Phylogenetic analysis of betanodavirus isolates from Australian finfish

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ABSTRACT: In Australia, disease caused by betanodavirus has been reported in an increasing number of cultured finfish since the first report of mortalities in 1990. Partial coat protein gene sequences from the T2 or T4 regions of 8 betanodaviruses from barramundi *Lates calcarifer*, sleepy cod *Oxyeleotris lineolata*, striped trumpeter *Latris lineata*, barramundi cod *Cromileptes altivelis*, Australian bass *Macquaria novemaculata* and gold-spotted rockcod *Epinephelus coioides* from several Australian states were determined. Analysis of the 606 bp nucleotide sequences of the T2 region of 4 isolates demonstrated the close relationship with isolates from the red-spotted grouper nervous necrosis virus (RGNNV) genotype and the Cluster Ia subtype. Comparison of a smaller 289 bp sequence from the T4 region identified 2 distinct groupings of the Australian isolates within the RGNNV genotype. Isolates from barramundi from the Northern Territory, barramundi, sleepy cod, barramundi cod and gold-spotted rockcod from Queensland, and striped trumpeter from Tasmania shared a 96.2 to 99.7% nucleotide identity with each other. These isolates were most similar to the RGNNV genotype Cluster Ia. Isolates from Australian bass from New South Wales and from barramundi from South Australia shared a 98.6% sequence identity with each other. However, these isolates only shared an 85.8 to 87.9% identity with the other Australian isolates and representative RGNNV isolates. The closest nucleotide identity to sequences reported in the literature for the New South Wales and South Australian isolates was to an Australian barramundi isolate (Ba94Aus) from 1994. These 2 Australian isolates formed a new subtype within the RGNNV genotype, which is designated as Cluster Ic.

KEY WORDS: Betanodavirus · Phylogeny · Nervous necrosis virus · Coat protein · Nodavirus · Molecular detection

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INTRODUCTION

Viral nervous necrosis (VNN) or viral encephalopathy and retinopathy (VER) is caused by viruses of the *Betanodavirus* genus of the Family *Nodaviridae* and has been reported from over 40 fish species worldwide (reviewed by Munday et al. 2002). The disease is characterised by a vacuolating necrosis of neuronal cells of the spinal cord, brain and retina. Mortalities are most commonly observed in cultured larvae, fry and juvenile marine finfish, although the disease has been reported in adult sea bass *Dicentrarchus labrax* (Le Breton et al. 1997) and a number of freshwater finfish species (Hegde et al. 2003, Athanassopoulou et al. 2004). In Australia, VNN has been associated with cultured barramundi *Lates calcarifer* since picorna-

like viral particles were first reported to be associated with degenerative areas of the brain and retina of larvae which had exhibited abnormal swimming behaviour (Glazebrook et al. 1990, Munday et al. 1992). The close antigenic relationship of the picorna-like virus in diseased barramundi to the betanodavirus of striped jack nervous necrosis virus (SJNNV) was reported after specific fluorescence was observed in barramundi tissue using anti-SJNNV rabbit serum in an indirect fluorescence antibody test (Munday et al. 1994). Amplification and sequencing of the T4 region of the coat protein gene of an isolate from barramundi from Australia (Ba94Aus) confirmed betanodavirus as the causative agent (Nishizawa et al. 1997). Virions are small (25 to 30 nm), non-enveloped, icosahedral in shape, and contain single-stranded, bipartite positive sense RNA. The larger RNA segment (RNA1, 3.1 kb) encodes a nonstructural protein of ~110 kDa, while the smaller RNA segment (RNA2, 1.4 kb) encodes the 42 kDa coat protein (Mori et al. 1992, Murphy et al. 1995, Nagai & Nishizawa 1999, Skliris et al. 2001). The RNA2 coat protein gene is the most common target for reverse transcription polymerase chain reaction (RT-PCR) as this represents the highly conserved area of the viral genome (Nishizawa et al. 1994, Thiéry et al. 1999, Grotmol et al. 2000, Skliris et al. 2001, Gagné et al. 2004, Gomez et al. 2004). While a number of studies have compared betanodavirus isolates by virus neutralization tests (Skliris et al. 2001, Chi et al. 2003, Mori et al. 2003, Shieh & Chi 2005) or cross-species infectivity trials (Arimoto et al. 1993, Totland et al. 1999), most comparative analyses of isolates have been based on phylogenetic analysis of complete or partial coat protein gene sequences derived from RNA2. Partial sequences have been generated using primers to target the T2, or the smaller, more variable internal T4 region of the coat protein gene described by Nishizawa et al. (1994). Originally, Nishizawa et al. (1995) determined (from sequencing of the T2 region of the coat protein genes) that piscine betanodaviruses isolated in Japan were significantly different from isolates from insects and proposed that these isolates be put into a new genus, *Betanodavirus*, in the Family *Nodaviridae*. Analysis of a further 25 finfish betanodavirus isolates identified 4 discrete genotypes that were defined as striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa et al. 1997). As additional isolates were identified in new species or from new geographic locations, there has been general agreement on these 4 genotypes (Dalla Valle et al. 2001, Skliris et al. 2001, Johnson et al. 2002, Chi et al. 2003, Hegde et al. 2003, Thiéry et al. 2004,

Cutrín et al. 2007). However, more recent nucleotide sequencing of the coat protein gene of additional betanodavirus isolates has led to proposals for an Atlantic cod nervous necrosis virus (ACNNV) genotype (Gagné et al. 2004) and a turbot betanodavirus (TNV) genotype (Johansen et al. 2004). Thiéry et al. (2004) have proposed a nonhost name derived nomenclature (Cluster I, II, III and IV).

