Aquaculture 509 (2019) 23–31

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Complete genome of Atlantic halibut reovirus (AHRV) associated with mortality in production of Atlantic halibut (*Hippoglossus hippoglossus*) fry

Renate Hvidsten Skoge*, Are Nylund, Kjetil Solheim, Heidrun Plarre

University of Bergen, Department of Biological Sciences, Fish Disease Research Group, 5020 Bergen, Norway

A B S T R A C T

The *Aquareovirus* (AQRV) member Atlantic halibut reovirus (AHRV) is associated with severe liver pathology and high fry mortality and constitutes a significant problem for Atlantic halibut (*Hippoglossus hippoglossus*) production in Norway. Halibut is a batch spawner and it has been suspected that AHRV may be transmitted via eggs since outbreaks of disease arise in successive batches of fry originating from the same group of brood fish. In this study, we present the complete genome of AHRV representing the first complete AQRV genome sequence from a marine cold-water fish species in the North Atlantic. The terminal 5′- and 3′-ends of the segments have the canonical conserved nucleotides 5′-GUUUUAU————UCAUC-3′. The 13 putative proteins encoded in the 11 AHRV genome segments share the highest amino acid identity with members of species AQRV A and B. Phylogenetic analysis of the most conserved proteins (VP1, VP2, VP3 and VP5) groups AHRV in a major clade together with the same two species. However, the differences in host and environment, and the amino acid sequence identity of the RdRp (~80%) compared to either AQRV A or B, suggest that this virus could possibly represent a novel species within the genus. Furthermore, we show that even though AHRV RNA cannot be detected by real time RT PCR in the first egg batches from asymptomatic Atlantic halibut brood fish, the viral RNA load is not only detectable, but appears to increase in successive spawning batches.

1. Introduction

Genus *Aquareovirus* (AQRV), family *Reoviridae*, contain seven assigned species (AQRV A – G) and a number of species yet to be approved. They have genomes consisting of 11 dsRNA segments, and have been isolated from mollusks and a wide variety of fish species (Hedrick et al., 1984; Winton et al., 1987; Attoui et al., 2002; Zhang et al., 2010; Ke et al., 2011; Wang et al., 2012; Ye et al., 2012; Fan et al., 2013; Pei et al., 2014; Yan et al., 2014; Schachner et al., 2014; Blindheim et al., 2015; Chen et al., 2015; Iwanowicz et al., 2016; Wu et al., 2016; Makhsous et al., 2017; Zainathan et al., 2017; Lupiani et al., 1995; Jaafar et al., 2008; Xu et al., 2013; Seng et al., 2005b; Seng et al., 2005a). Identification of species belonging to the Aquareovirus genus have traditionally been based on many factors such as number of genome segments, host range and disease symptoms, virion morphology, cross-hybridization, electropherotype analysis, serological comparison, ability to reassort during mixed infections, conserved terminal sequences and RNA and amino acid sequence analysis (King et al., 2012). During the last decade more emphasis has been put on analysis of the complete genome with a major emphasis on the RNA-dependent RNA polymerase (RdRp) gene (Attoui et al., 2002; Ke et al., 2011; Ye et al., 2012; Wang et al., 2012; Fan et al., 2013; Nibert and Duncan, 2013; Makhsous et al., 2017; Jaafar et al., 2008). Species segregation is recommended when the RdRp sequence has < 74% amino acid identity while sequence identity > 95% identity warrants species integration (King et al., 2012). However, there is considerable uncertainty and variability for delineation between species when the RdRp sequence identity is between 74 and 95%. In these instances, additional factors should still be considered.

Reovirus-associated mortality has been reported to take place in commercial fry production of Atlantic halibut (*Hippoglossus hippoglossus*) in Canada, Scotland and Norway (Cusack et al., 2001; Ferguson et al., 2003; Blindheim et al., 2015). The disease has been a dominating problem in halibut fry production in Norway the last 10 years. The virus, Atlantic halibut reovirus (AHRV), was identified in 2014 as a putative new AQRV species, subfamily *Spinareovirinae*, based on the nearly complete sequence of the RdRp gene. Phylogenetic analysis using the RdRp showed that AHRV were closest related to AQRV A and the unassigned turbot *Scophthalmus maximus* reovirus (SMReV) (Blindheim et al., 2015).

Atlantic halibut are batch spawners and up to 10 batches can be obtained from each fish during spawning in commercial hatcheries. Horizontal transmission of AHRV and long distance spreading via movement of infected fry has already been observed (Blindheim et al., 2015, A. Nylund Pers. obs.). However, disease outbreaks occur in successive batches of fry from the same brood stock population. During one of these outbreaks at a brood fish and hatchery site in 2013, the inlet water and live feed *Artemia* nauplii were tested and found to be

* Corresponding author.
E-mail address: Renate.skoge@uib.no (R.H. Skoge).

https://doi.org/10.1016/j.aquaculture.2019.04.080
Received 7 December 2018; Received in revised form 26 April 2019; Accepted 30 April 2019
Available online 07 May 2019

negative for AHRV (Blindheim et al., 2015). Since AHRV is present in several organs of infected halibut (Blindheim et al., 2015), the virus may pass from the liver and pancreas through the intestines and into the hindgut, or from the kidney via the urine to the hindgut. Thus, eggs may be contaminated from faeces or urine as they pass the anus. Given the repeated disease outbreaks, we therefore hypothesized that AHRV also can be transmitted vertically from asymptomatic Atlantic halibut brood fish to eggs.

In the present study, the complete genome of the Atlantic halibut reovirus is presented and the taxonomic position is rediscussed with respect to other characterized species of aquareoviruses. In addition, a new real time RT PCR assay was used to detect virus transmission from brood fish to offspring via eggs.

2. Material and methods

2.1. Cell culture

AHRV was cultured in CHSE-214 cells from homogenates of Atlantic halibut larvae suffering from mortalities at two different production sites in Norway as described previously (Blindheim et al., 2015).

2.2. Sampling of eggs from halibut brood fish

Atlantic halibut is a batch spawner and in commercial production each female may provide as much as 10 separate batches. The eggs from 25 females, that had contributed eggs to tanks with AHRV positive larvae, were tested by real time RT PCR. Eggs were transferred using a disposable pipette from a collection jug into a 2.0 ml Eppendorf tube, and then stored at −20 °C. Ten egg batches were collected from each female. The eggs were treated with the anti-fungal agent Pyceze (30 min, 50 mg/l) approx. 60 degree days after fertilization. The eggs were also tested for presence of two other viruses that have been associated with fry mortalities in halibut production, i.e. Infectious pancreas necrosis virus (IPNV, *Birnaviridae*) and Nervous necrosis virus (NNV, *Nodaviridae*).

2.3. RNA extraction

RNA was extracted from infected CHSE-214 cells and halibut eggs as described previously (Steigen et al., 2013), except that RNase free water heated to 70 °C was added to the RNA pellet, and the RNA samples were stored at −20 °C. RNA from infected CHSE-214 cells was used for Illumina sequencing, RT PCR, and Sanger sequencing (Big Dye v3.1).

2.4. Illumina sequencing

RNA from AHRV-infected CHSE-214 cells was sent to BaseClear (BaseClear Group, Netherlands) for Illumina sequencing (Illumina Casava pipeline version 1.8.3) and assembly (Økland et al., 2014). Paired-end sequences reads were generated using the Illumina HiSeq 2500 system. The sequences were obtained from a single extraction from cultured AHRV. Paired-end reads that could not be mapped to the Atlantic halibut genome were subsequently assembled into scaffold sequences. The scaffold sequences were filtered in three steps: a) removal of scaffolds with no open reading frame (ORF), b) removal of scaffolds with stop-codons in all six-frames for scaffolds below 200 bp, and c) removal of scaffolds with a BLAST hit against vertebrate organisms (threshold e-value = 0.01). This resulted in 19,768 sequences with an average sequence size of 186 bp and a total sum of 3,685,571 bp. Selected sequences were translated using ExPASy's online translation tool (http://web.expasy.org/translate/) and the BLASTP algorithm was used to identify similar sequences in the BLAST suite databases. Sequences matching all 11 segments from genus *Aquareovirus* were identified. These sequences were used as templates for production of primers used to confirm these virus sequences through Sanger sequencing and for sequencing of the termini of the eleven segments.

