Vol. 116: 1–9, 2015
doi: 10.3354/dao02903

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published September 17

FREE ACCESS

Molecular characterization of Tasmanian aquabirnaviruses from 1998 to 2013

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ABSTRACT: Tasmanian aquabirnaviruses (TABVs) have been isolated intermittently since 1998 from healthy Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* farmed in Macquarie Harbour, Tasmania, Australia. However, beginning in 2011, TABVs have been isolated from rainbow trout in association with mortality events. To determine if recent molecular changes in TABV were contributing to increased mortalities, next generation sequencing was undertaken on 14 TABVs isolated from 1998 to 2013. Sequencing of both genome segments and analysis of the 5 viral proteins they encode revealed that minimal changes had occurred in the past 15 yr. Of the amino acid changes detected only 1, alanine to aspartic acid at position 139 of the minor structural VP3 protein, was unique to the recent disease events. The most dramatic changes observed were in the length of the non-structural VP5 protein varying from 43 to 133 amino acids. However, the amino acid substitution in VP3 and variable VP5 length were unlikely to have resulted in increased TABV pathogenicity. The genome of a novel Australian aquabirnavirus, Victorian trout aquabirnavirus (VTAB) was also sequenced and compared to TABV isolates.

KEY WORDS: Aquabirnavirus · Genome · Atlantic salmon · Rainbow trout · Australia

INTRODUCTION

The *Aquabirnavirus* genus of the viral family *Birnaviridae* consists of viruses present in a diverse array of freshwater and marine fish and some invertebrates from broad geographic locations (Reno 1999). Infectious pancreatic necrosis virus (IPNV) is the type species of aquabirnavirus that cause significant disease, particularly in farmed salmonids (Roberts & Pearson 2005). The pathogenicity of isolates vary dramatically, with those not pathogenic to salmonids being named as aquabirnaviruses, to be distinguished from IPNV (Hill & Way 1995). Traditionally the typing of aquabirnaviruses has been based on serological relationships, with serotypes A1-A9 and B1 (Hill & Way 1995). However, more recently a classification system of 7 genogroups has become established based on the viral protein, VP2 (Blake et al. 2001, Cutrín et al. 2004, Nishizawa et al. 2005).

The aquabirnavirus genome consists of bisegmented, double-stranded RNA (dsRNA). Segment A has 2 partially overlapping open reading frames (ORFs). The larger ORF encodes a polyprotein (NH_2-pVP2-VP4-VP3-COOH) that is cleaved to produce proteins pVP2, VP3 and VP4 (Petit et al. 2000). Upon maturation, VP2 is the major capsid structural protein, VP3 is a minor capsid structural protein and VP4 is a protease (Delmas et al. 2012). The smaller ORF encodes VP5, a non-structural anti-apoptotic protein (Hong et al. 2002). Segment B has a single ORF that encodes VP1, an RNA-dependent RNA polymerase responsible for replication (Duncan et al. 1991).

In 1998, the first aquabirnavirus isolate from Australia, TAB98, was isolated from 'pinhead' Atlantic salmon *Salmo salar* samples collected during health surveillance in Macquarie Harbour, Tasmania (Crane et al. 2000). Tasmanian aquabirnaviruses (TABVs) have subsequently been isolated from sev-

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eral other healthy wild and farmed fish species inhabiting Macquarie Harbour. These viruses were collected from fish with no overt signs of disease and were not detected elsewhere in Tasmania. The nucleotide sequence for both segments of the original isolate, TAB98, were determined by Davies et al. (2010). Based on this sequence, TAB98 was found to cluster within Genogroup 5 along with New Zealand aquabirnaviruses that had been isolated from healthy Chinook salmon *Oncorhynchus tshawtscha*. Genogroup 5 consists of isolates from diverse geographical and host ranges such as pathogenic IPNV Sp116 isolated from Atlantic salmon in Norway (Shivappa et al. 2004).

A second novel aquabirnavirus was isolated, in 2010, from rainbow trout at a freshwater hatchery in Victoria (McCowan et al. 2015). The isolation of Victorian trout aquabirnavirus (VTAB) from the skin of dead female fingerlings was the first detection of an aquabirnavirus from mainland Australia. Based on limited sequence analysis of a 738 bp region of pVP2, which shared less than 80% identity with TABV and IPNV isolates, the authors described VTAB as a novel aquabirnavirus, as it was genetically and phylogenetically distinct from known aquabirnaviruses. The role of VTAB in the disease event in 2010 is unclear and the virus has not been detected since.

