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Ranavirus phylogeny and differentiation based on major capsid protein, DNA polymerase and neurofilament triplet H1-like protein genes

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ABSTRACT: In this study, we developed new methods for differentiation of ranaviruses based on polymerase chain reaction and restriction enzyme analysis of DNA polymerase and neurofilament triplet H1-like (NF-H1) protein gene. Using these methods, we were able to differentiate the 6 known ranaviruses — Bohle iridovirus (BIV), European catfish virus (ECV), epizootic haematopoietic necrosis virus (EHNV), European sheatfish virus (ESV), frog virus 3 (FV3) and Singapore grouper iridovirus (SGIV) — with 3 less characterised virus isolates: short-finned eel ranavirus (SERV), *Rana esculenta* virus Italy 282/I02 (REV 282/I02) and pike-perch iridovirus (PPIV). Doctor fish virus (DFV) and guppy virus 6 (GV6) were distinguished as a group from the other viruses. In addition, all 11 isolates were analysed and compared based on nucleotide sequences from 3 different genomic regions: major capsid protein (MCP), DNA polymerase and NF-H1. The partial DNA polymerase gene was sequenced from all analysed viruses. The complete sequence of the MCP and a fragment of the NF-H1 gene were obtained from BIV, ECV, EHNV, ESV, FV3, PPIV, REV 282/I02 and SERV. With the exception of GV6, DFV and SGIV, the sequence analyses showed only a few variations within the analysed viruses. The sequence data suggest that PPIV, REV 282/I02 and SERV are new members of the genus *Ranavirus*. The methods developed in this study provide tools to differentiate between closely related ranaviruses of different host and geographical origin.

KEY WORDS: Ranavirus · Iridovirus · Fish virus · Amphibian virus · EHNV · FV3 · ECV · BIV

INTRODUCTION

Ranaviruses are large double-stranded DNA viruses in the genus *Ranavirus* of the family *Iridoviridae*. The other genera within this family are *Iridovirus*, *Chloridovirus*, *Lymphocystivirus* and *Megalocytivirus* (Chinchar et al. 2005). Members of the family *Iridoviridae* infect fish, amphibians, reptiles, insects, crustaceans and molluscs. Diseases induced by members of the genera *Ranavirus* and *Megalocytivirus* are a growing concern in both aquaculture and in amphibian ecology. These viruses are found worldwide and cause high mortality in their host species (reviewed by Williams et al. 2005).

Ranaviruses infect fish, amphibians and reptiles, and often cause an acute, systemic disease. The disease can be severe, with necrosis of kidney and spleen and haemorrhages on the skin and internal organs (Chinchar 2002, Williams et al. 2005). Epizootic haematopoietic necrosis virus (EHNV) was the first ranavirus isolated from fish. It causes high mortality in red-fin perch *Perca fluviatilis* and high morbidity in rainbow trout *Oncorhynchus mykiss* in Australia (Langdon et al. 1986, 1988, Langdon & Humphrey 1987). Other pathogenic ranaviruses have been isolated during disease outbreaks among e.g. catfish *Ameiurus melas* and sheatfish *Silurus glanis* in Europe (Ahne et al. 1989, Pozet et al. 1992, Bovo et al. 1993, Bigarré et al. 2008), in largemouth bass *Micropterus salmoides* in North America (Plumb et al. 1996, Hanson et al. 2001) and in grouper (*Epinephelus* sp.) in Southeast Asia (Chua et al. 1994, Chou et al. 1998). Since the first detection of frog virus 3 (FV3; Rafferty 1965, Granoff et al. 1966), ranaviruses have been isolated in association with disease outbreaks among amphibians in most parts of the world (Fijan et al. 1991, Drury et al. 1995,

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Jancovich et al. 1997, Kanchanakhan 1998, Zupanovic et al. 1998, Bollinger et al. 1999, Zhang et al. 2001, He et al. 2002, Green et al. 2002, Greer et al. 2005, Fox et al. 2006).

The ranavirus virion contains a single linear dsDNA molecule that is 150 to 170 kbp in size. Genomes of ranaviruses are large and contain more than 100 open reading frames (ORFs), many of which are homologous to cellular genes (Jancovich et al. 2003, Chinchar et al. 2005, Tsai et al. 2005). The highly methylated genome is circularly permuted and terminally redundant and has a G+C content of approximately 54% (Willis & Granoff 1980, Goorha & Murti 1982, Willis et al. 1984, Murti et al. 1985, Jankovich et al. 2003).

Five ranavirus genomes have been completely sequenced to date: FV3, which represents the type species of the genus *Ranavirus* (Tan et al. 2004), *Ambystoma tigrinum* virus (ATV) associated with salamander mortalities in North America (Jancovich et al. 2003), tiger frog virus (TFV, He et al. 2002), Singapore grouper iridovirus (SGIV, Song et al. 2004) and grouper iridovirus (GIV, Tsai et al. 2005).

