

Identification of a Betanodavirus Isolated from Viral Nervous Necrosis Disease in Red-Spotted Grouper (*Epinephelus coioides*) Cultured in Thailand using PCR and Sequence Analysis

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ABSTRACT

A viral agent was successfully isolated from red-spotted grouper fry (*Epinephelus coioides*) exhibiting clinical signs of viral nervous necrosis (VNN) in southern Thailand in November 2001. Diseased fish (~1.5-2 cm in total length) were collected from the wild and nursery farms. The VNN disease usually occurred within a few days after stocking of the wild seed, mortality reaching 100%. Eyes and brains of diseased fish were pooled, extracted and filtered through 0.45 µm disposable filters. The filtrates were inoculated on to striped snakehead whole fry tissue or SSN-1 cells and incubated at 28°C. Cytopathic effect (CPE), recognized as shrunken and rounded cells, was first observed on day 3 post-inoculation. The infected cells continued to aggregate and the CPE was complete on day 7. Electron micrographs of the infected SSN-1 cells revealed icosahedral nucleocapsid virions, 25 nm in diameter. Viral harvests were resistant to chloroform or 5-iodo-2-deoxyuridine (IUDR) treatments, which indicated that they were naked and contained RNA genome. These characteristics classified the virus as a member of family *Nodaviridae*. Identification using reverse transcription polymerase chain reaction (RT-PCR) and sequence analysis of PCR product was conducted. RT-PCR amplification using primers designed from betanodavirus genotype RGNNV (GenBank #D38636.1) gave a single PCR product at 770 bp in agarose gel. The specific primers for genotype SJNNV gave RT-PCR products at 426 bp and other non-specific product bands. One 770 bp PCR product was sequenced in both directions. Nucleotide homology of new isolated nodavirus were 98% (720/730) similar to those of genotype RGNNV, 83% (606/729) to genotype BFNNV, 78% (574/735) to genotype SJNNV and 77% (568/735) to genotype TPNNV. It is therefore concluded that the new virus isolate is the first isolate of betanodavirus genotype RGNNV in Thailand.

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INTRODUCTION

Viral nervous necrosis virus (VNN) or viral encephalopathy and retinopathy (VER) has been reported in many cultured marine fish species worldwide. The etiological agent of the disease is a member of genus *Betanodavirus* that usually causes high mortality in fish fry. The affected fish exhibited darkening of body colour, no response to feed and swirling movement. Over 22 fishes species from 11 families have been found to be affected with large losses recorded in sea bass, (*Dicentrarchus labrax*), groupers (*Epinephelus akaara*, *E. fuscogutatus*, *E. malabaricus*, *E. septifasciatus*, *E. tauvina*), jacks (*Pseudocaranx dentex*, *Seriola dumerili*), parrotfish (*Oplegnathus fasciatus*) and flatfishes (*Verasper moseri*, *Hippoglossus hippoglossus*, *Paralichthys olivaceus*, *Scophthalmus maximus*) (OIE, 2000). In Thailand, the VNN clinical signs might have been first recognized in 1982 in wild and hatchery seeds of grouper (*Epinephelus coioides*). However, the causative agent could not be identified at that time (Danayadol *et al.*, 1995). The VNN disease was first confirmed when the disease caused severe losses in brown-spotted grouper fry (*E. malabaricus*) in mid-1990's in southern Thailand. The diseased fry showed vacuolation and necrotic cells in the brain and retina. Virions were also found in the affected tissue (Danayadol *et al.*, 1995). Almost every year this disease still occurs in grouper culture.

Betanodavirus isolation in cell culture has been found difficult in the past because of the lack of susceptible cell lines. Several methods have been developed to detect betanodaviruses such as enzyme-linked immunosorbent assay (ELISA) (Arimoto *et al.*, 1992), immunohistochemistry (Le Breton *et al.*, 1997), fluorescent antibody technique (FAT) (Nguyen *et al.*, 1994), reverse transcription-polymerase chain reaction (RT-PCR) (Nishizawa *et al.*, 1994) and *in situ* hybridization (Comps *et al.*, 1996). The first susceptible fish cell line for betanodavirus isolation was SSN-1 cell line, which was derived from snakehead fish, *Channa striata* (Frerichs *et al.*, 1996). Iwamoto *et al.* (1999) demonstrated that the SSN-1 cell line could support the growth of all betanodavirus genotypes.

In this report we described the isolation of a betanodavirus from wild and nursery farmed red-spotted grouper (*E. coioides*) using SSN-1 cell line. The isolated virus was identified using RT-PCR technique and sequence analysis.

MATERIALS AND METHODS

Fish samples

Moribund red-spotted grouper seed, ~1.5 -2.0 cm in total length, with clinical signs were collected from the wild and private grouper farms in southern Thailand during November-December 2001. Eyes and brain of fish specimens were divided in two parts, one part was fixed in 10% phosphate-buffered formalin for histopathological examination and the second part was used for viral isolation.