Within Australia, increased awareness of the disease, improvements in hatchery biosecurity and a reduction in stocking density have significantly reduced mortalities in subsequent batches of larvae compared to those initially observed (Glazebrook & Heasman 1992, Munday et al. 1992, Anderson et al. 1993). However, as aquaculture of new species has expanded, so has the occurrence of VNN. In this paper, we describe the phylogenetic comparison of partial coat protein gene sequences from the T2, or the smaller T4 region, of endemic Australian betanodavirus isolates from different finfish species from several geographic regions. We also discuss relationships with sequences reported in the literature.

MATERIALS AND METHODS

Betanodavirus isolates. Betanodavirus isolates from Queensland, Australia, were obtained from laboratory submissions during routine disease investigations between 1999 and 2005. Samples from the Northern Territory and Tasmania were obtained during a collaborative research project (Moody et al. 2004), while those from New South Wales and South Australia were obtained during investigations into VNN outbreaks in these states. With the exception of the striped trumpeter sample from Tasmania, all isolates were obtained from larvae or juvenile finfish that were exhibiting clinical signs of VNN. Confirmation of infection was determined by observation of vacuolation in the brain and retina by light microscopy and positive immunohistochemistry (IHCT) using sheep anti-barramundi nervous necrosis virus (BNNV) recombinant coat protein polyclonal antibodies. The striped trumpeter isolate was obtained from a broodstock blood sample after nested RT-PCR testing as described below. The location of betanodavirus isolates and species details are outlined in Table 1.

Sample preparation. Heads were aseptically removed from fish and homogenised in an equal volume of Medium 199 (Trace Scientific), supplemented with 500 U benzylpenicillin ml^{-1}, 500 µg streptomycin sulphate ml^{-1} and 2 µg amphotericin B ml^{-1}. The homogenate was clarified by centrifugation at 10 000 × g for 10 min. The striped trumpeter blood sample was frozen and thawed once, incubated with

an equal volume of sterile deionised water for 60 min at 5°C, and clarified by centrifugation at $10\,000 \times g$ for 10 min. Clarified supernatants were stored at −80°C until tested.

RT-PCR amplification of the T2 region. The T2 RT-PCR was established specifically for the phylogenetic analysis of selected isolates. RNA was extracted from 200 µl of clarified homogenate from 4 Queensland betanodavirus isolates (T99180614, T342514, T442286 and T540844) using the High Pure viral RNA kit (Roche) according to the manufacturer's instructions, incubated at 90°C for 5 min, then stored in ice prior to RT-PCR. RT-PCR was undertaken using the F1 (5′-GGA TTT GGA CGT GCG ACC AA-3′) primer described by Grotmol et al. (2000) and the NR3 (5′-GGA TTT GAC GGG GCT GCT CA-3′) primer described by Thiéry et al. (1999) to produce an amplicon of ~830 bp from the T2 region of the coat protein gene. Reverse transcription was conducted at 42°C for 60 min in a 20 µl volume containing 2 µl of 10× StrataScript buffer (Integrated Sciences), 20 U StrataScript RT (Integrated Sciences), 0.5 mM of each dNTP (Integrated Sciences), 5 U RNasin (Promega), 1.0 µM NR3 primer, RNase-free water and 9.5 µl RNA template. PCR amplification was carried out in a 50 µl reaction mix containing 5 µl of the RT reaction, 0.2 µM of each primer, 2.5 mM $MgCl_2$ and 25 µl HotStarTaq Master Mix (Qiagen). Thermal cycling was conducted in an Eppendorf Mastercycler programmed as follows: 1 cycle at 95°C for 15 min, 30 cycles at 95°C for 40 s, 50°C for 40 s and 72°C for 40 s, and 1 cycle at 72°C for 10 min. RT-PCR products were resolved using 1.5% TAE-buffered agarose gel electrophoresis.

RNA extraction and nested RT-PCR of the T4 region. The nested RT-PCR of the T4 region has been in routine diagnostic use at the Tropical and Aquatic Animal Health Laboratory for nodavirus detection since 1999. Over 215 positive and 140 negative reference samples have been used for test optimisation and validation (N. J. G. Moody unpubl. data). To improve efficiency, only nested RT-PCR products are analysed using agarose gel electrophoresis. RNA was extracted from 200 µl of clarified homogenate using the High Pure viral RNA kit (Roche) according to the manufacturer's instructions, incubated at 90°C for 5 min, then stored in ice prior to RT-PCR. RT-PCR was performed using the F2 (5′-CGT GTC AGT CAT GTG TCG CT-3′) and R3 (5′-CGA GTC AAC ACG GGT GAA GA-3′) primer set and the method described by Nishizawa et al. (1994) to amplify the T4 region of the coat protein gene, with the following modifications: reverse transcription was conducted at 42°C for 60 min in a 20 µl volume containing 2 µl of 10× StrataScript buffer (Integrated Sciences), 20 U StrataScript RT (Integrated Sciences), 0.5 mM of each dNTP (Integrated Sciences),