Marsh et al. (2002) studied 7 fish and amphibian ranaviruses originating from Europe, Australia and America (European catfish virus (ECV), European sheatfish virus (ESV), EHNV, Bohle iridovirus (BIV), Wamena virus, FV3 and Gutapo virus) and developed a rapid method based on PCR and restriction enzyme analysis (REA) for differentiating these viruses. This method is recommended by the Office International des Epizooties (OIE) in the current Manual for Diagnostic Tests for Aquatic Animals (Whittington & Hyatt 2006). The aim of the present study was to include more ranavirus isolates and to provide new PCR-REA differentiation methods based on DNA polymerase and neurofilament triplet H1-like (NF-H1) protein genes. In addition, this study was an attempt to increase our knowledge of some known and some previously uncharacterised ranavirus isolates, compare the obtained DNA sequences and, finally, to address the phylogenetic positioning of these viruses within the family *Iridoviridae*.

MATERIALS AND METHODS

Virus isolates. Eleven ranavirus isolates from different piscine and amphibian hosts were selected for the study. Table 1 summarises the origin of the virus isolates. Three of the isolates are less well characterised. The first of these, short-finned eel ranavirus (SERV), was isolated in Italy from short-finned eel *Anguilla australis* imported from New Zealand for food consumption (Bovo et al. 1999; the same isolate was referred to as New Zealand eel virus (NZeelV) by Bang Jensen et al. 2009). The second, *Rana esculenta* virus Italy 282/I02 (REV 282/I02), has not been formally described. REV 282/I02 was isolated from tadpoles of wild edible frog *Pelophylax esculentus* (formerly *Rana esculenta*) during a disease outbreak occurring 2 d after transfer of tadpoles to aquarium facilities (G. Bovo pers. comm.). The third, pike-perch iridovirus (PPIV), was originally isolated from pike-perch fry *Stizostedium lucioperca* (Tapiovaara et al. 1998). This is the first attempt to obtain viral DNA sequence from these 3 isolates.

All viruses were grown in epithelioma papulosum cyprini (EPC) cells (Fijan et al. 1983) in Eagle's minimum essential medium (MEM) at room temperature (22°C) according to the OIE Manual of Diagnostic Tests for Aquatic Animals (Whittington & Hyatt 2006). The viruses were harvested when the cytopathic effect (CPE) of the cells was complete. The passage levels of the studied virus isolates are presented in Table 1.

PCR. The viral DNA used as a template for PCR amplification was extracted from the ranavirus-infected EPC cells with a QiaAmp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. Several oligonucleotides were used in PCR reactions to amplify specific genetic regions from the ranavirus genomes (Table 2).

The major capsid protein gene (MCP) was amplified in 3 overlapping fragments using primers and reaction conditions published by Hyatt et al. (2000). Primers DNApol-F and DNApol-R were deduced from published sequences of the FV3 (Tan et al. 2004, GenBank accession number AY548484) and SGIV genome (Song et al. 2004, AY521625) for amplification of the partial DNA polymerase gene. A fragment of the neurofilament triplet H1-like protein (NF-H1) gene was amplified using primers NF-H1-F and NF-H1-R deduced from the FV3 genome (AY548484).

Amplification reactions with either DNA polymerase or NF-H1 primers were performed as follows: 35 cycles of 95°C for 1 min, 50°C (for DNA polymerase) or 55°C (for NF-H1) for 1 min and 72°C for 1 min. The reaction mixture for DNA polymerase or NF-H1 PCR 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 8.0), 1 mM $MgCl_2$ and 2 U of *Taq* polymerase (AmpliTaq Gold, Applied Biosystems). All PCR products were run on a 2% agarose gel to confirm the quality and correct size of the amplicons.

Sequencing and sequence analysis. Prior to sequencing, the PCR products were purified with a Min Elute PCR purification Kit (Qiagen). All PCR products were sequenced at least twice in both directions using the forward and reverse PCR primers. Sequencing was carried out with the Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

Table 1. Ranavirus isolates used in this study. UN: unknown

Virus	Acronym	Host	Isolated in	Isolate obtained from	Passage level	Source
Bohle iridovirus	BIV	Burrowing frog *Limnodynastes ornatus*	Australia	A. Hyatt[a]	5th	Speare & Smith (1992)
Doctor fish virus	DFV	Doctor fish *Labroides dimidatus*	North America/ fish imported from Asia	B. Hill[b]	7th	Hedrick & McDowell (1995)
Epizootic haematopoietic necrosis virus	EHNV	Red-fin perch *Perca fluviatilis*	Australia	R. Whittington[c]	UN	Langdon et al. (1986)
European catfish virus	ECV	European catfish *Ameiurus melas*	France	G. Bovo[d]	8th	Pozet et al. (1992)
European sheatfish virus	ESV	European sheatfish *Silurus glanis*	Germany	W. Ahne[e]	8th	Ahne et al. (1989)
Frog virus 3	FV3	Leopard frog *Rana pipiens*	North America	W. Ahne[e]	UN	Granoff et al. (1966)
Guppy virus 6	GV6	Guppy *Poecilia reticulata*	North America/ fish imported from Asia	B. Hill[b]	7th	Hedrick & McDowell (1995)
Pike-perch iridovirus	PPIV	Pike-perch *Stizostedion lucioperca*	Finland	H. Tapiovaara[f]	8th	Tapiovaara et al. (1998)
Rana esculenta virus 282/I02	REV 282/ I02	Edible frog *Pelophylax esculentus*	Italy	G. Bovo[d]	3rd	G. Bovo pers. comm.
Short-finned eel ranavirus	SERV	Short-finned eel *Anguilla australis*	Italy/ fish imported from New Zealand	G. Bovo[d]	6th	Bovo et al. (1999), Bang Jensen et al. (2009)
Singapore grouper iridovirus	SGIV	Grouper (*Epinephelus* sp.)	Singapore	N. Fook Kheong[g]	2nd	Chou et al. (1998)