Through the Tasmanian Salmonid Health Surveillance Program, more than 40 isolations of TABV from farmed Atlantic salmon *S. salar* and rainbow trout *O. mykiss* in Macquarie Harbour has occurred between 1998 and 2013. Prior to 2011, isolates were obtained from healthy fish as part of the routine surveillance program. However, from 2011, TABV has been isolated on several occasions from diseased rainbow trout from this location. In this study, next generation sequencing (NGS) was undertaken to generate consensus sequences for selected TABV isolates, from 1998 to 2013, to identify any molecular changes that may have occurred during that period and their potential contribution to pathogenicity. Representative isolates were selected based on the year isolated, host species and whether healthy or diseased fish. The genome of the novel VTAB was also sequenced to allow greater comparison with TABV isolates.

MATERIALS AND METHODS

Viruses

More than 40 TABVs have been isolated from Macquarie Harbour, confirmed by diagnostic RT-PCR (Davies et al. 2010) and amplicon sequencing, since the original isolation of TAB98 from farmed Atlantic salmon in 1998 (Crane et al. 2000). In 1998, after the initial isolation, a survey of wild fish in Macquarie Harbour identified TABV in several species, including Morid cod (*Pseudophycis* sp.). However, most TABV isolates up to 2011 have predominantly been isolated from fish health surveillance program samples of farmed Atlantic salmon and rainbow trout. No TABVs were isolated for a 4 yr period between 2003 and 2006. From late 2011 to 2013, TABVs were isolated from farmed rainbow trout populations that had exhibited increased mortalities. Fourteen TABV isolates from the previous 15 yr were cultured in chinook salmon embryo (CHSE-214) or bluegill fry (BF-2) cells until near 100% cytopathic effect (CPE) was observed before harvest. In addition, VTAB isolated from diseased farmed rainbow trout in 2010 in the rainbow trout gonad cell line, RTG-2 (McCowan et al. 2015), was also included. Isolates with passage history are listed in Table 1.

Nucleic acid extraction

Infected cell culture supernatants were clarified by centrifugation (425 × *g*) for 10 min. For each isolate, the supernatant was decanted from the cell debris pellet and filtered (0.45 µm) before high speed centrifugation (103 900 × *g*) for 3 h. The viral pellet was re-suspended in 300 µl phosphate-buffered saline (PBSA, pH 7.4). For random-primer conventional PCR, 140 µl of viral pellet was treated at 37°C with 100 U RNase I_f (New England Biolabs) for 16 h to reduce the CHSE-214 RNA content. Total nucleic acid was extracted using the QIAamp viral RNA mini kit (QIAGEN) and eluted in 30 µl. Co-extracted CHSE-214 genomic DNA was removed from 10 µl of the extracted nucleic acid by rigorous treatment at 37°C with 30 U of the Turbo DNA-free Kit (Ambion). For specific-primer conventional RT-PCR, total nucleic acid was extracted from 140 µl of re-suspended viral pellet or clarified viral culture supernatant with the QIAamp viral RNA mini kit and eluted in 50 µl.

Random PCR

The random PCR method to obtain complementary DNA (cDNA) from viral dsRNA was based on the sequence independent single primer amplification (SISPA) method described by Rosseel et al.

Table 1. Percent identity of Tasmanian aquabirnaviruses (TABV) and Victorian trout aquabirnavirus (VTAB) nucleotide sequences in comparison to the TAB98 isolate, the first aquabirnavirus isolated from Atlantic salmon in Australia. Segment A and B refers to the aquabirnavirus genome consisting of bisegmented, double-stranded RNA. Virus was isolated from healthy (H) or diseased (D) fish; for each isolate the first 2 digits represent the year of isolation and the final 5 digits refer to the case number. 'Passage history' refers to the number of passages in fish cell lines: chinook salmon embryo (CHSE-214), bluegill fry (BF-2), epithelioma papulosum cyprini (EPC), and rainbow trout gonad cell line (RTG-2). TAB98 nucleotide sequences obtained from GenBank accession EU672429 for Segment A and EU672430 for Segment B; the number of nucleotide differences for Segment A and B compared to TAB98 are given in brackets