2.5. Sequencing of the genome termini

dsRNA was purified from supernatant of AHRV-infected CHSE-214 cells using the Pure-link viral RNA/DNA kit (ThermoFisher) according to the manufacturer's recommendation. To allow sequencing of the termini of the AHRV genome segments, a DNA adapter-primer was ligated to both 3′-ends of the genome segments prior to cDNA synthesis. The adapter-primer (5′-Ph-CATCCACTAGTTCTAGAGCGGC-3′ddC; adapted from (Coutts and Livieratos, 2003)) was first adenylated using a 5′-DNA adenylation kit (NEB, E2610). The adenylated adapter-primer (5 pmol) was then ligated to 10 µl of purified dsRNA in a reaction containing 200 U T4 RNA ligase 2 truncated K227Q (NEB, M031), 40 U RNaseOUT (Invitrogen), 12.5% PEG8000, and 2 µl T4 RNA ligase 2 truncated K227Q reaction buffer, in a total of 20 µl. After incubation at 16 °C overnight, the reaction was stopped by incubation at 65 °C for 20 min. Adapter-ligated dsRNA was further denatured by boiling (95 °C) for 5 min with 15% DMSO, and cooled down on ice. cDNA synthesis was performed using SuperScript® III First-strand synthesis system for RT-PCR (Invitrogen), starting with 8 µl denatured adapter-ligated dsRNA and using a primer with the reverse complement sequence of the adapter-primer (revcomp primer: 5′-GCCGCTCTAGAAC TAGTGGATG). The reaction was performed according to the standard protocol, except that the reverse transcription reaction was incubated at 55 °C instead of 50 °C. The cDNA (1–10% of final volume) was used directly in PCR amplification (Phusion Green Hot Start II High-Fidelity PCR Master Mix, ThermoFisher) of the segment termini using the revcomp primer and a segment- and orientation-specific primer. Following a semi-nested PCR amplification using the revcomp primer and an internal segment- and orientation specific nested primer, the resulting amplicons were either gel-purified (QIAquick Gel Extraction Kit, Qiagen) or purified directly in solution (QIAquick PCR purification Kit, Qiagen). Some of the segments' termini were difficult to amplify. For these termini, forced primers were used instead of the revcomp primer, either in the first and/or nested PCR. These primers partially overlapped the adapter primer sequence, but provided a forced 5-′end of 5′-GTTTTA…-3′ or a forced 3′-end of 5′-…TCATC-3′, corresponding to the conserved terminal sequences (5′-forced primer: 5′-GCTCTAGAACTAG TGGATGGTTTTA and 3′-forced primer: 5′-GCTCTAGAACTAGTGGATG GATGA; the underlined sequences denote the incorporated forced ends). An overview of fully and forced sequenced termini can be seen in Table 2. Finally, the purified DNA was Sanger sequenced using the segment and orientation-specific primers.

2.6. Identification of protein coding genes

BLAST searches (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to find matching nucleotide and amino acids sequences to those present in the genome of AHRV. The Compute pI/Mw tool in ExPASy was used to calculate the theoretical pI (isoelectric point) and Mw (molecular mass) of the putative proteins coded by the ORFs in the genome of AHRV. In addition, searches for conserved domains within the proteins were done using Simple Modular Architecture Research Tool (http://smart.embl-heidelberg.de/) and MOTIF search (http://www.genome.jp/tools/motif/).

2.7. Phylogeny

After preliminary identification of sequences related to the AHRV segment sequences by BLAST searches, the Vector NTI Suite software package was used to obtain multiple alignments. To perform pairwise comparisons of AHRV, the multiple sequence alignment editor GeneDoc (available at: www.psc.edu/biomed/genedoc) was used for manual adjustment of the sequence alignments. Selected sequences from

members of the genera *Aquareovirus* and *Orthoreovirus*, already available on the EMBL nucleotide database, were included in the alignments. This resulted in sequence alignments of 1295, 1266, 1203 and 652 amino acids for the most conservative proteins VP1, VP2, VP3 and VP5 respectively. Phylogenetic relationships were determined using the maximum-likelihood (ML) method available in TREE_PUZZLE 5.2 (available at: http://www.tree-puzzle.de), employing the JTT model (VP1 and VP2) (Jones et al., 1992), VT model (VP3) (Muller and Vingron, 2000), and WAG model (VP5) (Whelan and Goldman, 2001) of amino acid substitution. Quartet puzzling was used to choose from the possible tree topologies and to simultaneously infer support values for internal branches. Quartet trees are based on approximate maximum likelihood values using the selected model of substitution. The robustness of each node was determined using 20,000 puzzling steps. Phylogenetic trees were drawn using TreeView (Page, 1996).

2.8. Real time RT PCR

A new real time RT PCR assay targeting the putative outer capsid protein gene (VP7) on AHRV segment eleven (AHRV-S11-F: GCTTTAT GCGACGCTCTCACT, AHRV-S11-probe: ATTTGTATATGCCCGG, AHRV-S11-R: GCCCCATTGTGATCCAGTTT) was developed for this study. The assay was used to screen egg batches from halibut brood fish. An assay targeting the elongation factor 1 alpha (EF1A1) from Atlantic halibut was used as internal control (Øvergård et al., 2010). The assays targeting Infectious pancreas necrosis virus (IPNV, *Birnaviridae*) and Nervous necrosis virus (NNV, *Nodaviridae*) have been published previously (Nylund et al., 2011; Korsnes et al., 2005).

The AgPath-ID™One step RT-PCR kit was used in this study and the real-time RT-PCR analyses were run by the Applied Biosystems 7500 Fast Real-Time PCR System. The efficacy test of the AHRV-S11 assay was performed according to the recommendations for a Standard AgPath-IDTM One-Step RT-PCR Kit (Life Technologies). The tests of the primers and probe were conducted in triplicates. The assay efficacy (E) were calculated using the following formula; $E = (10^{-1/slope})$ (Pfaffl, 2004). The regression line had a slope of −3.3018 and the efficiency (E) was calculated to be 2.00846.

3. Results

The complete genome sequence was determined for the isolate AHRV-241013 (Accession numbers: MH108635 - MH108645). The genome consists of 11 segments with a total size of 24,171 bp and a GC content ranging from 47.8% to 52.7% (percentages refer to segment means, Table 1). Each genome segment contains one ORF, except for segments seven and ten that encode two putative proteins. An overview of the different genome segments and the putative proteins they encode is presented in Table 1. The non-coding regions of the 11 segments range from 5 to 64 nucleotides at the 5′-end and from 33 to 147 nucleotides at the 3′-end, and the non-forced terminal non-coding 5′- and 3′-ends have seven and five conserved nucleotides (5′-GUUUUAU…UCAUC-3′), respectively (Table 2).

Table 2
5′ and 3′ end non-coding regions and terminal sequences. Underlined: incorporated in primer sequence (“forced” ends).*: sequence 5′-GTTTTA was incorporated in primer sequence.