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[c]University of Sidney, Australia
[d]Instituto Zooprofilattico delle Venezie, Italy
[e]University of Munich, Germany
[f]Finnish Food Safety Authority Evira, Finland
[g]Agri-Food and Veterinary Authority of Singapore, Republic of Singapore

Table 2. Primers used in PCR reactions and the predicted amplicon sizes. The primer position is presented relative to the FV3 genome (AY548484). MCP primers were published by Hyatt et al. (2000). Y = C + T

Target gene	Primer designation	Primer position	Amplicon size (bp)	Nucleotide sequence (5′ to 3′)
MCP	MCP-1	97318-97347	543	CAC CGT GTA TCT TAT AAT AAA AAG GAA ATG
	MCP-2R	97860-97842		GGC TCC GTC CTG GCC TGT G
	MCP-3	97774-97794	530	GAG GCC AAG CGC ACA GGC TAC
	MCP-4R	98303-98284		TTG GAG CCG ACG GAA GGG TG
	MCP-5	98244-98263	585	CGC AGT CAA GGC CTT GAT GT
	MCP-6R	98828-98807		AAA GAC CCG TTT TGC AGC AAA C
DNA polymerase	DNApol-F	67188-67208	560	GTG TAY CAG TGG TTT TGC GAC
	DNApol-R	67747-67728		TCG TCT CCG GGY CTG TCT TT
NF-H1	NF-H1-F	39068-39085	639	CCA AAG ACC AAA GAC CAG
	NF-H1-R	39706-39687		GTT GGT CTT TGG TCT CGC TC

Sequence data were analysed using Sequencing Analysis Software 5.1 (Applied Biosystems).

The multiple sequence alignments were done with ClustalX 1.81 (Thompson et al. 1997). Sequence pair percent identity values were calculated from multiple alignments by the MegAlign program from the DNASTAR Lasergene 7.1 application package. Maximum parsimony analyses were conducted with Mega 4.1 software (Tamura et al. 2007). The reliability of the phylogenetic analyses was tested by bootstrapping.

Restriction enzyme analyses (REA). The PCR products of the DNA polymerase gene were digested with the restriction enzyme *Bpm*I, and those of NF-H1 with *Alu*I, *Hae*III and *Afl*III (New England Biolabs). The REA reaction mixtures consisted of 20 to 30 µl of PCR product, 2.5 to 5 U of respective restriction enzyme, 4 µl of 10× restriction enzyme buffer, and sterile water to a final reaction volume of 40 µl. The *Bpm*I and *Afl*III reaction mixtures were supplemented with 0.5 µl bovine serum albumin (10 mg ml^{-1}). The reaction mixtures were incubated at 37°C for 1 to 4 h and analysed after electrophoresis through a 2% agarose gel.

RESULTS

Sequence data

MCP

The MCP gene was amplified in 3 overlapping fragments (Hyatt et al. 2000) from BIV, ECV, EHNV, ESV, FV3, SERV, PPIV and REV 282/I02. The estimated size of the contiguous sequence generated from the 3 amplicons was 1511 bp. No PCR products were obtained in the amplification reactions of guppy virus 6 (GV6), doctor fish virus (DFV) and SGIV. In all other analysed ranaviruses, the amplified MCP gene was 1392 bp in length based on the obtained sequences, and corresponded in size to published data on EHNV and BIV (Marsh et al. 2002) and FV3 (Mao et al. 1996, Tan et al. 2004). This is the first report on the complete MCP gene sequence of ECV, ESV, PPIV, REV 282/I02 and SERV.

Comparison of the MCP gene of the FV3 isolate used in this study to the published FV3 genomic sequence (Tan et al. 2004, AY548484) showed 1 nucleotide substitution at position 97992, resulting in the exchange of amino acid from histidine (H) to glutamine (Q). The BIV isolate used in this study also differed by 1 nucleotide from the published BIV MCP sequence (Marsh et al. 2002, AY187046). This nucleotide difference, located at position 20 from the beginning of the gene, leads to a change from leucine (L) to serine (S). The obtained MCP sequence of EHNV was identical to the published one (Marsh et al. 2002, AY187045). The overall MCP sequence identity among the analysed ranaviruses varied between 94.1 and 100%. ECV and ESV were identical, and SERV was the most divergent isolate. The homology of the MCP gene of different ranaviruses and the other members within the family *Iridoviridae* is presented in Table 3.