Virus	Fish species	Passage history	Segment A (2942 bp)	GenBank accession (Segment A)	Segment B (2605 bp)	GenBank accession (Segment B)
TABV						
98-00208 (H)	Morid cod	CHSE-214 ×3	99.8% (5)	KP268650	99.8% (6)	KP268665
01-01282 (H)	Rainbow trout	CHSE-214 ×3	99.6% (13)	KP268651	99.4% (15)	KP268666
02-00229 (H)	Atlantic salmon	CHSE-214 ×6	99.5% (16)	KP268652	99.5% (13)	KP268667
07-01693 (H)	Rainbow trout	CHSE-214 ×4	99.2% (24)	KP268653	99.2% (20)	KP268668
08-03923 (H)	Rainbow trout	BF-2 ×2	98.9% (32)	KP268654	99.3% (17)	KP268669
08-04494 (H)	Atlantic salmon	BF-2 ×2/ CHSE-214 ×2	99.0% (30)	KP268655	99.2% (21)	KP268670
09-01031 (H)	Atlantic salmon	EPC ×2/ CHSE-214 ×2	99.1% (27)	KP268656	99.3% (18)	KP268671
09-01288 (H)	Rainbow trout	CHSE-214 ×3	98.9% (31)	KP268657	99.2% (21)	KP268672
10-00221 (H)	Atlantic salmon	CHSE-214 ×4	98.8% (35)	KP268658	99.1% (23)	KP268673
10-00224 (H)	Rainbow trout	CHSE-214 ×4	98.8% (34)	KP268659	99.3% (18)	KP268674
11-04076 (H)	Atlantic salmon	CHSE-214 ×2	98.8% (35)	KP268660	99.0% (25)	KP268675
11-04755 (D)	Rainbow trout	CHSE-214 ×2	98.8% (34)	KP268661	99.0% (25)	KP268676
11-04973 (D)	Rainbow trout	CHSE-214 ×2	98.8% (35)	KP268662	99.0% (26)	KP268677
13-03567 (D)	Rainbow trout	CHSE-214 ×1	98.8% (34)	KP268663	99.1% (23)	KP268678
VTAB						
10-04677	Rainbow trout	RTG-2 ×2	79.5% (603)	KP268664	81.3% (487)	KP268679

(2011, 2013). RNase- and DNase-treated nucleic acid (7 µl) was heat-denatured in the presence of 2.5 µM random primer (FR20RV-12N) at 95°C for 5 min before quenching on ice. After the addition of 2 µl Superscript III/RNaseOUT enzyme mix and reaction mix (Superscript III first-strand synthesis supermix, Invitrogen) to a final volume of 20 µl, first-strand cDNA was synthesized. To first-strand cDNA, 5U Klenow and reaction buffer (Promega) were added with 200 µM dNTPs, 1 µM FR20RV-12N and molecular grade water to a final volume of 30 µl, before second-strand cDNA synthesis. To amplify the randomly generated DNA, 2 µM FR20RV and Phusion High-fidelity PCR master mix with HF buffer (NEB) were added to 5 µl second-strand cDNA to a final volume of 20 µl. First- and second-strand cDNA synthesis and cDNA amplification cycling conditions are described in Table 2. The amplified DNA was quantified with the Qubit dsDNA HS assay kit (Invitrogen) before 150 ng was purified of primers and random-primed amplicons shorter than approx. 250 bp with 0.6× AMPure XP beads (Agencourt). The eluted DNA was quantified by Qubit.

Specific RT-PCR

Specific RT-PCR primers were designed to amplify Segments A and B by conventional PCR for next generation sequencing (Table 2). In 25 µl reactions, the Superscript III One-Step RT-PCR System with Platinum Taq High Fidelity (Life Technologies) with 180 nM of each primer was used to amplify the desired amplicons from 2 µl total nucleic acid. Amplicons were visualised after electrophoresis on SYBR Safe stained 1.5% agarose gels, excised and purified using the QIAquick gel extraction kit (Qiagen). For next generation sequencing, purified Segment A and B amplicons for each isolate were quantified by NanoDrop before being combined in a 1:1 ratio and the mixed segments quantified by Qubit.

The complete VP5 ORF for each isolate was amplified by conventional PCR with Superscript III One-step RT-PCR with Platinum Taq (Life Technologies) and specific primers (Table 2). Each amplicon was purified by the QIAquick gel extraction kit and Sanger-sequenced using the forward and reverse primers by direct product sequencing using the

Table 2. PCR primers and conditions used in this study of Tasmanian aquabirnaviruses (TABV) and Victorian trout aquabirnavirus (VTAB). SISPA: sequence independent single primer amplification method. NA: not applicable

