50 fish (10 from 5 tanks) did not reveal any pathological changes.

Discussion

Currently, EHNV is the only ranavirus listed by the OIE as notifiable for fish. In addition to EHNV, there are also other known pathogenic ranavirus isolates, e.g. ESV and ECV [16–18]. The common denominator stringing these events together is the relatively high water temperature under which the fish were cultured. Several other ranavirus isolations have been made, many of which appear to be haphazard isolations from symptomless fish, indicating that ranavirus isolates are not always virulent [1]. The panel of isolates investigated here contains isolates from outbreaks, from routine survey of apparently healthy fish, and three amphibian isolates: FV3, the reference strain for ranaviruses, BIV and REV 282/I02. The objective was to compare two Danish isolates to a panel of ranavirus in terms of their reactivity in IFAT, sequence of MCP, DNApol, RNase III, RNR-α and RNR-β and relative pathogenicity to rainbow trout under experimental challenge conditions.

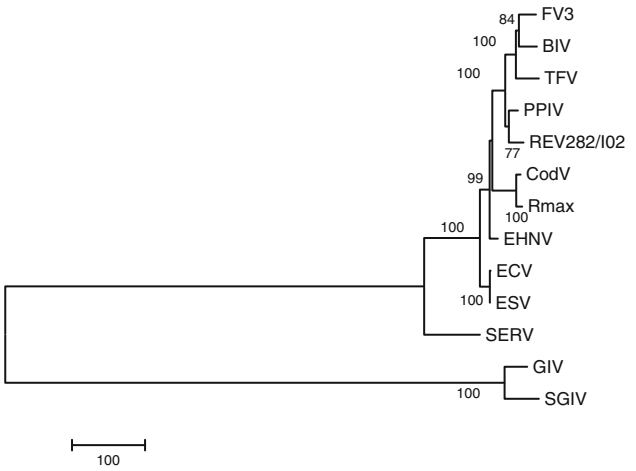

Fig. 2 Maximum parsimony analysis of the concatenated nucleotide sequences (3,959 bp) of RNR-α, DNApol, RNR-β, RNase III and MCP genes of ranaviruses. Numbers at the nodes of the tree indicate bootstrap values of 1,000 replicates; values under 70 are not shown. *Scale bar* indicates 100 nucleotide substitutions. The GenBank accession numbers of sequences used in the analysis are presented in Table 4

Both CodV and Rmax reacted with all of the polyclonal antisera against the various ranavirus but not the monoclonal antiserum against RSIV and the negative-control antisera. The high degree of serological cross-reaction between ranavirus isolates renders IFAT based on polyclonal antisera a suitable technique for fast identification of ranaviruses [34]. The technique does not allow for separating isolates into notifiable and non-notifiable or into pathogenic versus non-pathogenic groups. A panel of specific monoclonal antibodies could be a possible alternative to achieve this, and by implementation in an ELISA, this would be an easy, fast and reliable technique to differentiate these isolates. This is, of course, assuming that sufficient epitope differences exist among the isolates to produce differentiating antibodies. Alternatively, PCR may lead the way towards differential diagnosis with the design of specific primers to differentiate according to pathogenicity of strains. A real-time PCR method has been developed to differentiate between Australian and European ranavirus isolates [52]. Other methods such as restriction enzyme analysis (REA) can also differentiate strains [26–53], but is not commonly implemented in routine diagnostic laboratories in Europe.