	5′ NCR		3′ NCR	
Segments	Length (bp)	Terminal sequences	Terminal sequences	Length (bp)
1 (VP1)	16	GTTTTAT—	—TCATC	45
2 (VP2)	14	GTTTTAT—	—TCATC	33
3 (VP3)	21	GTTTTAT—	—TCATC	38
4 (NS1)	21	GTTTTAT—	—TCATC	147
5 (VP4)	19	GTTTTAT—	—TCATC	34
6 (VP5)	28	GTTTTAT—	—TCATC	68
7 (NS2/NS3)	13	GGTTTAA—*	—TCATC	67
8 (VP6)	12	GTTTTAT—	—TCATC	55
9 (NS4)	5	GTTTTAT—	—TCATC	43
10 (NS5/NS6)	64	GTTTTAT—	—TCATC	47
11 (VP7)	25	GTTTTAT—	—TCATC	62
Consensus		GG/TTTTAT/A—	—TCATC	

3.1. Proteins encoded by segments S1-S3

The three largest segments (1–3) from AHRV encode one protein each, and based on matches with other aquareoviruses they are named VP1, VP2 and VP3, respectively (Table 1). VP1 share sequence identity with the core-spike protein from other aquareoviruses, i.e. a protein that function as an mRNA capping enzyme (Tables 1 & 3). Four conserved amino acids were present in the N-terminal part of VP1: Lysine L_{175} and L_{198}, and histidine H_{229} and H_{238}. BLASTn and SMART sequence analysis of VP2 gave a significant match for the ORF in AHRV segment two with the RNA-dependent RNA polymerase (RdRp; λ-2) from several aqua- and orthoreoviruses (Table 3). The putative AHRV RdRp shows the highest amino acid identity (80.5%) with the unclassified turbot reovirus (SMReV), a possible member of AQRV A, closely followed by the two AQRV A members *Etheostoma fonticola* aquareovirus (EFAV; 80.4%) and *Micropterus salmoides* reovirus (MsReV; 80.3%), and the AQRV B member Fall chinook aquareovirus (GSH1; 79.1%). VP3 share amino acid identity with a putative NTPase/RNA helicase core protein and contains a zinc finger domain, $_{113}$LKCNQCGAEFSSMSQLSEHIRTEH$_{136}$ (Tables 1 & 3).

Table 1
Characteristics of the 11 genome segments and predicted proteins functions in AHRV.

Genome	Accession	Gene			Putative proteins				Predicted function
segment	No.	Length bp	GC %	ORF	protein	Length aa	Mw (KDa)	pI	
Seg-1	MH108635	3946	50.1	3885	VP1	1295	141.4	6.30	core-spike protein λ-2, capping enzyme
Seg-2	MH108636	3871	51.0	3825	VP2	1275	141.2	8.03	RNA-dependent RNA polymerase, λ-3
Seg-3	MH108637	3692	52.3	3663	VP3	1211	131.7	6.14	inner capsid protein λ-1,RNA helicase, NTPase
Seg-4	MH108638	2556	52.7	2388	NS87	796	86.5	5.65	Nonstructural protein, formation of viral inclusion bodies
Seg-5	MH108639	2240	50.0	2187	VP4	729	81.3	7.27	NTPase, minor core protein,
Seg-6	MH108640	2052	47.8	1956	VP5	652	69.1	5.20	Outer capsid, major virion structural protein
Seg-7	MH108641	1370	51.3	564	NS21	188	21.2	8.55	Fusion-associated small transmembrane protein, FAST protein
				798	NS30	266	30.4	5.22	Nonstructural protein
Seg-8	MH108641	1309	50.4	1242	VP6	414	45.7	7.17	Core protein
Seg-9	MH108643	1122	52.3	1074	NS40	358	38.9	6.10	NS protein, involved in the formation of viral inclusion bodies
Seg-10	MH108644	1035	51.1	924	NS34	308	34.0	6.19	Nonstructural protein
				813	HP30	271	30.2	5.66	Hypothetical protein
Seg-11	MH108645	978	51.0	981	VP7	297	31.7	8.28	Outer capsid protein

Table 3
Amino acid sequence identity of the putative proteins encoded by the 11 segments in the genome of AHRV (isolate AHRV-241013) compared to the closest relatives present in the GenBank. GRCV (Green River chinook virus), GSH1 (Fall chinook aquareovirus), MsReV (*Micropterus salmoides* reovirus), EFAV (*Etheostoma fonticola* aquareovirus), SMReV (*Scophthalmus maximus* reovirus), CSRV (Chum salmon reovirus), GSRV (Golden shiner reovirus) and American grass carp reovirus (AGCRV). np = not present, nm = no match, and - = complete sequence not available.

AHRV		AQRV B			AQRV A					AQRV C		AQRV G	
Segment	Protein	Seg	GRCV	GSH1	Seg	MsReV	EFAV	SMReV	CSRV	Seg.	GSRV	Seg.	AGCRV
S1	VP1	S1	68.9	62.5	S1	60.5	60.6	59.3	59.8	S1	43.9	S1	45.0
S2	VP2	S2	–	79.1	S2	80.3	80.4	80.5	76.9	S2	57.6	S2	56.7
S3	VP3	S3	80.3	76.6	S3	74.4	74.3	73.3	66.1	S3	52.3	S3	51.8
S4	NS87	S4	–	31.9	S4	37.3	34.3	34.5	–	S4	23.4	S4	25.1
S5	VP4	S5	67.5	57.3	S5	59.5	59.1	59.9	–	S5	35.9	S5	36.6
S6	VP5	S6	72.5	73.2	S6	69.8	73.2	68.9	64.4	S6	54.6	S6	54.8
S7	NS21	S7	42.6	43.1	S7	46.8	45.2	47.3	39.9[a]	S7	21.8	S7	22.3
S7	NS30	S7	49.2	42.5	S7	37.6	38.0	37.2	28.2[a]	S7	28.2[a]	S7	24.8
S8	VP6	S8	65.5	59.7	S8	59.4	58.7	58.2	58.5	S8	42.3	S8	41.8
S9	NS40	S9	66.2	52.8	S9	58.9	57.8	57.8	56.4	S9	39.4	S9	38.3
S10	NS34	S11	–	nm	S11	nm	nm	nm	nm	S11	16.6	S11	15.3
S10	HP30	–	–	np	–	np	np	np	np	–	np	–	np
S11	VP7	S10	28.6[a]	47.1	S10	34.7	35.7	36.0	33.7		15.3[a]		15.8

[a] Partial sequence.

3.2. Proteins encoded by segments S4-S6

The putative proteins encoded by segments 4, 5 and 6 have been named NS87, VP4 and VP5 based on comparison with other AQRV proteins. The putative AHRV NS87 protein has one coiled-coil region at amino acid positions 570–611 and shows the highest amino acid identity (37.3%) to segment four from EFAV (Table 3). NS proteins are believed to be involved in the formation of viral inclusion bodies (Table 1). BLASTp search using the amino acid sequence encoded in segment five give a significant match to VP4 from other aquareoviruses, i.e. a protein believed to be a minor core proteins with NTPase function, sharing the highest amino acid identity (67.5%) with the AQRV B member Green River chinook virus (Table 3). MOTIF search using the amino acid sequences of the putative VP4 from AHRV give a match to the reovirus core protein μ-2 that is thought to play a role in the formation and structural organization of reovirus inclusion bodies. BLASTp search using the amino acid sequence encoded in segment six give a significant match to VP5, a major virion structural protein (outer capsid) that play a role in host cell membrane penetration in other aquareoviruses (Tables 1 & 3). VP5 from AHRV show the highest amino acid identity to putative members of species AQRV A (EFAV, 73.2%) and B (GSH1, 73.2%). A putative cleavage site, $_{35}$DLKPGVL**NP**NGKL$_{47}$, found in other reoviruses and all sequenced aquareoviruses, is also present in AHRV VP5. This cleavage site is located between the asparagine and proline residues (position 42–43), and the amino acids flanking this site is conserved in AQRV A (Threadfin reovirus (TFV), CSRV, EFAV, SMReV and MsReV), and AQRV B (GSH1).