PCR Primer	Sequence (5′–3′)	PCR conditions Cycles	Time	Temp (°C)	Amplicon size (bp)
Random PCR SISPA					
FR20RV-12N[a]	GCC GGA GCT CTG CAG ATA TCN NNN NNN NNN NN	First-strand cDNA			NA
FR20RV[a]	GCC GGA GCT CTG CAG ATA TC	1×	10 min	25	
			50 min	50	
			5 min	85	
		Second-strand cDNA			
		1×	60 min	37	
			10 min	75	
		cDNA-amplification			
		1×	30 s	98	
		40×	15 s	98	
			30 s	48	
			2 min	72	
		1×	10 min	72	
TABV SegA RT-PCR					
TABV-SegAFor	CAA CCA CAA AAT CTA TAT CAA TGC AAG	1×	30 min	50	2971
			2 min	94	
TABV-SegARev	GAT CGG TCA GGA AAG AGA GTA G	40×	30 s	94	
			30 s	52	
			3 min	68	
		1×	7 min	68	
TABV SegB RT-PCR					
TABV-SegBFor	ACC ACG ACT GTT TAT GTA TGC AC	As per TABV SegA RT-PCR			2648
TABV-SegBR2	CTG ATG AGT CCA TCC AGG TC				
TABV SegA VP5 RT-PCR					
TABV-AF2	AGC CCT TTC TAA CAA ACA ACC AC	1×	30 min	50	471
			2 min	94	
TABPa8R[b]	GAC ATC AGG CTG TTG TAG G	40×	30 s	94	
			30 s	53	
			45 s	68	
		1×	7 min	68	
VTAB SegA RT-PCR					
VAB-SegAFor	CAT GAA CAC AAA CAA AGC AAC CG	As per TABV SegA RT-PCR			2945
TABV-SegARev	As above	(but 50°C annealing temperature)			
VTAB SegB RT-PCR					
VAB-SegBFor	ACC ACG ACT GTT TAC GTA TGC AC	As per TABV SegA RT-PCR			2656
VAB-SegBRev	CGG TGT TGA GTC CAG TCA TGT C	(but 50°C annealing temperature)			
VTAB SegA VP5 RT-PCR					
TABV-AF3	GAG GAG CTC TCC GTC GATG	As per TABV SegA VP5 RT-PCR			497
TABPa8R[b]	As above	(but 50°C annealing temperature)			

[a]Rosseel et al. (2013); [b]Blake et al. (2001)

BigDye Terminator v3.1 Cycle Sequencing chemistry and 3130xl Genetic Analyzer (Life Technologies) according to the manufacturer's instructions. Chromatogram analysis and consensus sequence generation were conducted using Geneious (Biomatters).

Next generation sequencing (NGS)

The NGS platform used in this study was the MiSeq Desktop Sequencer (Illumina). To prepare sequencing-ready libraries, 4 ng of bead-purified random-primer amplified DNA or combined Segment A and B specific-primer amplified DNA were fragmented and tagged by the Nextera XT DNA Sample Preparation Kit (Illumina) according to the manufacturer's instructions. The quantity of each purified DNA library was assessed by Qubit and the average fragment size determined with the Bioanalyzer High Sensitivity DNA Kit (Agilent). Subsequently, 2 nM of each DNA library (up to 14 individual libraries) was denatured with 0.1 N sodium hydroxide and pooled before being diluted to 14 pM in hybridization buffer. The pooled denatured DNA library was sequenced using an Illumina MiSeq Reagent Kit v2 (300 cycle) with MiSeq Control Software (Illumina). Approximately 1 to 2 million, 150 bp paired reads were obtained for each individual DNA library, dependent on the number of denatured libraries present in the pool added to the flow cell.

NGS sequence assembly and analysis

Random primer tag sequences were removed from the MiSeq paired read raw data with a dedicated iPython Anaconda script. The trimmed sequences were assembled and contigs generated for Segments A and B of each isolate by read-mapping to TAB98 Segments A and B (EU672429 and EU672430) using Geneious (Biomatters). Sanger-sequenced VP5 ORF PCR amplicons were used to complete Segment A contigs before the sequence of VP1 to VP5 were determined and translated. Sequence alignments and pairwise distance calculations for the percent nucleotide and translated amino acid identity were undertaken with Geneious Alignment algorithms. All finalized sequences for TABV and VTAB Segment A and B were submitted to GenBank (Table 1). Phylogenetic analyses using the neighbor-joining method with a bootstrap consensus tree being inferred from 10000 replicates were conducted using MEGA5 (Tamura et al. 2011). Evolutionary distances were calculated using the Tamura-Nei model.

RESULTS

Segments A and B of TABV isolates 98-00208 and 01-01282 that covered all ORFs were assembled from SISPA random-primer PCR sequences. Each segment had an average coverage of >3000×. Comparison of the nucleotide sequences for each segment from both isolates with TAB98 revealed >99.4% identity, despite isolation from different fish species and up to 3 yr separation in isolation date (Table 1). Based on the high sequence similarity observed between these isolates, specific-primer Segment A and B RT-PCRs were performed on the remaining 12 isolates before NGS. The specific-primer segment RT-PCR approach was also performed to confirm the TABV 98-00208 genome sequence; the assembled sequences were identical to those obtained by SISPA random RT-PCR. The specific segment amplification approach resulted in average segment coverages ranging from 8900 to 83 000× for the 13 isolates sequenced. The final Segment A and B sequences provided complete coverage of the 3 ORFs and represented >94% of each genome, but each segment lacked ~150 bp of sequence that include the 5′ and 3′ termini.

Nucleotide alignments and percent identity calculation for both Segments A and B with the original TAB98 showed a high degree of similarity (≥98.8%) for all the isolates sequenced (Table 1). Interestingly, despite TABV 13-03567 being isolated from rainbow trout 15 yr after the original TAB98 isolation from Atlantic salmon, Segments A and B shared 98.8 and 99.1% nucleotide identity, respectively. The TABV 11-04973 isolate had the highest number of nucleotide differences (61) of the 5547 bp of the genome that was compared with TAB98, a difference of only 1.1%.