5 U RNasin (Promega), 1.0 µM R3 primer, RNase-free water and 9.5 µl RNA template. PCR amplification was carried out in a 50 µl reaction mix containing 5 µl of the RT reaction, 0.2 µM of each primer, 2.5 mM $MgCl_2$ and 25 µl HotStarTaq Master Mix (Qiagen). Thermal cycling was conducted in an Eppendorf Mastercycler programmed as follows: 1 cycle at 95°C for 15 min, 30 cycles at 95°C for 40 s, 50°C for 40 s and 72°C for 40 s, and 1 cycle at 72°C for 10 min. Nested PCR was performed using the NF2 (5′-GTT CCC TGT ACA ACG ATT CC-3′) and NR3 (5′-GGA TTT GAC GGG GCT GCT CA-3′ primer set and the method described by Thiéry et al. (1999), with the following modifications: amplification was carried out in a 50 µl reaction mix containing 1 µl of the RT-PCR reaction, 1.0 µM of each nested primer, 25 µl HotStarTaq Master Mix (Qiagen) and RNase-free water. Thermal cycling was conducted in an Eppendorf Mastercycler programmed as follows: 1 cycle at 95°C for 15 min, 25 cycles at 94°C for 40 s, 50°C for 40 s and 72°C for 40 s, and 1 cycle at 72°C for 10 min. Nested RT-PCR products were resolved using 2% TAE-buffered agarose gel electrophoresis.

Sequencing and phylogenetic analysis. Amplicons generated by amplification of the T2 region of 4 isolates (T99180614, T342514, T442286 and T540844) were ligated into the pGEM-T Easy Vector system (Promega) and cloned using JM109 competent cells according to the manufacturer's instructions. Clones were checked for the correct insert using the T2 PCR, and plasmids were amplified and purified for sequencing. Nucleotide sequencing was conducted using the Big Dye 3.1 sequencing chemistry (Applied Biosystems) with M13 forward and reverse primers. Three clones were sequenced for each isolate. The nucleotide sequences were analysed using Sequencher V4.0.5 (Gene Codes), and the consensus sequence for each isolate was determined from replicate sequences in the forward and reverse directions (National Centre for Biotechnology Information GenBank accession numbers for these sequences are provided in Table 1). Nucleotide sequence information for 4 betanodavirus isolates (T156700, T256901, T451722 and T452934) were obtained by direct sequencing of the amplicon produced from the nested RT-PCR targeting an internal region of the T4 region of the coat protein gene. Representative sequences of non-Australian derived betanodavirus isolates (Table 1) were obtained from BLASTn searches of GenBank for comparison and all sequences were manually aligned using GeneDoc (Nicholas et al. 1997). Phylogenetic trees were determined using the neighbour-joining method, with the bootstrap consensus tree being inferred from 1000 replicates in MEGA 3.1 (Kumar et al. 2004). Evolutionary distances were computed using the maximum composite likelihood method.

RESULTS

RT-PCR amplification of partial T2 and T4 regions

The T2 RT-PCR produced amplicons of the expected size of ~832 bp for the 4 Queensland isolates (T99180614, T342514, T442286 and T540844) tested (data not shown). The nested RT-PCR protocol produced amplicons of the expected size (~295 bp; Fig. 1) for the 7 diseased fish samples tested and the blood sample from the broodstock striped trumpeter.

Sequence and phylogenetic analysis

Partial nucleotide sequences of the T2 or T4 region of the betanodavirus coat protein gene from 8 Australian betanodavirus isolates were determined in this study. The GenBank accession numbers are contained in Table 1. Sequences covering 832 bp of the T2 region

were determined for T99180614, T342514 and T442286, while a 606 bp sequence was determined for T540844; thus, all sequences were reduced to 606 bp for comparison. These sequences shared a 96.9 to
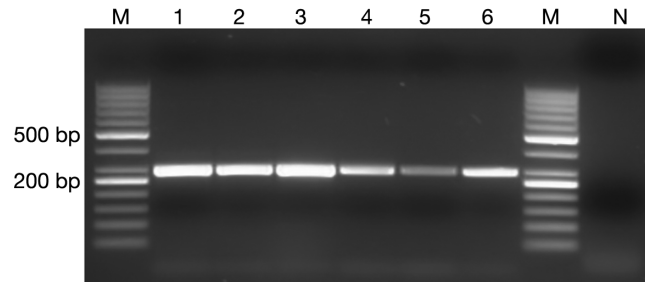

Fig. 1. Nested RT-PCR results (partial T4 region) for Australian betanodavirus isolates obtained from finfish. Lane M: GeneRuler 50 bp DNA ladder (MBI Fermentas); Lane 1: T342514; Lane 2: T99180614; Lane 3: T442286; Lane 4: T451722; Lane 5: T452934; Lane 6: T450844; Lane N: negative control