3.3. Proteins encoded by segments S7

Segment seven encodes two putative proteins (named NS21 and NS30) in overlapping open reading frames where the first (NS21) show a moderate identity of 21.8% (GSRV, AQRV C) and 47.3% (SMReV, AQRV A) to FAST proteins from other aquareoviruses (Table 3). A putative transmembrane region, $_{37}$WALPPLCICCCCLVCTGLGIYAI$_{59}$, was detected in NS21 using SMART search. NS30 shows an amino acid identity between 37.2% (SMReV) to 49.2% (GRCV) to non-structural proteins from species AQRV A and B (Table 3). The functions of these proteins are unknown.

3.4. Proteins encoded by segments S8–S11

BLASTp search using the putative protein encoded by segment eight from AHRV gives a match with a core protein, VP6, from aquareoviruses. VP6 from AHRV shows the highest amino acid identity (65.5%) to AQRV B (GRCV) (Table 3). SMART search of the ORF of segment nine from AHRV shows that this putative NS protein (NS40) exhibits ssRNA-binding activity. The NS40 protein from AHRV shows the highest amino acid identity to members of AQRV A (59.4% to MsRV) and B (66.2% to GRCV).

Segment 10 from AHRV encodes two putative proteins, NS34 (ORF1) and a hypothetical protein, HP30 (ORF2), where the second reading frame is inside the first. SMART search did not reveal any known motifs in NS34, while BLASTp showed a match between NS34 from AHRV and non-structural proteins from AQRV G and C with an amino acid identity ranging from 15.3% to 16.6%, respectively (Table 3). BLASTp search using the amino acid sequences of HP30 (ORF2) did not give a match with any known protein, but SMART search indicates a transmembrane region, $_{48}$LIWMLSGMILLLVATLILVIHFL$_{70}$, and a potential membrane-associated zinc ribbon motif in the N-terminal part. BLASTp and SMART search identified the putative protein encoded by segment 11 in AHRV as a major capsid protein (VP7) from aquareoviruses, and the highest identities are found among putative members of AQRV A (SMReV, 36.0%) and B (GSH1, 47.1%) (Table 3).

3.5. Phylogenetic position of AHRV

All putative proteins sequences encoded in the genome of AHRV, matching (> 50.0% identity) similar proteins from other aquareoviruses, were used for construction of phylogenetic trees. All trees show that AHRV belongs within the genus *Aquareovirus* (not presented). Fall chinook aquareovirus (GSH1) and Green river aquareovirus (GRCV), both presumed to belong to species AQRV B, are the closest relatives in the analyses of the majority of the putative protein sequences from AHRV. Analysis of VP1 places AHRV as a sister group to GRCV (AQRV B) and GSH1, with AQRV A as a slightly more distant relative (Fig. 1). The amino acid identity ranges from 59.3% (SMReV, AQRV A) to 68.9% (GRCV, AQRV B) (Table 3). The analysis of VP2 groups AHRV closer to AQRV A with GSH1 (putative AQRV B) as a slightly more distant relative, and the amino acids identities ranges from 76.9% (CSRV, AQRV A) and 79.1 (GSH1, AQRV B) to 80.5 (SMReV, AQRV A) (Fig. 2, Table 3). The AHRV VP3 does also show a relatively high amino acid identity to members of AQRV A (MsReV, 74.4%) and AQRV B (GRCV, 80.3%) (Table 3). Phylogenetic analysis of VP3 groups GSH1 (putative AQRV B) as a sister group to two other clades; AQRV A and a clade consisting of AQRV B and AHRV (Fig. 3). In the phylogenetic analysis of the putative protein from segment six, VP5

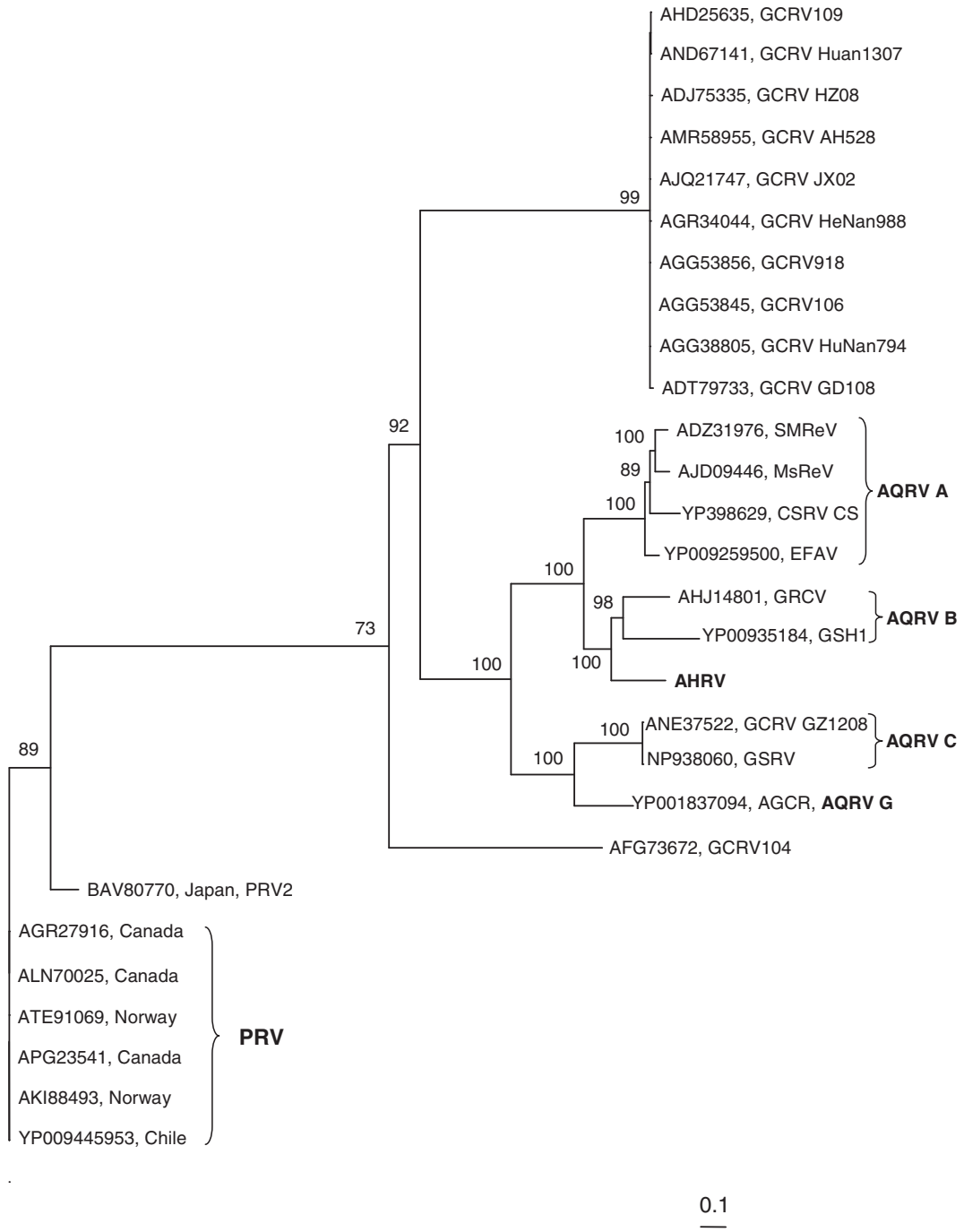

Fig. 1. The phylogenetic position of AHRV based on analysis of the putative protein, VP1 (core spike protein, capping enzyme), encoded on segment one, compared to the closest members within genus *Aquareovirus* using fish orthoreoviruses (PRV and PRV2) as outgroup. Four described species of *Aquareovirus* (AQRV A, B, C and G), several related grass carp reoviruses (GCRV), and a unique GCRV (104), are included. The support values are frequencies (%) at which a given branch appeared in 20,000 bootstrap replications.