In comparison to TAB98, less than 1.5% difference in amino acid (aa) sequences was identified between VP1, pVP2, VP3 and VP4 of the 14 TABV isolates sequenced (Table 3). In 1998, only 4 aa differences (0.2%) were detected between the original TAB98 isolate from Atlantic salmon and the wild cod isolate (98-00208). The latest isolate sequenced, TABV 13-03567, had a total of 18 aa differences (1.0%) when compared to TAB98. The largest difference observed in the VP1 protein (844 aa) was a total of 7 aa, found in isolate TABV 09-01288, 10-00221 and 11-04973. Regarding the other Segment A encoded proteins, 2 aa differences in VP4, 3 in VP3 and 5 in VP2 were the most detected in the TABV isolates sequenced (selected from the 15 yr time period).

The greatest variation in the VP5 amino acid sequence was 6 aa differences in TABV 08-03923, 11-04076 and 11-04755 that equated to less than a 5% change in sequence (Table 3). The length of the translated VP5 protein, however, varied dramatically. TAB98 and TABV 98-00208 had a VP5 protein encoded by 133 aa whereas 01-01282 and 02-00229 were truncated to just 43 aa due to a premature stop codon. A different premature stop codon also truncated VP5 of the other 11 TABVs (isolated from 2007 to 2013) to 128 aa. Sanger sequencing of the entire VP5 ORF of the 14 isolates confirmed the premature stop codons and the aa differences that had been identified.

Single IPNV isolates representing Genogroups 1 to 6 and a marine birnavirus representing Genogroup 7 were compared at the protein level with the most recent TABV isolate 13-03567 (Table 4). As expected, the highest identity was with Genogroup 5 as described by Davies et al. (2010). Isolate 13–03567 when compared with IPNV 1146 from Genogroup 5 had 94% or greater identity with viral proteins VP1 to VP4 and 81.3% for VP5. Genogroups 1 and 6 had the most aa changes for pVP2 to VP4 when compared with isolate 13-03567. Phylogenetic analysis of pVP2 nucleotide sequences was conducted due to VP2 encoding the major capsid protein that determines host range and has the greatest number of aquabirnavirus sequences (from all 7 genogroups) available on GenBank. The pVP2 of all 14 TABV isolates clearly clustered phylogenetically into 1 distinct node

Table 3. Percent identity of Tasmanian aquabirnaviruses (TABV) and Victorian trout aquabirnavirus (VTAB) proteins in comparison to the TAB98 isolate, the first aquabirnavirus isolated from Atlantic salmon in Australia. TAB98 protein sequences obtained from GenBank accession EU672430 for the viral protein VP1 and EU672429 for VP2 to VP5. Numbers in brackets indicate the number of amino acid (aa) differences compared to TAB98

Virus	Fish species	VP1 (%) (844 aa)	pVP2 (%) (449 aa)	VP3 (%) (217 aa)	VP4 (%) (252 aa)	VP5 (%) (133 aa)
TABV						
98-00208	Morid cod	99.8 (2)[a]	99.8 (1)	99.5 (1)	100 (0)	100 (0)[b]
01-01282	Rainbow trout	99.6 (3)	99.3 (3)	99.5 (1)	100 (0)	97.7 (1)[c]
02-00229	Atlantic salmon	99.5 (4)	99.1 (4)	99.5 (1)	100 (0)	97.7 (1)[c]
07-01693	Rainbow trout	99.5 (4)	98.9. (5)	99.5 (1)	99.6 (1)	98.4 (2)[a]
08-03923	Rainbow trout	99.3 (6)	99.3 (3)	99.1 (2)	99.6 (1)	95.3 (6)[a]
08-04494	Atlantic salmon	99.3 (6)	99.1 (4)	99.1 (2)	99.6 (1)	96.9 (4)[a]
09-01031	Atlantic salmon	99.5 (4)	98.9 (5)	99.5 (1)	99.6 (1)	97.7 (3)[a]
09-01288	Rainbow trout	99.2 (7)	98.9 (5)	99.1 (2)	99.6 (1)	96.1 (5)[a]
10-00221	Atlantic salmon	99.2 (7)	99.3 (3)	98.6 (3)	99.6 (1)	96.1 (5)[a]
10-00224	Rainbow trout	99.3 (6)	98.9 (5)	99.1 (2)	99.6 (1)	97.7 (3)[a]
11-04076	Atlantic salmon	99.3 (6)	99.1 (4)	99.1 (2)	99.6 (1)	95.3 (6)[a]
11-04755	Rainbow trout	99.3 (6)	99.1 (4)	98.6 (3)	99.6 (1)	95.3 (6)[a]
11-04973	Rainbow trout	99.2 (7)	99.1 (4)	98.6 (3)	99.2 (2)	96.9 (4)[a]
13-03567	Rainbow trout	99.3 (6)	99.1 (4)	98.6 (3)	100 (0)	96.1 (5)[a]
VTAB						
10-04677	Rainbow trout	89.6 (88)	89.8 (46)	80.6 (42)	85.3 (37)	66.2 (45)[b]