Based on the phylogenetic analysis (Fig. 1), PPIV and REV 282/I02 clustered together with FV3 and other

Table 3. Nucleotide sequence percent identity values based on MCP gene of viruses within the family *Iridoviridae*. *: sequences obtained in this study. Full names and accession numbers of previously published sequences as in legend to Fig. 1; other virus names are given in Table 1

	BIV*	BIV	EHNV	ECV*	ESV*	FV3*	FV3	SERV*	PPIV*	REV 208/I02*
BIV	99.9									
EHNV	97.9	97.9								
ECV*	97.2	97.1	99.0							
ESV*	97.2	97.1	99.0	100						
FV3*	98.8	98.8	97.9	97.4	97.4					
FV3	98.8	98.7	97.8	97.3	97.3	99.9				
SERV*	94.2	94.1	95.4	95.8	95.8	94.3	94.2			
PPIV*	98.3	98.2	98.7	98.1	98.1	98.3	98.3	94.6		
REV 282/I02*	98.5	98.4	98.9	98.3	98.3	98.4	98.3	94.7	99.4	
TFV	98.5	98.5	97.9	97.2	97.2	98.6	98.5	94.1	98.3	98.4
ATV	96.5	96.5	98.2	97.6	97.6	96.5	96.4	93.9	97.3	97.5
GIV	71.1	71.0	71.0	71.4	71.4	71.0	71.0	71.8	71.2	71.0
SGIV	70.5	70.5	70.6	71.0	71.0	70.5	70.4	71.5	70.8	70.6
ISKNV	54.8	54.7	55.5	55.4	55.4	55.3	55.2	55.6	55.4	55.4
RSIV	54.9	54.8	55.4	55.3	55.3	55.2	55.2	55.3	55.2	55.4
IIV-3	49.7	49.7	50.2	50.4	50.4	50.0	49.9	50.2	50.0	50.1
LCDV	51.4	51.3	50.9	51.1	51.1	51.1	51.1	51.1	50.8	51.1
TIV	48.2	48.1	48.1	48.1	48.1	48.3	48.3	48.2	48.2	48.2
CIV	47.9	47.8	47.4	47.4	47.4	47.7	47.7	47.5	47.6	47.8

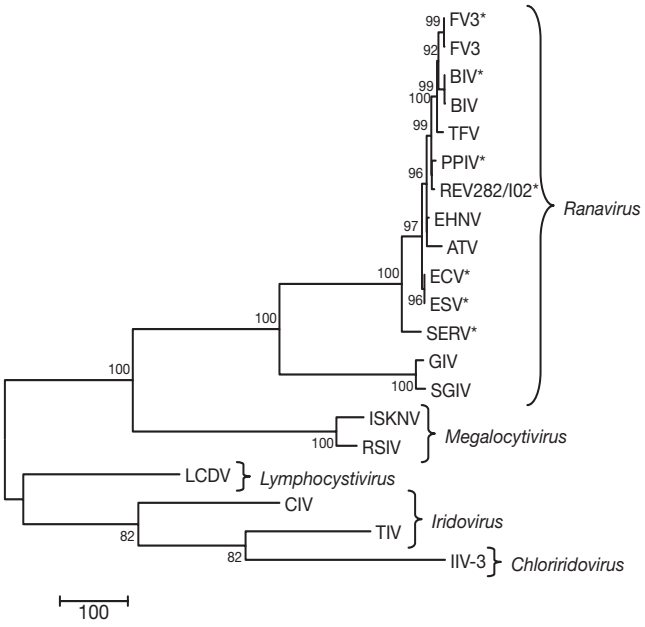

Fig. 1. Maximum parsimony analysis based on complete MCP gene sequences of members of the family *Iridoviridae*. *: sequences obtained in this study. Published sequences: BIV (AY187046), FV3 (AY548484), EHNV (AY187045), TFV (tiger frog virus, AF389451), ATV (*Ambystoma tigrinum* virus, NC_005832), GIV (grouper iridovirus, AY666015), SGIV (NC_006549), ISKNV (infectious spleen and kidney necrosis virus, NC_003494), RSIV (Red Sea bream iridovirus, AY310918), LCDV (*Lymphocystis* disease virus, AY380826), CIV (Chilo iridovirus, AF303741), TIV (Tipula iridescent virus, M33542), IIV-3 (invertebrate iridescent virus 3/*Aedes taeniorhynchus* iridescent virus, NC_008187). Other virus names are given in full in Table 1. Numbers at the nodes of the tree indicate bootstrap values of 1000 replicates; values under 70 are not shown. Scale bar indicates 100 nucleotide substitutions. Curly brackets indicate genera

ranaviruses originating from frogs. SERV was most related to ECV and ESV. EHNV was closely aligned to ATV. The analysed ranaviruses differ clearly from members of the genus *Megalocytivirus* (ISKNV and RSIV), *Chloriridovirus* (IIV-3/*Aedes taeniorhynchus* iridescent virus, *Lymphocystivirus* (LCDV) and *Iridovirus* (TIV and CIV).

The obtained MCP gene sequences were deposited in GenBank (BIV: FJ358613, ECV: FJ358608, ESV: FJ358609, FV3: FJ459783, PPIV: FJ358610, REV 282/I02: FJ358611, SERV: FJ358612).

DNA polymerase

Partial DNA polymerase gene was amplified from all 11 virus isolates. Based on the obtained sequences, the PCR products varied in size from 554 (DFV, GV6) to 560 bp (BIV, ECV, ESV, EHNV, FV3, SERV, PPIV, REV 282/I02 and SGIV). The overall sequence identity of analysed ranaviruses within the family *Iridoviridae* varied between 65.9 and 100% (Table 4). The obtained sequences of FV3 and SGIV were identical to the published sequences (Tan et al. 2004, AY548484; Song et al. 2004, NC_006549). The partial DNA polymerase sequences of ECV and ESV were identical to each other, as were those obtained from DFV and GV6. DFV, GV6 and SGIV were the most divergent isolates. In the phylogenetic analysis (Fig. 2), PPIV and REV 282/I02 were most closely related to FV3. SERV was closely positioned with ECV, EHNV, ESV and ATV. DFV and GV6 grouped apart from the other ranaviruses in a manner similar to SGIV and GIV. The partial DNA polymerase sequences were submitted to GenBank (BIV:

Table 4. Nucleotide sequence percent identity values based on the partial DNA polymerase gene of viruses within the family *Iridoviridae*. *: sequences obtained in this study. Full names and accession numbers of sequences as in legend to Fig. 1; other virus names are given in full in Table 1

	BIV*	EHNV*	ECV*	ESV*	FV3	SERV*	PPIV*	REV 208/I02*	DFV*	GV6*	SGIV
EHNV*	98.7										
ECV*	99.0	99.7									
ESV*	99.0	99.7	100								
FV3	99.1	99.0	99.3	99.3							
SERV*	96.8	97.2	97.5	97.5	97.1						
PPIV*	99.1	99.0	99.3	99.3	99.4	97.1					
REV 282/I02*	98.7	98.5	98.8	98.8	99.3	96.6	99.3				
DFV*	71.6	71.6	71.6	71.6	71.6	70.6	71.3	71.3			
GV6*	71.6	71.6	71.6	71.6	71.6	70.6	71.3	71.3	100		
SGIV	66.1	65.9	66.2	66.2	66.7	66.4	66.4	66.7	69.4	69.4	
TFV	98.8	99.0	99.3	99.3	99.1	96.8	99.1	98.7	71.3	71.3	66.1
ATV	98.5	99.3	99.6	99.6	98.8	97.4	98.8	98.4	71.3	71.3	66.4
GIV	66.5	66.4	66.7	66.7	67.1	66.8	66.8	67.1	69.7	69.7	98.7
ISKNV	50.1	49.9	50.1	50.1	49.9	49.9	49.9	49.7	49.6	49.6	49.1
IIV-3	30.3	30.3	30.3	30.3	30.1	30.4	30.6	30.3	28.1	28.1	30.1
LCDV	48.7	48.8	48.8	48.8	48.8	49.0	48.7	49.0	52.8	52.8	52.5
CIV	26.5	26.5	26.5	26.5	26.6	26.0	26.5	26.5	27.6	27.6	27.6

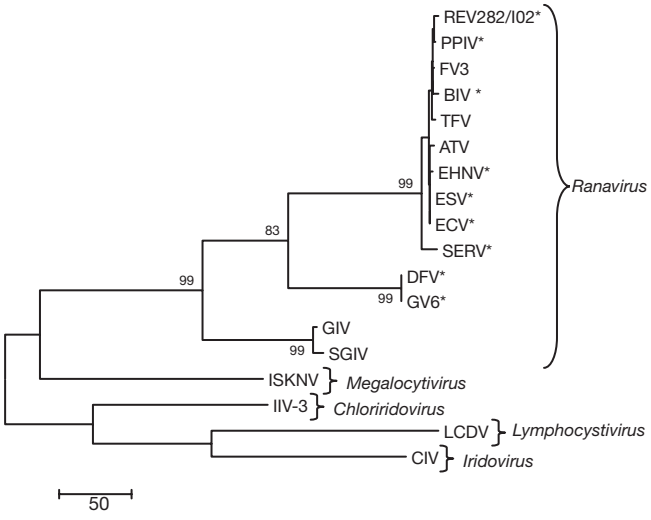

Fig. 2. Maximum parsimony analysis based on partial DNA polymerase gene sequences of members of the family *Iridoviridae*. *: sequences obtained in this study. Full names and accession numbers of published sequences as in legend to Fig. 1; other virus names are given in full in Table 1. Numbers at the nodes of the tree indicate bootstrap values of 1000 replicates; values under 70 are not shown. Scale bar indicates 50 nucleotide substitutions. Curly brackets indicate genera

FJ374280, ECV: FJ374277, EHNV: FJ374274, ESV: FJ374278, SERV: FJ374279, PPIV: FJ374276, REV 282/I02: FJ374275, DFV: FJ374281, GV6: FJ374282).

Neurofilament triplet H1-like protein (NF-H1)

Partial NF-H1 gene was amplified from all viruses with the exception of DFV, GV6 and SGIV. Based on the obtained sequences, the sizes of the NF-H1 PCR products varied depending on the different virus isolate: 714 (BIV), 597 (EHNV), 864 (ECV), 759 (ESV), 639 (FV3), 612 (PPIV), 579 (REV 282/I02) and 588 bp (SERV). This region contained a small repetitive sequence of 15 nucleotides in ECV and ESV (5′ CCA GCG AGA AAG TCT-3′, corresponding to amino acids PARKS). The analysed NF-H1 fragment of ECV contained 18, and that of ESV 11 of these consecutive repitions. Two and 3 similar short sequences were also found in FV3 and BIV, respectively. In addition, BIV had 3 succeeding repetitions of a 24-nucleotide sequence (5′-GGA GCG GAC TAC ATC TCT CAG GGA-3′, corresponding to amino acids GADYISQG) at the end of its partial NF-H1 sequence. The published sequence of TFV (He et al. 2002, AF389451) represented 6 repetitions of 12 nucleotides (5′-CCC AGA AAG TCT-3′, corresponding to amino acids PRKS) in the analysed NF-H1 fragment. The repeats in the NF-H1 region resulted in large gaps in the multiple sequence alignment of the analysed viruses. The first gap of more than 200 bp was caused by 15 repeat units of ECV, 8 of ESV and 4 of TFV. Another gap of 72 bp was caused by three 24-nucleotide repeat units of BIV. These gap-forming repeat units were excluded from the respective sequences in order to obtain alignment of sequences of similar length and to avoid overestimation of sequence differences. The same alignment was used both in calculations of sequence identity and in the phylogenetic analysis. The nucleotide identity between the isolates varied from 77.2 to 100% (Table 5). ECV and ESV were identical when the repetitive sequences were excluded. The obtained FV3 sequence was identical to the published one (Tan et al. 2004, AY548484). Based on the phylogenetic analysis, BIV, TFV and FV3 form a cluster, PPIV and REV 282/I02 group together and SERV lies apart from the other isolates (Fig. 3). The GenBank accession numbers for the obtained NF-H1 sequences are BIV: FJ391462, ECV: FJ391464, EHNV: FJ391466, ESV: FJ391465, SERV: FJ391463, PPIV: FJ391467 and REV 282/I02: FJ391461.