(outer capsid protein), places AHRV as a sister group to AQRV A and B (Fig. 4). The amino acid identity ranges from 64.4% (CSRV, AQRV A) to 73.2% (EFAV and GSH1, AQRV A and B).

3.6. Screening of eggs from halibut brood fish

Based on a suspicion that halibut brood fish could be disease-free carriers of AHRV, the real time RT PCR assay targeting AHRV segment 11 was used to test all 10 egg batches from 25 females. In addition, the eggs were screened for NNV and IPNV. NNV was not detected in any of the egg batches. The eggs from one of the brood fish (A-2014) were positive for both IPNV and AHRV, while the eggs from two other brood fish were positive for either IPNV or AHRV (B-2017). AHRV was not detected in the first egg batch of either of the two brood fish. However, except for the second batch from the brood fish from 2014, the subsequent batches were all AHRV positive for both brood fish. Based on the Ct values the amount of AHRV target RNA increases moderately in the successive egg batches (Table 4).

4. Discussion

Here we present the first complete genome of an aquareovirus from

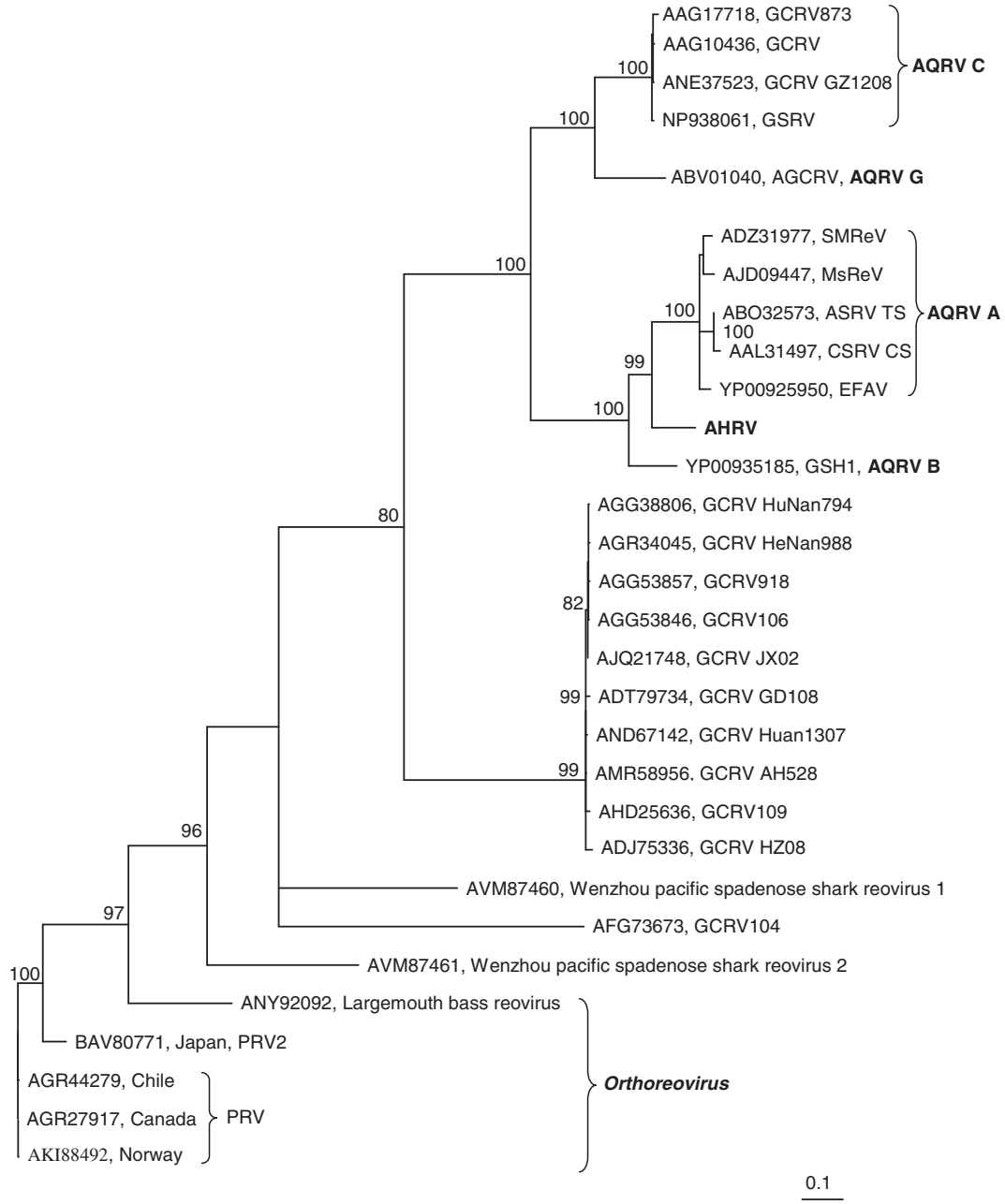

Fig. 2. The phylogenetic position of AHRV based on analysis of the putative protein, VP2 (RNA-dependent RNA polymerase protein), encoded on segment two, compared to the closest members within genus *Aquareovirus* using fish orthoreoviruses (PRV, PRV2 and Largemouth bass reovirus, LMBRV) as outgroup. Four described species of *Aquareovirus* (AQRV A, B, C and G), several related grass carp reoviruses (GCRV), and a unique GCRV (104), are included. Two reoviruses from Wenzhou pacific spadenose shark are also included. The support values are frequencies (%) at which a given branch appeared in 20,000 bootstrap replications.

a marine cold-water fish species in the North Atlantic, AHRV from Atlantic halibut. The first and last nucleotide of each AHRV genome segment are inverted complements, as seen for other *Aquareoviruses*. The genome ends of AHRV exhibit the canonical 5′ and 3′ ends found in species AQRV A, B, and G (AGCRV) (Ke et al., 2011; Chen et al., 2015; Makhsous et al., 2017; Jaafar et al., 2008), except for the 5′-end of segment seven (5′-GGUUUAA…). However, this difference could be a PCR artifact since the sequences obtained for this terminus were forced and thus should have had the sequence 5′-GTTTTA incorporated in the PCR primer sequence.

The genome segments from other members in genus *Aquareovirus* generally have a GC content above 50.0% (King et al., 2012). The GC content of AHRV segment six, encoding the putative outer capsid protein (VP5), is 47.8%, while all the other segments are at or above 50.0%. In a study of flaviviruses, that also included representative members of other virus families, among them two members of the *Reoviridae*, the GC content was found to be consistently higher for nucleotides encoding conserved amino acids, compared to non-coding nucleotides (Klitting et al., 2016). Whether the lower GC content of AHRV segment six reflects areas of less amino acid conservation in VP5 remains to be investigated and would require sequence information from more closely related aquareoviruses.