VP5 translated protein [a]128, [b]133 or [c]43 aa

Table 4. Percent identities between Tasmanian aquabirnavirus TABV 13-03567 and aquabirnaviruses Genogroups 1–7 for viral proteins VP1 to VP5. IPNV: infectious pancreatic necrosis virus. Numbers of amino acid (aa) differences compared to TABV 13-03567. NA: not available

Aquabirnavirus	Geno-group	Species	Location	GenBank accession (VP1)	VP1 (%) (844 aa)	GenBank accession (polyprotein)	pVP2 (%) (449 aa)	VP3 (%) (217 aa)	VP4 (%) (252 aa)	GenBank accession (VP5)	VP5 (%) (128 aa)
IPNV West Buxton	1	Trout	USA	AAC71004.1	89.8 (86)	AAC71003.1	88.6 (51)	82.5 (38)	84.1 (40)	AAC71002.1	64.3 (47)
IPNV 578	2	Turbot	Spain	CAD32986.1	96.2 (32)	CAD32970.1	92.7 (33)	87.6 (27)	92.9 (18)	CAD32978.1	74.2 (33)
IPNV Canada 1	3	Trout	Canada	NA	NA	AF342732_1	92.4 (34)	89.9 (22)	94.4 (14)	NA	NA
IPNV Canada 2	4	Trout	Canada	NA	NA	AF342733_1	90.2 (44)	87.1 (28)	91.7 (21)	AAA92627.1	74.2 (33)
IPNV 1146	5	Trout	Spain	CAD32980.1	96.4 (30)	CAD32964.1	95.3 (21)	94.0 (13)	98.4 (4)	CAD32972.1	81.3 (24)
IPNV He	6	Pike	Germany	NA	NA	AF342730_1	86.4 (61)	83.9 (35)	84.5 (39)	NA	NA
Marine birnavirus Y-6	7	Yellowtail	Japan	AAN04570.1	90.6 (79)	AAP37161.1	91.8 (46)	83.9 (35)	84.1 (40)	AAP37160.1	64.1 (46)

within Genogroup 5 (Fig. 1). This node was clearly separate from IPNV isolates of Genogroup 5 that clustered in a separate distinct node. The only other aquabirnaviruses to cluster in the same node as the 14 TABV isolates were from Macquarie Harbour (TAB98 and TAB02) and New Zealand (NZ06 and NZ10), further supporting the Australian and New Zealand isolates having a common ancestor. Analysis of the nucleotide sequences obtained for Segments A and B and the translated amino acid sequences of the 5 viral proteins resulted in similar topographies (data not shown).

A comparative analysis of TABV isolates obtained from rainbow trout farms showing mortalities (11-04755, 11-04973 and 13-03567) and from healthy Atlantic Salmon (11-04076) was performed. The analysis revealed 11 loci with differing amino acids in all 5 proteins. Of these, 8 loci had the same aa present in the 3 isolates but not TABV 11-04076. However, only alanine (A) at position 139 of VP3 of the 3 isolates was unique when compared to all the other sequenced TABV isolates which had an aspartic acid (D) at this position.

The VTAB 10-04677 isolated from rainbow trout in Victoria was distinct from TABV with approx. 20% of its genome differing from that of TAB98 (Table 1). Of the 5 VTAB viral proteins, VP1 and pVP2 shared the greatest identity (>89%) with TAB98, with the lowest identity (66%) present in the 133 aa VP5 (Table 3). In comparison to representatives of each aquabirna-

virus genogroup, VTAB was most similar to marine birnavirus Y-6 (Genogroup 7) isolated from yellowtail in Japan for proteins VP1, pVP2, VP4 and VP5 (Appendix). However, VTAB VP3 was most similar to IPNV West Buxton isolated from trout in USA (Genogroup 1). Phylogenetically analysis of pVP2 demonstrated that VTAB was distinctly different from all aquabirnavirus isolates examined, with the closest association to Genogroups 1 and 7 (Fig. 1). Similar to TABV, further phylogenetic analysis of Segment A and B and translated viral protein sequences resulted in similar topographies (data not shown).