Differentiation of ranaviruses by REA

REA based on the DNA polymerase and NF-H1 fragments was performed in order to differentiate the

Table 5. Nucleotide sequence percent identity values based on the partial neurofilament triplet H1-like protein gene of ranaviruses. *: sequences obtained in this study. Full names and accession numbers of previously published sequences as in legend to Fig. 1; other virus names are given in full in Table 1. Repeat units of BIV, ECV, ESV and TFV were omitted from the analysis

	BIV*	EHNV*	ECV*	ESV*	FV3	SERV*	PPIV*	REV 282/I02*
EHNV*	83.0							
ECV*	87.8	91.0						
ESV*	87.8	91.0	100					
FV3	95.6	82.7	86.2	86.2				
SERV*	78.6	77.7	80.8	80.8	77.2			
PPIV*	93.2	88.6	84.2	84.2	93.3	78.6		
REV 282/I02*	87.5	83.0	78.8	78.8	86.8	79.9	93.2	
TFV	93.2	81.3	83.8	83.8	91.9	77.2	91.3	86.8

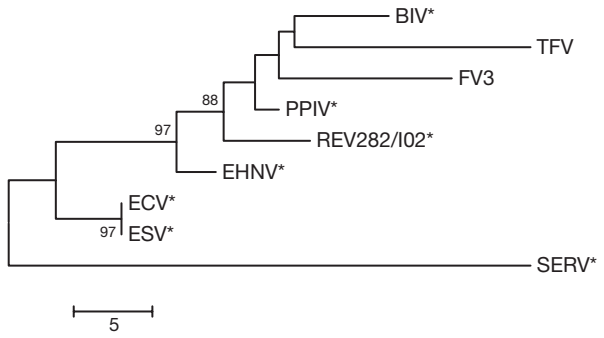

Fig. 3. Maximum parsimony analysis based on partial NF-H1 gene sequences of ranaviruses. *: sequences obtained in this study. Full names and accession numbers of published sequences as in legend to Fig. 1; other virus names are given in full in Table 1. Numbers at the nodes of the tree indicate bootstrap values of 1000 replicates; values under 70 are not shown. Scale bar indicates 5 nucleotide substitutions

analysed virus isolates. The DNA polymerase PCR products were digested with *Bpm*I, and NF-H1 PCR products were digested with *Afl*III, *Alu*I and *Hae*III. Based on the sequences obtained in this study, the predicted restriction fragments are presented in Table 6.

The *Bpm*I digestion of DNA polymerase PCR products produces an identical pattern for DFV and GV6 that differentiates them as a group from the other isolates (Fig. 4A). SERV and SGIV could also be differentiated with this enzyme. Further differentiation of BIV, EHNV, ECV, ESV, FV3, SERV, REV 282/I02 and PPIV was achieved by digesting NF-H1 PCR products with *Alu*I (Fig. 4B), *Hae*III (Fig. 4C) and *Afl*III (Fig. 4D). The *Alu*I digestion produces distinct patterns for BIV, ECV, ESV, SERV and REV 282/I02. Digestion with *Hae*III generates clearly dissimilar patterns for EHNV, FV3, BIV, ECV, ESV and SERV and with

Table 6. Restriction enzyme recognition sites and predicted fragment sizes after restriction of the DNA polymerase and NF-H1 PCR products. R = A/G, Y = C/T. Virus names are given in full in Table 1