In addition to PCR and REA, differentiation of ranaviruses can be accomplished by sequencing a genomic region [26, 37, 54]. To date, five ranavirus genomes have been completely sequenced: FV3 [51], Ambystoma tigrinum virus (ATV) [55], TFV [56], SGIV [57] and GIV [58]. Complete or partial sequences of specific viral genes, e.g. MCP and DNApol, have been reported for several ranaviruses isolated from both amphibian and fish species [5, 26, 37, 53, 54, 58–64]. Ranaviruses are a genetically homogenous group, with only a few divergent isolates: GIV, SGIV, doctorfish virus (DFV) and guppy virus 6 (GV6) [26, 37, 57, 59, 63, 65]. In this study, novel sequence data from all ten viruses studied, including the two Danish isolates CodV and Rmax, were obtained. The sequence data from five different gene regions of each virus isolate were combined and analysed as a concatenated sequence. Combining data, e.g. sequences from several genes, reduces the sampling variation in the phylogenetic analysis and provides more accurate phylogeny [66]. Based on the sequences analysed, differentiation of the isolates studied was possible, even though ECV and ESV differed by only one nucleotide in the entire concatenated sequence region. CodV and Rmax were very closely related to each other, and in the maximum parsimony phylogenetic analysis, they clustered together with EHNV.

EHNV has repeatedly been diagnosed in cultured rainbow trout in Australia with a low infectivity rate and high case mortality [67]. None of the nine isolates in the current study proved virulent to rainbow trout under the experimental conditions applied (15°C water temperature and both bath and i.p. exposure) even though virus was isolated from dead fish in the third week of the experiment, indicating that infection in a few cases did become established. Challenge trials with the cod and turbot ranavirus isolates in cod and turbot could reveal a different level of pathogenicity than what was observed here.

Temperature seems to be a contributing factor in the virulence of these viruses, affecting viral growth in vitro [21] and the immune defence of fish [68]. Whittington and Reddacliff [69] found that rainbow trout were not susceptible to EHNV infection by bath, but they could reproduce the disease by i.p. inoculation with $10^{5.6}$ TCID50 at water temperatures from 8 to 21°C. The incubation period increased with decreasing temperature and increased to 32 days at 8–10°C. Possibly, the experimental infection in this study induced only subclinical infection at 15°C in the majority of the population, or infection was not established. Persistent infection with EHNV in i.p.-inoculated rainbow trout was also detected by Whittington et al. [67] in a subclinically affected rainbow trout 63 days after exposure, which renders rainbow trout efficient vectors for an infection, especially when transferred from colder to warmer climates. Challenge trials with ESV in sheatfish cultured at 24°C resulted in 100% mortality 8 days after bath challenge and 11 days after exposure via co-habitation [70]. The pathogenicity of ranavirus isolates on commonly cultured warm-water species of European fish could be detrimental and should be tested.

Brunner et al. [71] found that the infectious dose and the genetic background of the experimental animals as well as their life-history stage influenced the virulence of ranavirus

infections in tiger salamander (*Ambystoma tigrinum*) under experimental conditions. Possibly, the doses we used in this study combined with the relatively low temperatures could have "concealed" the virulence of the isolates tested. Alternatively, the strain of rainbow trout used may be resistant to the isolates in the panel, or the experimental conditions were somehow not conducive to establishing an infection. Our findings are in accordance with other challenge studies of European stock of rainbow trout with ranavirus [72, 73], in which it was found that rainbow trout and red-fin perch (*Perca fluviatilis*) cultured under northern European conditions are relatively well protected against ranaviruses due to the low temperatures at which they are normally cultured.

Under the challenge conditions applied in this study, none of the isolates appeared to be highly pathogenic to rainbow trout. The results of IFAT and sequencing investigations firmly placed both Danish isolates, CodV and Rmax, within the genus *Ranavirus*, and we suggest the name Ranavirus maxima for the turbot isolate.

Acknowledgments The present work was partly funded by the 6th framework program of European Union contract number SSPE-CT-2005-006459, project RANA. Drs. Ahne, Bovo, Hyatt, de Kinkelin, Larsen, Nakajima, Owens and Whittington are acknowledged for providing iridovirus strains and antibodies. Thanks are due to all our technicians, but especially Nicole Nicolajsen.

Conflict of interest statement The authors declare that they have no conflict of interest.

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