Table 1. Details of nodavirus isolates used in the present study

Strain designation	GenBank accession	Host species	Location (Year)	Source
T156700	EF591367	Barramundi *Lates calcarifer*	NT, Aust. (2001)	Present article
T256901	EF591370	Striped trumpeter *Latris lineata*	TAS, Aust. (2002)	Present article
T342514	EF591372	Barramundi *L. calcarifer*	QLD, Aust. (2003)	Present article
T442286	EF591371	Barramundi cod *Cromileptes altivelis*	QLD, Aust. (2004)	Present article
T451722	EF591365	Australian bass *Macquaria novemaculata*	NSW, Aust. (2004)	Present article
T452934	EF591366	Barramundi *L. calcarifer*	SA, Aust. (2004)	Present article
T540844	EF591369	Goldspotted rockcod *Epinephelus coioides*	QLD, Aust. (2005)	Present article
T99180614	EF591368	Sleepy cod *Oxyeleotris lineolata*	QLD, Aust. (1999)	Present article
03-160	AJ698113	Senegalese sole *Solea senegalensis*	Spain (2003)	Thiéry et al. (2004)
ACNNV	AF445800	Atlantic cod *Gadus morhua*	Canada (1999)	Johnson et al. (2002)
AH95NorA	AJ254641	Halibut *Hippoglossus hippoglossus*	Norway (1995)	Grotmol et al. (2000)
AHNNV	AF160473	Halibut *H. hippoglossus*	Norway (1995)	Aspenhaug et al. (1999)
Ba94Aus	–	Barramundi *L. calcarifer*	Australia (1994)	Nishizawa et al. (1997)
BB09	AJ698094	Sea bass *Dicentrarchus labrax*	France (2003)	Thiéry et al. (2004)
BF93Hok	D38635	Barfin flounder *Verasper moseri*	Japan (1993)	Gagné et al. (2004)
Cod02ac2	AY547547	Atlantic cod *Gadus morhua*	Canada (2002)	Gagné et al. (2004)
DGNNV	AF245004	Giant grouper *E. lanceolatus*	Taiwan	Lin et al. (2001)
DlEV	Y08700	Sea bass *Dicentrarchus labrax*	Greece	Sideris (1997)
Dlev-Fr	U39876	European seabass *D. labrax*	France (1997)	Gagné et al. (2004)
ETNNV	AF318942	Greasy grouper *E. tauvina*	Singapore	Tan et al. (2001)
GNNV	AF499774	Guppy *Poicelia reticulata*	Singapore (2000)	Hegde et al. (2003)
JF93Hir	D38527	Japanese flounder *Paralichthys oliveatus*	Japan (1993)	Nishizawa et al. (1997)
MGNNV	AF245003	Malabar grouper *E. malabaricus*	Taiwan	Lin et al. (2001)
RG91Tok	D38636	Red-spotted grouper *E. akaara*	Japan (1991)	Nishizawa et al. (1997)
SJOri	D30814	Striped jack *Pseudocaranx dentex*	Japan (1991)	Nishizawa et al. (1997)
SGWak97	AY324870	Sevenband grouper *E. septemfasciatus*	Japan (1997)	Iwamoto et al. (2004)
TNV	AJ608266	Turbot *Scophthalmus maximus*	Norway	Johansen et al. (2004)
TP93Kag	D38637	Tiger puffer *Takifugu rupripes*	Japan (1993)	Nishizawa et al. (1997)
V67	AJ698104	Sea bass *D. labrax*	France (1995)	Thiéry et al. (2004)
X199	AJ698103	Meagre *Argyrosomus regius*	France (1999)	Thiéry et al. (2004)
Y235	AJ698105	Sea bass *D. labrax*	France (1991)	Thiéry et al. (2004)
Y55	AJ698102	Sea bass *D. labrax*	France (2000	Thiéry et al. (2004)
YGNNV	AF283554	Yellow grouper *E. awoara*	Taiwan	Lai et al. (2001)
Z233	AJ698111	Convict surgeonfish *Acanthurus triostegus*	Tahiti (2001)	Thiéry et al. (2004)

99.7% identity with each other. Phylogenetic analysis of the partial T2 region nucleotide sequences, with representative isolates from the different genotypes obtained from GenBank, identified these Australian betanodavirus isolates as members of the RGNNV genotype (Fig. 2). Interestingly, the marine isolates (T342514, T442286 and T540844) clustered together with other marine isolates within this genotype and shared a 98.8 to 99.0% nucleotide identity with a representative isolate of the RGNNV genotype, RG91Tok. In contrast, the isolate from the freshwater species (T99180614) clustered with an isolate (JF93Hir) from the Japanese flounder. These 2 isolates shared a 97.5% nucleotide sequence identity.