PCR target	Restriction enzyme	Recognition site	Virus	Recognition sites in target sequence	Predicted fragment sizes after restriction (bp)
DNA polymerase	*Bpm*I	$CTGGAG(N)_{16}$	EHNV	310, 526	34, 216, 310
			FV3	310, 526	34, 216, 310
			BIV	310, 526	34, 216, 310
			ECV	310, 526	34, 216, 310
			ESV	310, 526	34, 216, 310
			REV 282/I02	310, 526	34, 216, 310
			PPIV	310, 526	34, 216, 310
			SERV	310	250, 310
			DFV	520	34, 520
			GV6	520	34, 520
			SGIV	–	560
NF-H1	*Alu*I	AGCT	EHNV	18, 287, 450	18, 147, 163, 269
			FV3	18, 302, 465	18, 163, 174, 284
			BIV	18, 305, 468	18, 163, 246, 287
			ECV	18, 554, 717	18, 147, 163, 536
			ESV	18, 449, 612	18, 147, 163, 431
			SERV	18, 88, 278, 441	18, 70, 147, 163, 190
			REV 282/I02	18, 242	18, 224, 337
			PPIV	18, 275, 438	18, 163, 174, 257
NF-H1	*Hae*III	GGCC	EHNV	237, 253, 474	16, 123, 221, 237
			FV3	249, 265	16, 249, 374
			BIV	252, 268, 492	16, 222, 224, 252
			ECV	501, 741	123, 240, 501
			ESV	396, 636	123, 240, 396
			SERV	222, 238, 357, 465	16, 108, 119, 123, 222
			REV 282/I02	211, 429	150, 211, 218
			PPIV	222, 238, 462	16, 150, 222, 224
NF-H1	*Afl*III	ACRYGT	EHNV	293, 527	70, 234, 293
			FV3	–	639
			BIV	644	70, 644
			ECV	560, 794	70, 234, 560
			ESV	455, 689	70, 234, 455
			SERV	–	588
			REV 282/I02	509	70, 509
			PPIV	542	70, 542

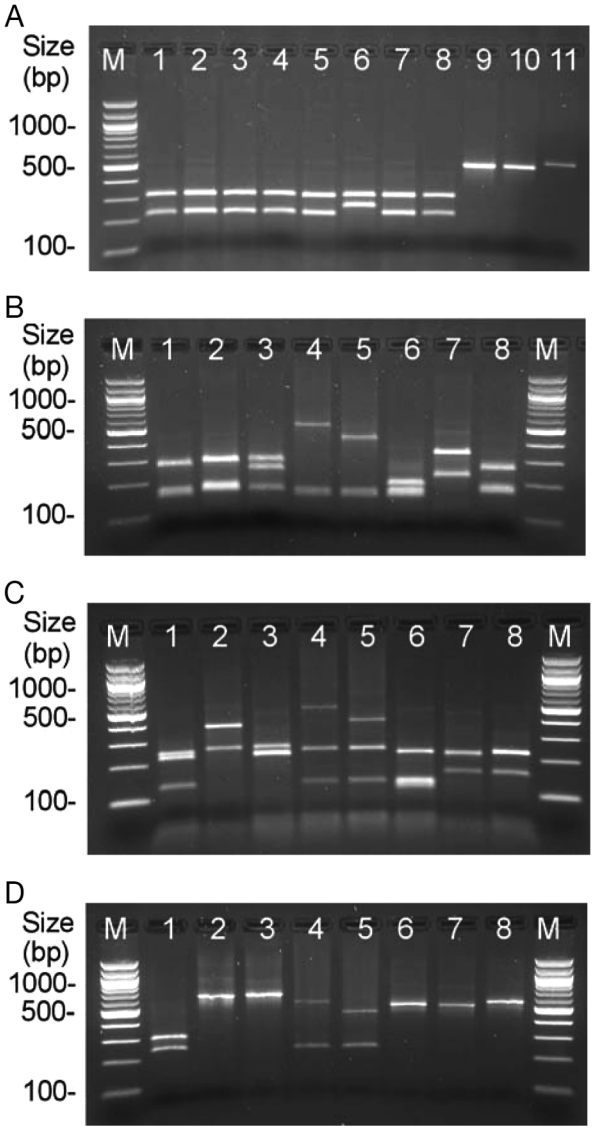

Fig. 4. Restriction enzyme analysis (REA) of ranaviruses. (A) *Bpm*I REA of DNA polymerase PCR products. (B) *Alu*I REA of NF-H1 PCR products. (C) *Hae*III REA of NF-H1 PCR products. (D) *Afl*III REA of NF-H1 PCR products. Lane contents as follows, M: size marker; 1: EHNV; 2: FV3; 3: BIV; 4: ECV; 5: ESV; 6: SERV; 7: REV 282/I02; 8: PPIV; 9: DFV; 10: GV6; 11: SGIV. Virus names given in full in Table 1

*Afl*III for EHNV, ECV and ESV. In order to differentiate PPIV, digestions with both *Alu*I and *Hae*III are required. Table 7 shows ways of distinguishing each virus isolate by the different restriction enzyme reactions.

DISCUSSION

In this study, sequence data of 3 gene regions (MCP, DNA-polymerase and NF-H1) were obtained from 3 new virus isolates, PPIV, REV 282/I02, and SERV. In addition, novel sequence data from other members of the genus *Ranavirus* were acquired.

The MCP gene has been commonly used to define ranavirus taxonomy and to differentiate between virus isolates, and the complete MCP sequences of some of the isolates included in this study are already known (BIV, EHNV: Marsh et al. 2002, FV3: Mao et al. 1996, SGIV: Song et al. 2004). In addition to the previously published data on ECV and ESV (Hyatt et al. 2000), we determined complete MCP gene sequence from these isolates.

Differences of 1 nucleotide were detected both in the BIV and FV3 MCP gene compared to the published sequences. A possible reason for this discrepancy could be mutation of the virus isolate during cell culture passage. Another possibility is that the original isolate consisted of genetic variants, which after selection in cell culture could result in differing subpopulations. Similarly, small sequence differences in the MCP gene of DFV and GV6 in 2 separate studies could also explain why no PCR products were acquired in this study, contrary to results published by Hyatt et al. (2000).