The genome of AHRV consists of 11 segments as in other aquareoviruses, but differs slightly in the genomic structure from other full genome sequenced members of this genus. While the number of ORFs for other aquareoviruses varies between 11 and 12, the putative number of ORFs for AHRV is 13. AHRV has two ORFs in segment seven as described for SMReV and MsReV (possible members of AQRV A) (Ke

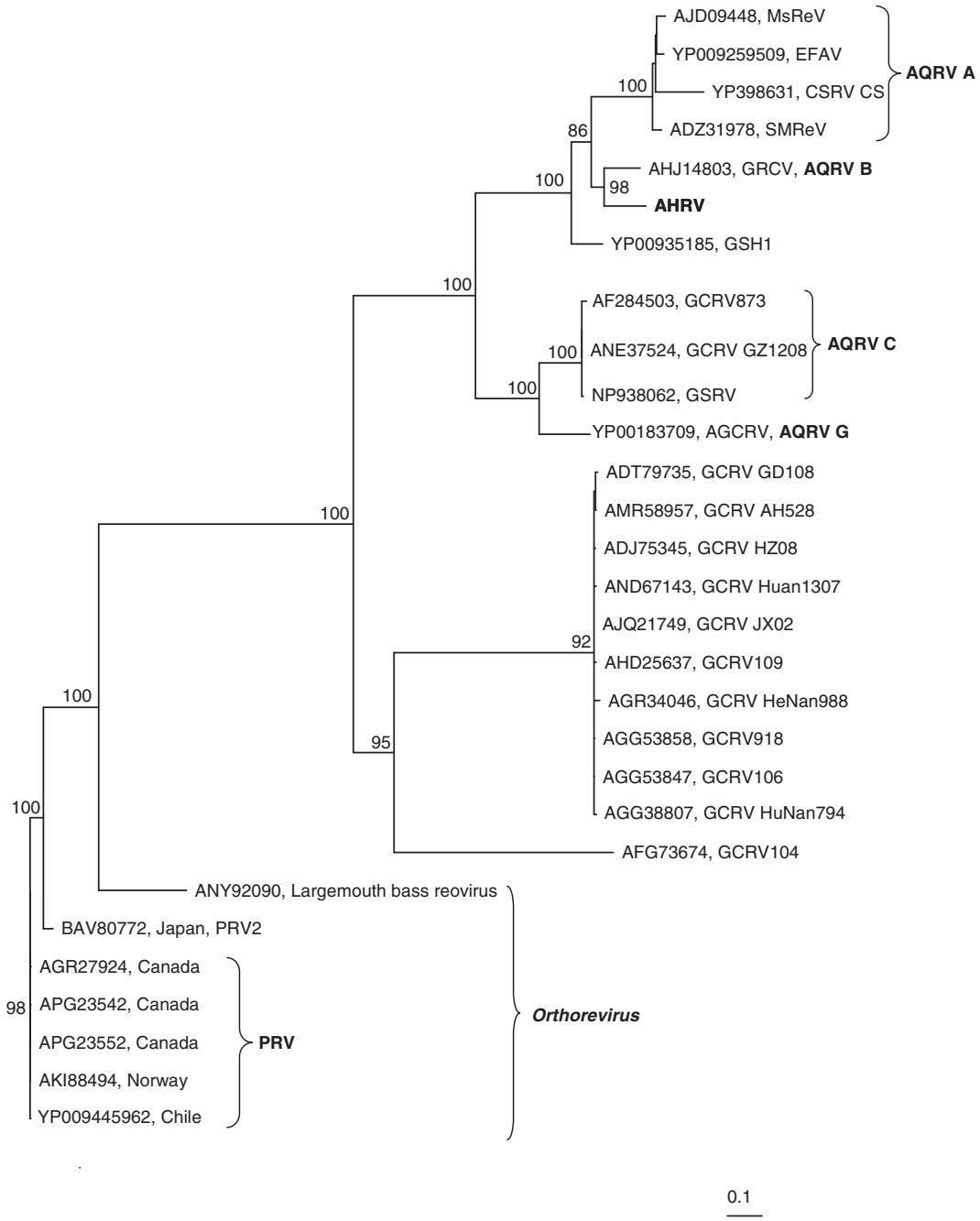

Fig. 3. The phylogenetic position of AHRV based on analysis of the putative protein, VP3 (inner capsid protein), encoded on segment three, compared to the closest members within genus *Aquareovirus* using fish orthoreoviruses (PRV, PRV2 and LMBRV) as outgroup. Four described species of Aquareovirus (AQRV A, B, C and G), several related grass carp reoviruses (GCRV), and a unique GCRV (104), are included. The support values are frequencies (%) at which a given branch appeared in 20,000 bootstrap replications.

et al., 2011; Chen et al., 2015), Fall chinook aquareovirus (AQRV B) (Makhsous et al., 2017), grass carp reovirus (GCRV) and Golden shiner reovirus (GSRV) (AQRV C) (Attoui et al., 2002), and American grass carp reovirus, AGCRV (AQRV G)(Jaafar et al., 2008). In contrast, Chum salmon reovirus (CSRV, AQRV A) and the Grass carp reoviruses GCRV-GD108, GCRV-HZ08 and GCReV-109 only have one ORF encoded on segment seven (Ye et al., 2012; Zhang et al., 2010; Wang et al., 2012; Pei et al., 2014). The two putative proteins encoded in AHRV segment seven, NS21 and NS30, have overlapping ORFs. This is also found in species AQRV A (MsReV and SMReV) and B (GRCV and GSH1), while species AQRV C (GSRV, and Channel catfish reovirus, CCRV-730) and G (AGCRV) have two non-overlapping ORFs (Attoui et al., 2002; Ke et al., 2011; Chen et al., 2015; Iwanowicz et al., 2016; Makhsous et al., 2017; Jaafar et al., 2008; Xu et al., 2013). This, in addition to the higher amino acid sequence identity of the proteins encoded on this segment, support a closer connection between AHRV and species AQRV A and B compared to species AQRV C and G.

AHRV segment 10 with its two ORFs differ from all other genome sequenced aquareoviruses, except for CSRV (AQRV A) and GCRV104 that both encodes two putative proteins on segment 11 (Winton et al., 1987; Fan et al., 2013). There is a slight amino acid identity between NS34 encoded on AHRV segment 10 and the protein encoded on segment 11 from GSRV (AQRV C) and AGCRV (AQRV G) (Table 3).