Sequences of 289 bp were determined after nested RT-PCR amplification of isolates T156700, T256901, T451722 and T452934. Comparison of all the Australian betanodavirus isolates using sequences derived from the T4 region replicated the results obtained for the T2 region sequences and determined that these 4 isolates were also more closely related to members of the RGNNV genotype than representative isolates from any other genotype (Fig. 3, Table 2). However, sequence analysis of these additional isolates demonstrated that the endemic Australian betanodavirus isolates comprised 2 distinct groups within the RGNNV genotype. One group corresponding to the Cluster Ia subtype described by Thiéry et al. (2004) contained the isolates from barramundi (T342514), barramundi cod (T442286), gold-spotted
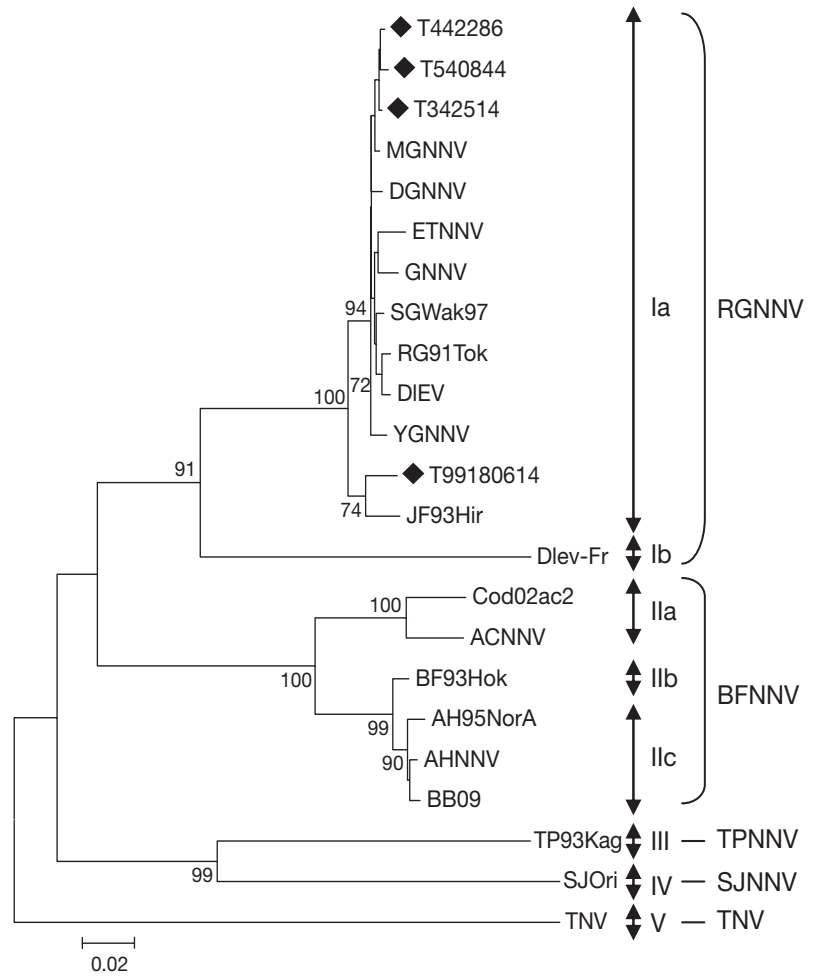

Fig. 2. Unrooted phylogenetic tree produced from the partial betanodavirus nucleotide sequences of the coat protein T2 region showing genotype groupings. See Table 1 for isolate information. (◆) Australian isolates from the present study. Phylogeny was determined using the neighbour-joining method in MEGA 3.1. Numbers indicate bootstrap percentages from 1000 resamplings. Nomenclature of genotypes is based on Nishizawa et al. (1997) and Thiéry et al. (2004). RGNNV: red-spotted grouper nervous necrosis virus; BFNNV: barfin flounder nervous necrosis virus; SJNNV: striped jack nervous necrosis virus; TNV: turbot betanodavirus

Table 2. Percent nucleotide sequence identities of the Australian nodavirus isolates and representative isolates from the literature, based on the 289 bp sequence of the partial T4 region of the coat protein gene. Isolate details are given in Table 1