Based on the MCP and DNA polymerase sequences, the identity of BIV, ECV, EHNV, ESV, FV3, PPIV, REV 282/I02 and SERV varied between 94.1 and 100% (Tables 3 & 4). The differences between isolates were larger, based on the NF-H1 region, even after the gap-forming short repetitive sequences of BIV, ECV, ESV and TFV were omitted from the alignment. The repeat units represent a problem with respect to finding the ideal multiple sequence alignment, especially when

Table 7. Ranavirus differentiation by REA of DNA polymerase and NF-H1 PCR products. U: a unique restriction pattern was obtained with the respective enzyme. C: a combination of *Alu*I and *Hae*III digestion of NF-H1 PCR products is needed for differentiating PPIV. (–): virus cannot be differentiated with respective enzyme. DFV and GV6 have an identical pattern. Virus names given in full in Table 1

PCR target	Enzyme	EHNV	FV3	BIV	ECV	ESV	SERV	REV282/I02	PPIV	DFV&GV6	SGIV
DNA polymerase	*Bpm*I	–	–	–	–	–	U	–	–	U	U
NF-H1	*Alu*I	–	–	U	U	U	U	U	C	–	–
	*Hae*III	U	U	U	U	U	U	–	C	–	–
	*Afl*III	U	–	–	U	U	–	–	–	–	–

some sequences contain a different number of repeats than others. It is not obvious how to align the repeat units, and one solution is to exclude them (Higgins 2003). The function of the short repeats within the partial NF-H1 region is unknown. In a genomic analysis of iridoviruses, Jancovich et al. (2003) found numerous 14 bp repeat sequences scattered throughout the genome of ATV. Repeat regions and palindromes appear to be common in iridovirus genomes. Their role could be related to the viral transcription or homologous recombination that has been observed in iridoviruses (Williams et al. 2005). In the phylogenetic analysis (Figs. 1 to 3), REV 282/I02 and PPIV grouped together with FV3 and other ranaviruses originating from frogs. SERV was the most distinct isolate among BIV, ECV, EHNV, ESV, FV3, PPIV and REV 282/I02. The sequence data obtained in this study suggest that PPIV, REV 282/I02 and SERV are new members of the genus *Ranavirus*.

The current ranavirus classification is based on comparisons at the genomic level, restriction enzyme profiles, virus protein profiles and host-specificity (Chinchar et al. 2005). Ranaviruses share at least 1 common antigen, as polyclonal antibodies against EHNV detect many of the viruses (Hedrick et al. 1992, Hengstberger et al. 1993, Hedrick & McDowell 1995, Ahne et al. 1998, Hyatt et al. 2000). In previous studies, different ranaviruses showed a high degree of similarity in the MCP and other genomic regions (Mao et al. 1997, Hyatt et al. 2000, Marsh et al. 2002). Due to the genetic similarity of ranaviruses, many known isolates could be considered members of the same species. However, classification of members of the genus *Ranavirus* into different species has been favoured, as ranaviruses do have clearly different hosts and geographical ranges (Hyatt et al. 2000, Chinchar 2002, Marsh et al. 2002, Chinchar et al. 2005, Williams et al. 2005). Similarly, in our study, PPIV and REV 282/I02 shared more than 98% homology in the MCP and DNA polymerase genes with BIV, ECV, EHNV, ESV and FV3, and therefore, they all could be argued to belong to the same species. Further knowledge on host specificity will be necessary to define the classification of these isolates.

The results of the phylogenetic positioning of DFV, GV6 and SGIV presented here are in agreement with previous studies, which indicate that these isolates form a separate group apart from the other ranaviruses (Mao et al. 1997, 1999a, Hyatt et al. 2000, Qin et al. 2003, Song et al. 2004, Eaton et al. 2007).

As knowledge about the host range of different ranaviruses accumulates, differentiation between virus isolates becomes increasingly important. Several approaches using REA of viral genomic DNA have been used in the characterisation and differentiation of ranaviruses (Hengstberger et al. 1993, Mao et al. 1997, 1999a,b, Ahne et al. 1998, Hyatt et al. 2000). Marsh et al. (2002) introduced a REA method based on the MCP gene for differentiation of ranaviruses from fish and amphibian hosts and different geographical regions. This method is currently recommended by the OIE (Whittington & Hyatt 2006). Pallister et al. (2007) developed a real-time PCR method to differentiate between Australian and European ranavirus isolates.

The new REA assays developed in this study provide new means of differentiating ranaviruses. The REA of the partial DNA polymerase gene allowed us to group DFV and GV6, along with SGIV, apart from the other viruses. The differentiation of the more closely related ranaviruses was accomplished using the REA of the NF-H1 region. Even ECV and ESV, which represent very close variants of the same virus (Mao et al. 1997, Ahne et al. 1998, Hyatt et al. 2000, Pallister et al. 2007), were separated from each other.

Several distinguishable ranavirus isolates are present in most geographical regions of the world (Williams et al. 2005). Some ranaviruses have a broad host range and can infect both fish and amphibians (Moody & Owens 1994, Mao et al. 1999a). The presence of multiple virus isolates with different pathogenicity in the environment of known and unknown hosts creates a need for specific differentiation of ranaviruses. Differentiation of viruses by REA of the NF-H1 region could be useful in epidemiological studies where several closely related virus isolates are present in the same environment.

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