The sizes of the different segments of AHRV are to a large extent

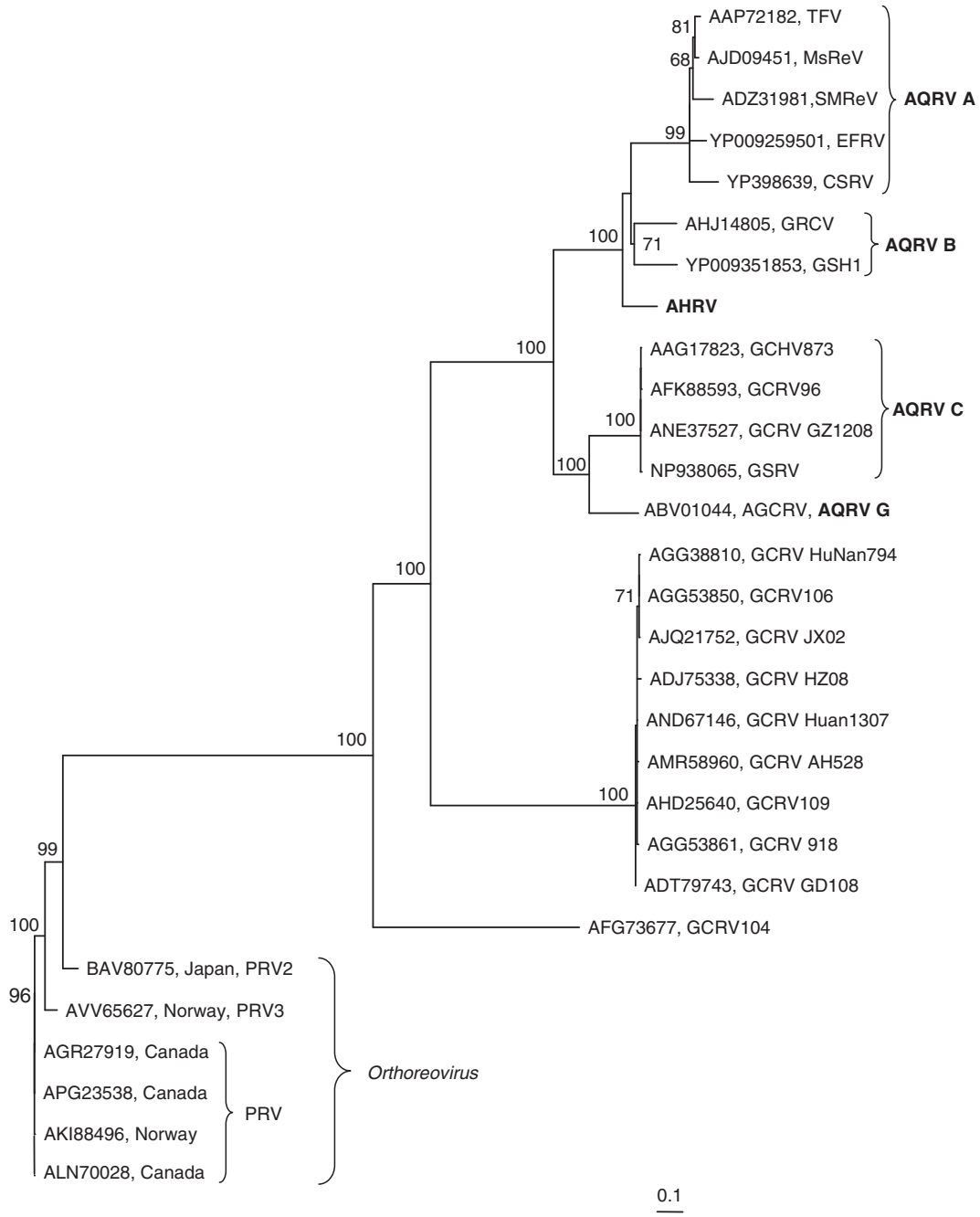

Fig. 4. The phylogenetic position of AHRV based on analysis of the putative protein, VP5 (outer capsid protein), encoded on segment six, compared to the closest members within genus *Aquareovirus* using fish orthoreoviruses (PRV, PRV2 and PRV3) as outgroup. Four described species of Aquareovirus (AQRV A, B, C and G), several related grass carp reoviruses (GCRV), and a unique GCRV (104), are included. The support values are frequencies (%) at which a given branch appeared in 20,000 bootstrap replications.

similar to that of other aquareoviruses, but VP7 is encoded on segment 11 in AHRV as opposed to segment 10 for other aquareoviruses with the exception of GCReV-109 (Pei et al., 2014). Segment 11 of the AHRV (978 bp) is 11.7% larger than the average size of segment 11 from other aquareoviruses, but smaller than the size of segment 11 (1027 bp) from GCRV-109 (Table 1). The sizes of segment 11 from all sequenced AQRV A and B are < 790 bp, while segment 10 from these two species, encoding VP7, range from 979 bp (AQRV B) to 987 (AQRV A), i. e. a similar size as segment 11 that encoding VP7 in AHRV (Table 1). Hence, the sizes of the segments encoding VP7 in AHRV and species AQRV A and B are similar, while AHRV segment 10, encoding NS34 and HP30, is larger than that of these two species.

The RdRp from isolates of the same species should have an amino acid identity > 95%, while the identity between species should be in the range from 57 to 74% (King et al., 2012). The amino acid identity of AHRV RdRp compared to AQRV A and B is over 74%, but not by much. Phylogenetic analysis based on amino acid sequences from the majority of the ORFs also places AHRV in a position close to species AQRV A and AQRV B, suggesting that AHRV could belong to one of these two species. However, occupancy of a particular ecological niche is a part of what defines a virus species (Van Regenmortel, 1989), and a previous study found that viruses from hosts in saline environments have more genomic structural similarities than the viruses from hosts in freshwater (Chen et al., 2015). Although aquareoviruses have been isolated and

Table 4
Testing of different egg batches from two AHRV positive brood fish halibut (A-2014 and B-2017). The first batch of eggs from brood fish A and B were sampled on the 15th and the 9th of January while the last batches were sampled the 14th and 6th of February, respectively. ELF = the Ct-values of the elongation factor obtained from the eggs, AHRV = Ct values for the assay targeting segment 11 from AHRV, IPNV = assay targeting Infectious pancreas necrosis virus, and NNV = assay targeting nervous necrosis virus.

	A-2014				B-2017			
Batch no:	ELF	AHRV	IPNV	NNV	ELF	AHRV	IPNV	NNV
1.	24,9	Neg	Neg	Neg	24,5	Neg	Neg	Neg
2.	24,7	Neg	Neg	Neg	23,3	36,8	Neg	Neg
3.	24,4	38.4	36.4	Neg	23,2	36,9	Neg	Neg
4.	24,7	37.3	Neg	Neg	23,6	34,8	Neg	Neg
5.	24,2	34.5	35.6	Neg	25,9	34,7	Neg	Neg
6.	25,2	32.4	32.6	Neg	23,6	34,1	Neg	Neg
7.	24,3	33.1	32.4	Neg	24,4	34,6	Neg	Neg
8.	25,8	33.4	33.9	Neg	24,2	34,1	Neg	Neg
9.	25,5	31.1	31.9	Neg	24,2	33,1	Neg	Neg
10.	25,8	30.7	29.2	Neg	25,4	31,1	Neg	Neg

characterized from other marine species, AHRV is the first fully genome sequenced aquareovirus from a cold-water marine fish in the North Atlantic, and thus from a different environment compared to freshwater fish species and warmwater marine fish species.

In conclusion, based on the differences in target host and environment, the differences in genome segments and putative amino acid sequences, and that the amino acid sequence identities of the RdRp is below 80.5% with respect to the closest relatives (AQRV A and B), AHRV might represent a new species within genus *Aquareovirus*. Moreover, detection of AHRV RNA in eggs of asymptomatic Atlantic halibut brood fish suggests transmission of this virus from parent to offspring and warrants further investigation. A precautionary approach that might reduce outbreaks of disease caused by AHRV would be to test all egg batches from brood fish by real time RT PCR before putting eggs into production.

Funding

This work was supported by the Fish Disease Research Group, University of Bergen.

References

Attoui, H., Fang, Q., Mohd Jaafar, F., Cantaloube, J.F., Biagini, P., De Micco, P., De Lamballerie, X., 2002. Common evolutionary origin of aquareoviruses and orthoreoviruses revealed by genome characterization of Golden shiner reovirus, Grass carp reovirus, Striped bass reovirus and golden ide reovirus (genus *Aquareovirus*, family Reoviridae). J. Gen. Virol. 83, 1941–1951.

Blindheim, S., Nylund, A., Watanabe, K., Plarre, H., Erstad, B., Nylund, S., 2015. A new aquareovirus causing high mortality in farmed Atlantic halibut fry in Norway. Arch. Virol. 160, 91–102.

Chen, Z.Y., Gao, X.C., Zhang, Q.Y., 2015. Whole-genome analysis of a novel fish Reovirus (MsReV) discloses Aquareovirus genomic structure relationship with host in saline environments. Viruses 7, 4282–4302.

Coutts, R.H.A., Livieratos, I.C., 2003. A rapid method for sequencing the 5′- and 3′-termini of double-stranded RNA viral templates using RLM-RACE. J. Phytopathol. 151, 525–527.

Cusack, R.R., Groman, D.B., Mackinnon, A.M., Kibenge, F.S., Wadowska, D., Brown, N., 2001. Pathology associated with an aquareovirus in captive juvenile Atlantic halibut Hippoglossus hippoglossus and an experimental treatment strategy for a concurrent bacterial infection. Dis. Aquat. Org. 44, 7–16.

Fan, Y., Rao, S., Zeng, L., Ma, J., Zhou, Y., Xu, J., Zhang, H., 2013. Identification and genomic characterization of a novel fish reovirus, Hubei grass carp disease reovirus, isolated in 2009 in China. J. Gen. Virol. 94, 2266–2277.