| Isolate	T352514	T442286	T540844	T99180614	T156700	T256901	T452934	T451722
RG91Tok	98.6	98.9	98.6	96.1	97.9	98.6	85.8	85.8
Ba94Aus	83.7	83.4	83.4	83.7	84.4	83.1	94.8	94.8
Dlev-Fr	83.7	84.0	83.7	85.4	84.4	84.1	82.3	81.7
BF93Hok	76.8	77.1	76.8	75.7	76.4	76.8	75.1	75.7
AHNNV	76.4	76.8	76.4	75.4	76.1	76.5	75.4	76.1
ACNNV	74.3	74.0	74.4	75.4	73.7	73.7	75.4	75.4
SJOri	66.7	66.4	66.0	65.7	65.4	66.1	67.1	66.4
TP93Kag	66.7	66.4	66.1	66.7	65.4	66.7	67.1	67.1
TNV	68.8	68.8	68.5	67.8	69.2	68.8	68.2	68.2
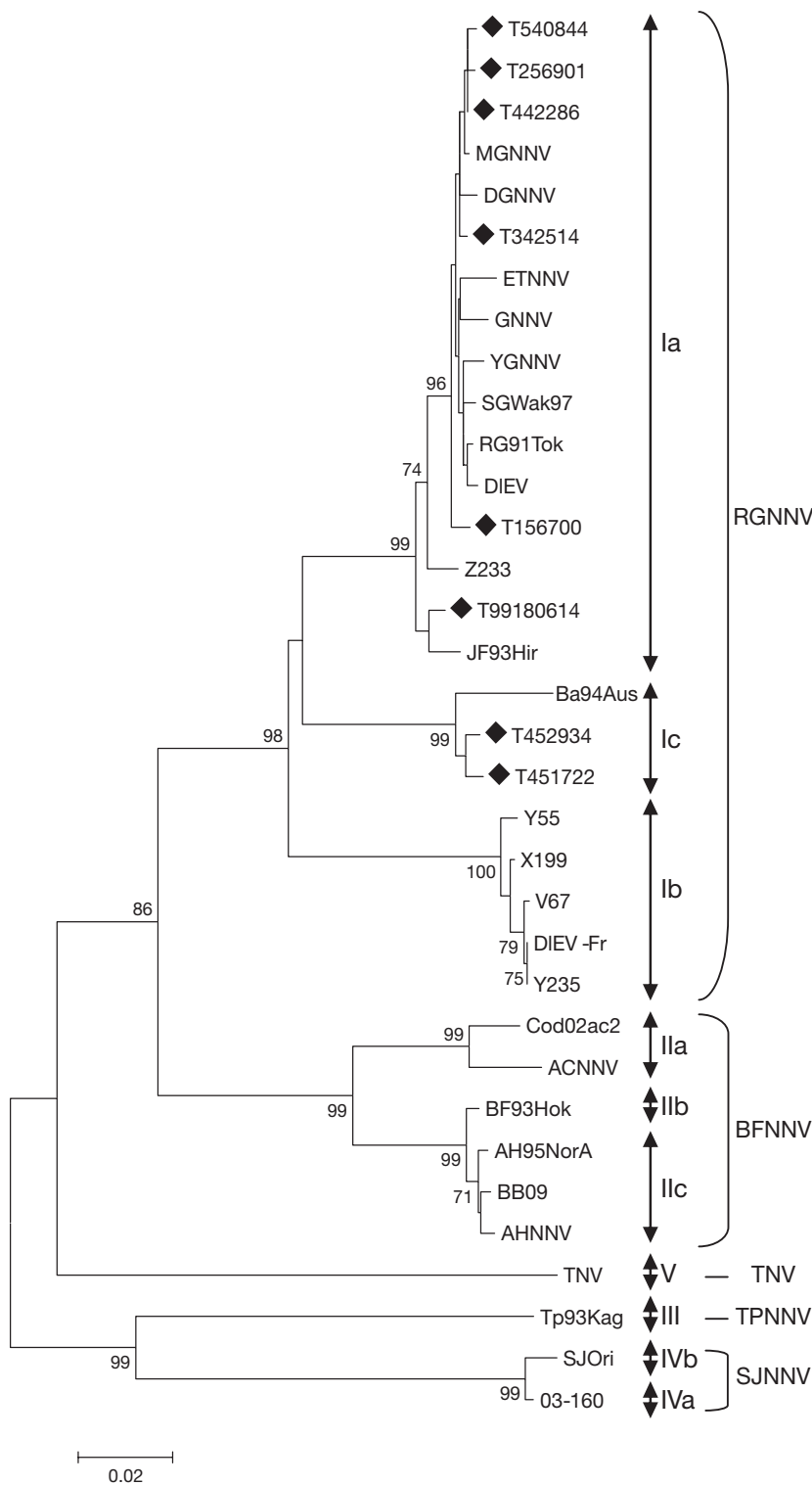
Fig. 3. Unrooted phylogenetic tree produced from the partial betanodavirus nucleotide sequences of the coat protein T4 region showing genotype groupings. See Table 1 for isolate information. (◆) Australian isolates from the present study. Phylogeny was determined using the neighbour-joining method in MEGA 3.1. Numbers indicate bootstrap percentages from 1000 resamplings. Nomenclature of genotypes is based on Nishizawa et al. (1997) and Thiéry et al. (2004). See Fig. 2 for full virus names

rockcod (T540844) and sleepy cod (T99180614) from Queensland, barramundi from the Northern Territory (T156700), and striped trumpeter from Tasmania (T256901). All shared a 96.1 to 99.7% nucleotide homology with each other and with RG91Tok (Table 2). In contrast, while the 2 isolates from Australian bass from New South Wales (T451722) and barramundi from South Australia (T452934) shared 98.6% nucleotide and 96.8% deduced amino acid identity with each other, they only shared an 85.8 to 87.9% nucleotide and 67.3 to 72.9% deduced amino acid identity with the other Australian isolates (Table 3). T451722 and T452934 shared a much lower nucleotide identity with RG91-Tok of only 85.8% (Table 2). Both were most closely related to an Australian barramundi isolate (Ba94Aus) reported by Nishizawa et al. (1997), and these 3 isolates produced a distinct group within the RGNNV genotype (Fig. 3) corresponding to a new subtype, Cluster Ic.