DISCUSSION

In this study, the complete coding regions of 14 TABVs (selected from 1998 to 2013 isolations) and the novel VTAB were sequenced by NGS. To enable the assembly of the complete coding regions of Segments A and B of TABV and VTAB isolates a combination of SISPA random-primer RT-PCR and specific-primer RT-PCR approaches were performed. The SISPA technique, while labour-intensive, allowed for entire dsRNA genomes to be sequenced. The method developed should be effective for the sequencing of other RNA viruses that can be cultured *in vitro*. However, after initial sequencing revealed that the TABV genomes being studied were highly similar, a much more rapid specific-primer RT-PCR was developed. This method was efficient and effective as the dsRNA genome being amplified was relatively short and only bisegmental. This specific-primer RT-PCR approach would be more complicated and potentially problematic if the viruses being studied had more segments (e.g. reoviruses) or the genomes were more diverse in sequence.

Discrete amino acids of the VP2 major capsid protein have been identified as key virulence factors for IPN viruses in salmonid species (Bruslind & Reno 2000, Song et al. 2005). In addition, the reversion of a less virulent IPNV isolate to a more virulent isolate in persistently infected and stressed Atlantic salmon has been associ-

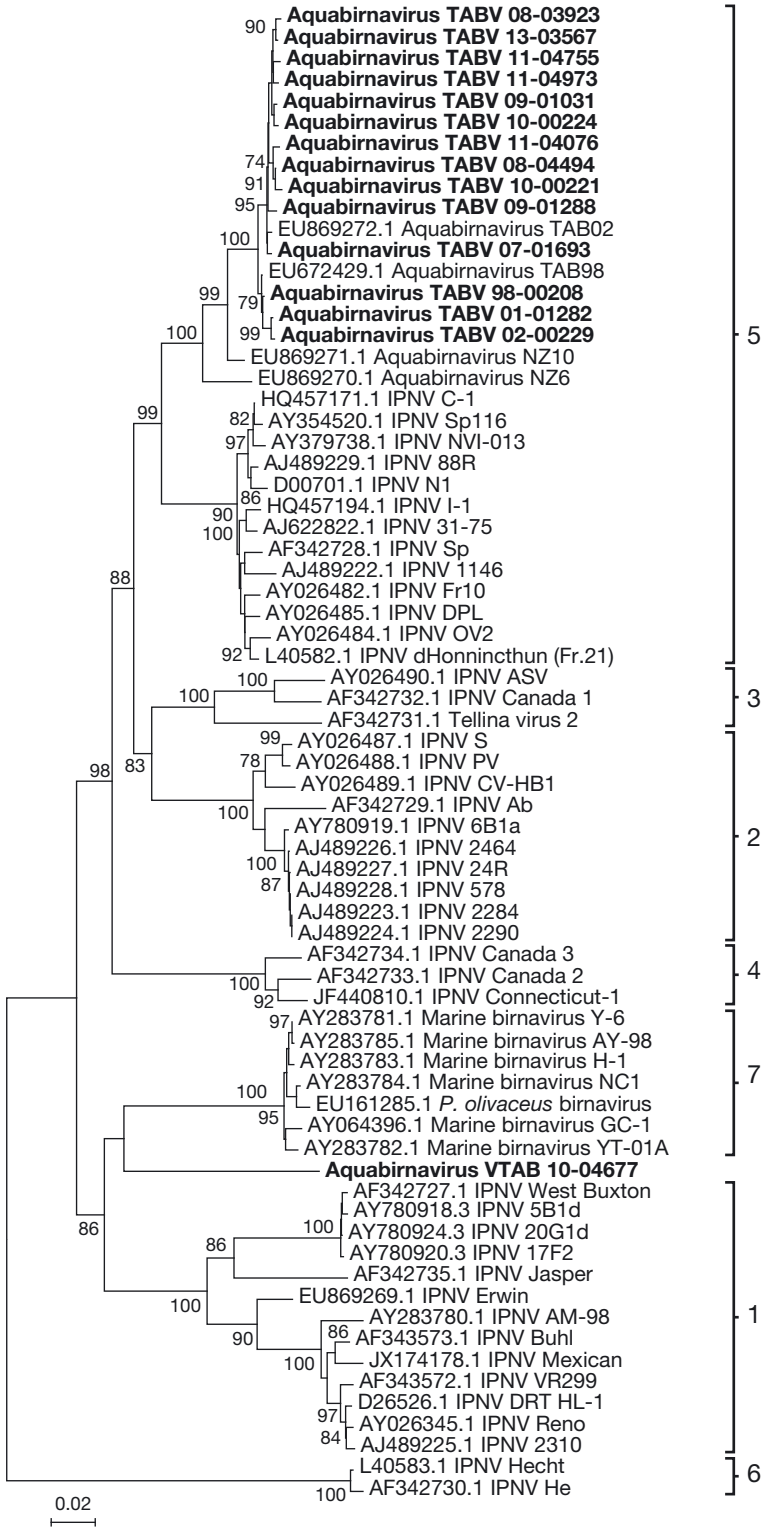

Fig. 1. Unrooted phylogenetic tree of aquabirnaviruses over the viral protein pVP2 region (1344 bp) of Segment A, inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches (only when >70%). Sequences generated from this study are highlighted in **bold**. IPNV: infectious pancreatic necrosis virus