Ferguson, H.W., Millar, S.D., Kibenge, F.S., 2003. Reovirus-like hepatitis in farmed halibut (Hippoglossus hippoglossus). Vet. Rec. 153, 86–87.

Hedrick, R.P., Rosemark, R., Aronstein, D., Winton, J.R., Mcdowell, T., Amend, D.F., 1984. Characteristics of a new reovirus from channel catfish (Ictalurus punctatus). J. Gen. Virol. 65 (Pt 9), 1527–1534.

Iwanowicz, L.R., Iwanowicz, D.D., Adams, C.R., Lewis, T.D., Brandt, T.M., Cornman, R.S., Sanders, L., 2016. Complete genome sequence of a novel *Aquareovirus* that infects the endangered fountain darter, *Etheostoma fonticola*. Genome Announc. 4.

Jaafar, F.M., Goodwin, A.E., Belhouchet, M., Merry, G., Fang, Q., Cantaloube, J.F., Biagini, P., De Mieco, P., Mertens, P.P.C., Attoui, H., 2008. Complete characterisation of the American grass carp reovirus genome (genus *Aquareovirus*: family Reoviridae) reveals an evolutionary link between aquareoviruses and coltiviruses. Virology 373, 310–321.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282.

Ke, F., He, L.B., Pei, C., Zhang, Q.Y., 2011. Turbot reovirus (SMReV) genome encoding a FAST protein with a non-AUG start site. BMC Genomics 12, 323.

King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J., 2012. Virus Taxonomy. Classification and Nomenclature of Viruses. Academic Press, London.

Klitting, R., Gould, E.A., De Lamballerie, X., 2016. G + C content differs in conserved and variable amino acid residues of flaviviruses and other evolutionary groups. Infect. Genet. Evol. 45, 332–340.

Korsnes, K., Devold, M., Nerland, A.H., Nylund, A., 2005. Viral encephalopathy and retinopathy (VER) in Atlantic salmon *Salmo salar* after intraperitoneal challenge with a nodavirus from Atlantic halibut Hippoglossus hippoglossus. Dis. Aquat. Org. 68, 7–15.

Lupiani, B., Subramanian, K., Samal, S.K., 1995. Aquareoviruses. Annu. Rev. Fish Dis. 5, 175–208.

Makhsous, N., Jensen, N.L., Haman, K.H., Batts, W.N., Jerome, K.R., Winton, J.R., Greninger, A.L., 2017. Isolation and characterization of the fall *Chinook aquareovirus*. Virol. J. 14, 170.

Muller, T., Vingron, M., 2000. Modeling amino acid replacement. J. Comput. Biol. 7, 761–776.

Nibert, M.L., Duncan, R., 2013. Bioinformatics of recent aqua- and orthoreovirus isolates from fish: evolutionary gain or loss of FAST and fiber proteins and taxonomic implications. PLoS One 8, e68607.

Nylund, S., Andersen, L., Saevareid, I., Plarre, H., Watanabe, K., Arnesen, C.E., Karlsbakk, E., Nylund, A., 2011. Diseases of farmed Atlantic salmon *Salmo salar* associated with infections by the microsporidian *Paranucleospora theridion*. Dis. Aquat. Org. 94, 41–57.

Økland, A.L., Nylund, A., Overgard, A.C., Blindheim, S., Watanabe, K., Grotmol, S., Arnesen, C.E., Plarre, H., 2014. Genomic characterization and phylogenetic position of two new species in Rhabdoviridae infecting the parasitic copepod, salmon louse (*Lepeophtheirus salmonis*). PLoS One 9, e112517.

Øvergård, A.C., Nerland, A.H., Patel, S., 2010. Evaluation of potential reference genes for real time RT-PCR studies in Atlantic halibut (*Hippoglossus hippoglossus* L.); during development, in tissues of healthy and NNV-injected fish, and in anterior kidney leucocytes. BMC Mol. Biol. 11, 36.

Page, R.D., 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.

Pei, C., Ke, F., Chen, Z.Y., Zhang, Q.Y., 2014. Complete genome sequence and comparative analysis of grass carp reovirus strain 109 (GCReV-109) with other grass carp reovirus strains reveals no significant correlation with regional distribution. Arch. Virol. 159, 2435–2440.

Pfaffl, M.W., 2004. Quantification strategies in real-time PCR. In: A-Z of Quantitative PCR. International University Line, La Jolla, CA, USA.

Schachner, O., Soliman, H., Straif, M., Schilcher, F., El-Matbouli, M., 2014. Isolation and characterization of a novel reovirus from white bream Blicca bjoerkna. Dis. Aquat. Org. 112, 131–138.

Seng, E.K., Fang, Q., Sin, Y.M., Lam, T.J., 2005a. Molecular characterization of a major outer capsid protein encoded by the *Threadfin aquareovirus* (TFV) gene segment 10 (S10). Arch. Virol. 150, 2021–2036.

Seng, E.K., Fang, Q., Sin, Y.M., Lam, T.J., 2005b. Molecular cloning, DNA sequence analysis, and expression of cDNA sequence of RNA genomic segment 6 (S6) that encodes a viral outer capsid protein of threadfin aquareovirus (TFV). Virus Genes 30, 209–221.

Steigen, A., Nylund, A., Karlsbakk, E., Akoll, P., Fiksdal, I.U., Nylund, S., Odong, R., Plarre, H., Semyalo, R., Skar, C., et al., 2013. 'Cand. Actinochlamydia clariae' gen. nov., sp. nov., a unique intracellular bacterium causing epitheliocystis in catfish (*Clarias gariepinus*) in Uganda. PLoS One 8, e66840.

Van Regenmortel, M.H.V., 1989. Applying the species concept to plant viruses. Arch. Virol. 104, 1–17.

Wang, Q., Zeng, W., Liu, C., Zhang, C., Wang, Y., Shi, C., Wu, S., 2012. Complete genome sequence of a reovirus isolated from grass carp, indicating different genotypes of GCRV in China. J. Virol. 86, 12466.

Whelan, S., Goldman, N., 2001. A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Mol. Biol. Evol. 18, 691–699.

Winton, J.R., Lannan, C.N., Fryer, J.L., Hedrick, R.P., Meyers, T.R., Plumb, J.A., Yamamoto, T., 1987. Morphological and biochemical properties of four members of a novel group of reoviruses isolated from aquatic animals. J. Gen. Virol. 68 (Pt 2), 353–364.

Wu, M., Cui, K., Li, H., He, J., Chen, H., Jiang, Y., Ren, J., 2016. Genomic characterization and evolution analysis of a mutant reovirus isolated from grass carp in Anhui. Arch. Virol. 161, 1385–1387.

Xu, J., Zeng, L.B., Luo, X.S., Wang, Y., Fan, Y.D., Gong, S.Y., 2013. Reovirus infection emerged in cultured channel catfish, Ictalurus punctatus, in China. Aquaculture 372, 39–44.

Yan, X.Y., Wang, Y., Xiong, L.F., Jian, J.C., Wu, Z.H., 2014. Phylogenetic analysis of newly isolated grass carp reovirus. Springerplus 3, 190.

Ye, X., Tian, Y.Y., Deng, G.C., Chi, Y.Y., Jiang, X.Y., 2012. Complete genomic sequence of a reovirus isolated from grass carp in China. Virus Res. 163, 275–283.

Zainathan, S.C., Carson, J., Crane, M.S., Williams, L.M., Hoad, J., Moody, N.J., Gudkovs, N., Crameri, S., Hyatt, A.D., Young, J., et al., 2017. Preliminary characterization of *Tasmanian aquareovirus* (TSRV) isolates. Arch. Virol. 162, 625–634.

Zhang, C., Wang, Q., Shi, C., Zeng, W., Liu, Y., Wu, S., 2010. Molecular analysis of grass carp reovirus HZ08 genome segments 1-3 and 5-6. Virus Genes 41, 102–104.