All Australian betanodavirus sequences contained a 6 nucleotide deletion when compared with representative isolates of the SJNNV (SJOri) and TPNNV (TP93Kag) genotypes (Nishizawa et al. 1997) and a recently reported sequence from TNV (Johansen et al. 2004). High bootstrap support values were obtained for the division into the major groupings for the phylogenetic analysis, and construction of phylogenetic trees using maximum parsimony and minimum evolution methods for nucleic acid sequences and deduced amino acid sequences resulted in very similar results (data not shown).

DISCUSSION

In this paper, we describe the sequence comparisons and phylogenetic relationships of partial nucleic acid and deduced amino acid sequences from the T2 or T4 region of the coat protein gene from 8 betanodavirus isolates from 6 economically impor-

Table 3. Percent nucleotide sequence (upper right) and deduced amino acid sequence (lower left) identities of the Australian nodavirus isolates, based on the 289 bp sequence of the partial T4 region of the coat protein gene. Isolate details are given in Table 1

Isolate	T352514	T442286	T540844	T99180614	T156700	T256901	T452934	T451722
				Nucleotide identity (%)				
T342514		99.7	99.3	96.2	98.6	99.3	86.5	86.5
T442286	98.9		99.6	96.5	99.0	99.7	86.2	86.2
T540844	97.9	99.0		96.2	98.6	99.3	85.8	85.8
T99180614	91.6	92.6	91.6		96.9	96.2	87.2	87.9
T156700	96.8	97.9	96.8	91.7		98.6	86.5	86.5
T256901	97.8	99.0	97.9	90.6	96.8		85.8	85.8
T452934	70.5	69.5	68.4	71.9	69.5	68.4		98.6
T451722	69.5	68.4	67.3	72.9	68.4	67.4	96.8	
				Deduced amino acid identity (%)				

tant cultured fish species in Australia. With the exception of the striped trumpeter isolate (T256901), which was obtained using nested RT-PCR testing of broodstock blood, all isolates were from clinically affected fish. The presence of betanodavirus had been determined based on observation of a vacuolating neuronal necrosis in histological sections, previously positive nested RT-PCR test results and/or positive IHCT using polyclonal antibodies raised against the recombinant coat protein prepared from a Queensland barramundi betanodavirus isolate (Moody et al. 2004). All of the Australian betanodavirus isolates were of the RGNNV genotype proposed by Nishizawa et al. (1995, 1997), although they occurred in 2 distinct clusters. Those in Cluster Ia, which are described by Thiéry et al. (2004), contained isolates from Queensland (T342541, T540844 and T99180614), the Northern Territory (T156700), and Tasmania (T256901) (Fig. 3). The similarity of the barramundi isolates from Queensland and the Northern Territory is not surprising given their geographic proximity, the natural host range of wild barramundi (Grey 1987), and the degree of translocation of stocks within this region of Australia. The similar geographic distributions of barramundi cod and gold-spotted rockcod probably account for the similarity to these barramundi isolates. They are all from tropical marine fish species. The sleepy cod isolate, although from a tropical species, was obtained from a freshwater facility with no association with marine finfish; no previously reported VNN and no subsequent mortalities have been reported for this species. The source of infection and distribution of this isolate is unknown. The inclusion of the striped trumpeter isolate from Tasmania in the RGNNV genotype, Cluster Ia subtype, is also interesting as this is a cooler water, temperate species (Morehead et al. 2000) and most isolates in this genotype are from tropical species.

The Australian bass isolate from New South Wales (T451722) and the barramundi isolate from South Aus-

tralia (T452934) also fell within the RGNNV genotype, although these isolates formed a second distinct cluster, Cluster Ic, with Ba94Aus (Fig. 3). This indicates that within Australia, at least 2 distinct strains of betanodavirus are present and barramundi are susceptible to both. The distinct clustering observed in these isolates is similar to the grouping of isolates from France (X199, Y55, V67 and Y235) that formed a separate subgroup within the RGNNV genotype, which was designated as Cluster Ib (Thiéry et al. 2004). Clustering of isolates within genotypes has also been described after phylogenetic analysis of SJNNV isolates from the Iberian Peninsula, where 2 distinct clusters designated as IVa and IVb were reported (Cutrín et al. 2007). Nylund et al. (2008) identified 3 clusters, which are designated as Atlantic halibut nervous necrosis virus (AHNNV), cod nervous necrosis virus (GMNNV) and ACNNV, within the BFNNV genotype after phylogenetic analysis of betanodavirus isolates from Norway. The 3 BFNNV clusters are very similar to the IIa, IIb and IIc clusters within the BFNNV genotype described by Thiéry et al. (2004), and adopted in Fig. 3. While the T4 nested RT-PCR successfully amplified the appropriate sequence for all isolates tested, the resulting placement of the Australian betanodavirus isolates into 2 distinct subtypes of the RGNNV genotype was unexpected. This highlights the need to routinely sequence amplicons, especially when diagnosis is based solely on results from PCR-based tests, to ensure that any genetic variation can be determined. This is particularly important when nodavirus infection is suspected in a new species or from a new geographic location.