ated with VP2 amino acid variations (Gadan et al. 2013). However, none of the amino acid changes detected in TABV VP2 were unique to the TABV isolates from 2011 to 2013 associated with rainbow trout disease events. The only unique change (D139A) common to the isolates associated with disease occurred in VP3, the minor internal structural protein. To date, substitutions in VP3 sequences have not been attributed to changes in aquabirnavirus virulence. It is therefore likely that a combination of factors related to the host and environment could have been sufficient to result in increased incidence of disease rather than distinct molecular changes in the virus.

The VP5 protein may be involved in anti-apoptosis (Hong et al. 2002) but has been found to be highly variable in amino acid sequence and not required for viral replication or persistence (Santi et al. 2005). The length of VP5 can vary dramatically in IPNV (i.e. 28 aa to 133 aa) but the functional significance of such changes remains unclear (Skjesol et al. 2011). The effect of the premature stop codons detected within TABV in this study are also unknown but did not correlate with the disease events of 2011 to 2013.

Within the 15 yr period of study, the TABV genomes were remarkably similar with low genetic diversity regardless of whether the isolates were from Atlantic salmon or rainbow trout. The TABV isolates sequenced from Macquarie Harbour were monophyletic and demonstrated that, thus far, only 1 viral population exists from a single ancestral origin. Therefore, the genetic diversity observed probably originated from point mutations associated with the error-prone nature of RdRp replication of RNA viruses without proofreading capabilities (Duffy et al. 2008). In one of the few longitudinal studies of an aquabirnavirus, low genetic diversity was also observed within IPNV in Mexico over a 6 yr period from 4 rainbow trout-producing regions (Barrera-Mejía et al. 2011).

The VTAB isolate was associated with deaths in freshwater rainbow trout, but it is unknown if the virus was responsible for the disease or was an opportunistic isolation from the disease investigation (McCowan et al. 2015). Intriguingly, the virus has not been isolated since 2010, and its host range is unknown. Our phylogenetic analysis indicated that the virus was distinct from TABV, and despite some relatedness to Genogroups 1 and 7, the VTAB genome is sufficiently different to propose that the virus is the type strain for a new Genogroup 8.

Acknowledgements. We thank Ivano Broz for development of the iPython Anaconda script and the CSIRO, AAHL DSR Sequencing Laboratory for generating Sanger sequences. This research was supported by funding from Fisheries Research and Development Corporation on behalf of the Australian Government (FRDC Project 2010-032).

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Appendix. Percent identities between Victorian trout aquabirnavirus VTAB 10-04677 and aquabirnaviruses Genogroups 1–7 for viral proteins VP1 to VP5. Number of amino acid (aa) differences compared to VTAB 10-04677. IPNV: infectious pancreatic necrosis virus; NA: not available

Aquabirnavirus	Geno-group	Species	Location	GenBank accession (VP1)	VP1 (%) (844 aa)	GenBank accession (polyprotein)	pVP2 (%) (449 aa)	VP3 (%) (217 aa)	VP4 (%) (252 aa)	GenBank accession (VP5)	VP5 (%) (133 aa)
IPNV West Buxton	1	Trout	USA	AAC71004.1	94.2 (49)	AAC71003.1	89.8 (46)	88.9 (24)	86.1 (35)	AAC71002.1	75.9 (32)
IPNV 578	2	Turbot	Spain	CAD32986.1	90.6 (79)	CAD32970.1	89.1 (49)	81.1 (41)	84.9 (38)	CAD32978.1	63.9 (48)
IPNV Canada 1	3	Trout	Canada	NA	NA	AF342732_1	89.5 (47)	79.7 (44)	82.5 (44)	NA	NA
IPNV Canada 2	4	Trout	Canada	NA	NA	AF342733_1	88.4 (52)	80.6 (42)	83.7 (41)	AAA92627.1	66.9 (44)
IPNV 1146	5	Trout	Spain	CAD32980.1	90.3 (82)	CAD32964.1	90.9 (41)	79.3 (45)	84.5 (39)	CAD32972.1	66.9 (44)
IPNV He	6	Pike	Germany	NA	NA	AF342730_1	84.4 (70)	80.2 (43)	79.4 (52)	NA	NA
Marine birnavirus Y-6	7	Yellowtail	Japan	AAN04570.1	95.1 (41)	AAP37161.1	92.0 (36)	86.2 (30)	89.3 (27)	AAP37160.1	78.2 (29)

Editorial responsibility: V. Gregory Chinchar, Jackson, Mississippi, USA

Submitted: February 4, 2015; Accepted: July 3, 2015
Proofs received from author(s): August 26, 2015