The phylogenetic analysis of partial coat protein gene sequences in this paper agrees with the classification of betanodavirus isolates into at least 5 genotypes (Figs. 2 & 3) similar to those of previous studies (Nishizawa et al. 1997, Dalla Valle et al. 2001, Skliris et al. 2001, Johnson et al. 2002, Chi et al. 2003, Hegde et al. 2003, Gagné et al. 2004, Johansen et al. 2004,

Thiéry et al. 2004, Cutrín et al. 2007). These genotypes include the 4 (BFNNV, SJNNV, RGNNV and TPNNV) originally proposed by Nishizawa et al. (1995) and an additional genotype, TNV, proposed by Johansen et al. (2004). Based on the classification system described by Thiéry et al. (2004), the TNV genotype corresponds to Cluster V (Figs. 2 & 3). Two Australian isolates form a new subtype, Cluster Ic (Fig. 3). Within the *Betanodavirus* genus, the RGNNV genotype contains isolates from the widest host range (over 20 different species) with the greatest geographic distribution (over 10 countries, or regions, throughout the northern and southern hemispheres) including Asia, Europe, the Mediterranean, the USA (Nishizawa et al. 1995, 1997, Curtis et al. 2001, Dalla Valle et al. 2001, Lin et al. 2001, Skliris et al. 2001, Chi et al. 2003, Hegde et al. 2003, Thiéry et al. 2004) and Australia. The detection of representatives of the other betanodavirus genotypes has been more limited although reporting of isolates in these genotypes is increasing, possibly due to increased awareness of betanodavirus, availability of more sensitive test protocols, and an increase in the number of species cultured. Members of the BFNNV genotype have been detected from at least 5 predominantly colder water species in France, Japan, North America, Norway and Scotland (Nishizawa et al. 1997, Grotmol et al. 2000, Starkey et al. 2000, Johnson et al. 2002, Thiéry et al. 2004). Isolates of the SJNNV genotype were originally found only in 4 species from Japan (Nishizawa et al. 1997) but have subsequently been reported from 5 species in Spain and Portugal (Thiéry et al. 2004, Cutrín et al. 2007). The TPNNV genotype is represented by only one isolate from *Takifugu rubripes* from Japan (Nishizawa et al. 1997) as is the TNV genotype which is represented by an isolate from an outbreak in *Scophthalmus maximus* from Norway (Johansen et al. 2004). Amplification and sequencing of the T2 region of additional betanodavirus isolates, including those in this study that could only be sequenced from the partial T4 region, will assist in the determination of genotypic boundaries. Due to improved biosecurity measures and subsequent elimination of the infection, or resource constraints, no confirmatory sampling, testing and sequencing could be undertaken from the facilities where the samples were originally obtained. Sequencing of additional isolates from the same facilities and regions within Australia as those reported in this paper will enhance our knowledge of strain variability within and among species, and clarify the relationships between isolates of the different subtypes within the RGNNV genotype.

Investigations of the relationships between betanodavirus isolates comparing viral RNA1 sequences were limited to isolates from Japan and Singapore, with results being in agreement with RNA2 phylogeny (Mori et al. 1992, Nagai & Nishizawa 1999, Tan et al. 2001). However, RNA1 sequences for additional isolates from Europe and the Pacific region have recently been determined and compared (Névarez et al. 2004, Toffolo et al. 2007). Results show that while the RNA1 and RNA2 sequences were of the same genotype for the majority of isolates studied, reassortment between genotypes had occurred in a small number of isolates. As a result, depending on the gene used in the phylogenetic analysis (RNA1 or RNA2), these isolates could be attributed to different genotypes. Therefore, sequencing of both RNA1 and RNA2 is required to completely characterise new isolates. Amplification and sequence comparison of RNA1 of the Australian isolates described in this study is important to clarify the relationships between isolates in the different clusters within the RGNNV genogroup. Sequencing of both genes could also provide early warning of the presence of a previously undetected genotype. However, until a consensus has been reached regarding which RNA strand is more appropriate for phylogenetic classification, and more RNA1 sequences are available, the current standard for phylogenetic determination using RNA2 sequences should be maintained.

Further biological characterisation of the isolates will add important information about the characteristics of the different genotypes. Cross-species infectivity trials have identified differences in susceptibility of certain species to isolates of different genotypes (Totland et al. 1999, Grotmol et al. 2000) and Iwamoto et al. (2004) reported that this host specificity was determined by RNA2. Different optimum growth temperatures in cell culture have been observed for isolates of different genotypes (Iwamoto et al. 2000), and results from neutralisation studies using genotype-specific monoclonal or polyclonal antibodies have identified serotypes that are similar to the genotypes identified by phylogenetic analysis based on the RNA2 gene (Skliris et al. 2001, Chi et al. 2003, Mori et al. 2003). These characteristics have yet to be determined for the majority of the Australian betanodavirus isolates. Full molecular and biological characterisation of betanodavirus isolates, including complete sequencing of the 2 segments of the genome, requires cross-species infectivity trials as well as temperature sensitivity and neutralisation studies. Knowledge of these features will enable optimisation of detection tests and improved management procedures to reduce the impact of these viruses to established and emerging aquaculture facilities and to the environment. More complete characterisation will also assist in determining whether the distinct Clusters Ia, Ib (Thiéry et al. 2004) and Ic, which are observed within the RGNNV genotype, are really subtypes or should be considered as different genotypes.

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