Viral isolation

Brains and eyes of diseased fish were pooled, homogenised in pre-cooled pestles and mortars and diluted to 10⁻¹ in Hank's balanced salt solution (HBSS, Gibco,UK) supplemented with 2% fetal bovine serum (FBS). The homogenate was then centrifuged at 2000 X g for 15 min at 4°C. The supernatant was diluted further 1:2 then filtered through 0.45 µm disposable

filters. The tissue extracts were inoculated on to fresh SSN-1 cells in 24 well plates (Nunc, Denmark). The cells were maintained in Leibovitz-15 (L-15) medium (Gibco, UK) containing 10% FBS at 28°C. Cytopathic effect (CPE) was observed daily for 2 weeks. Two blind passages were performed in fresh SSN-1 cells after days 7-10 from the previous inoculation.

Viral characterisation

Viral morphology. 200 µl viral suspension with an original titre 10^8 TCID₅₀ ml⁻¹ was used to inoculate on to a pre-formed monolayer of SSN-1 cells in 25 cm² cell culture flask at 28°C. After 24 h, the infected cells were removed by a scraper (Nunc, Denmark), pelleted by centrifugation and fixed in 4% paraformaldehyde. The fixed cell pellets were rinsed with 0.1 M phosphate buffer pH 3.0 and post-fixed in 1% osmium tetroxide (OsO₄), dehydrated and embedded in epoxy resin. Blocks of cell pellets were cut at 80 nm and stained using uranyl acetate-lead citrate and examined with a JEM-100 electron microscope.

Envelope testing. Sensitivity of the viral isolate to lipid solvents was determined by the method of Burleson *et al.* (1992). One milliliter of viral suspension (10^8 TCID₅₀ ml⁻¹) was combined with either 0.5 ml chloroform or 0.5 ml HBSS and incubated for 15 min. Virus titrations were determined from aqueous phase of the treated preparation and from the HBSS-treated viral control.

Type of nucleic acid: The effect of 5-iodo-2-deoxyuridine (IUDR) on viral replication was determined by the method of Rovozzo and Burke (1973). Briefly, a pre-formed monolayer of SSN-1 cells in a 96-well plate was treated with 10⁻⁴ M IUDR in HBSS or with HBSS alone for 4 h. Then the viruses were inoculated on to the cells with a series of 10-fold dilutions. After 3 h incubation, the supernatant containing IUDR was removed and 0.2 ml of L-15 medium was added to each well. The plate was incubated at 28°C for 7 day and the viral titre determined.

Virus identification

Total RNA extraction and RT-PCR: Virus-infected SSN-1 cells were extracted using QIAmp Viral RNA Mini kit (Qiagen, UK). The total RNA was used directly for RT-PCR detection using an OneStep RT-PCR kit (Qiagen, UK). One primer set SJNNV426 (forward 5'CGT-GTC-AGT-CAT-GTG-TCG-CT and reverse 5' CGA-GTC-AAC-ACG-GGT-GAA-GA) was used as suggested by Nishizawa *et al.* (1994). Another primer set RGNNV770 (forward 5'GGG-ACA-GGA-ACA-GAC-GGA-TA, reverse 5' AAC-AGG-CAG-CAG-GAT-TTG-AC) was designed from the betanodavirus genotype RGNNV nucleotide sequence from GenBank accession #D38636. The expected RT-PCR products using primers SJNNV426 and RGNNV770 were 426 bp and 770 bp respectively.

Sequence analysis: One 770 bp PCR product was cut from 1% low-melting agarose gel then purified by silica adsorption using a QIAquick gel extraction kit (Qiagen, UK). The purified DNA was sequenced in both directions using primer set RGNNV770 and ABI cycle sequencer dRhodamine Big Dye kit. The reaction product was analysed on an ABI model 377 automated sequencer (Bioservice Unit NSTDA, Bangkok). Nucleotide sequences of the PCR product were compared to the known sequences in the GenBank using BLAST program (Altschul *et al.*, 1997).

RESULTS

Fish samples

The moribund grouper fry exhibited dark body coloration and lethargy (Fig.1). Fish swam up and down with corkscrew swimming motion through the water column. The digestive tracts were devoid of food and had no obvious pathological lesions. Histopathological examination revealed vacuoles in the plexiform layer of retina and in various parts of brains (Fig. 1).

Virus isolation

CPE began to develop in SSN-1 cells at day 3 post-inoculation with tissue extract of diseased grouper fry. The infected cells shrunk, rounded up and then aggregated. The CPE advanced and was complete on day 7 post-inoculation (Fig. 2). The CPE can be transmitted after subculture to fresh SSN-1 cells.

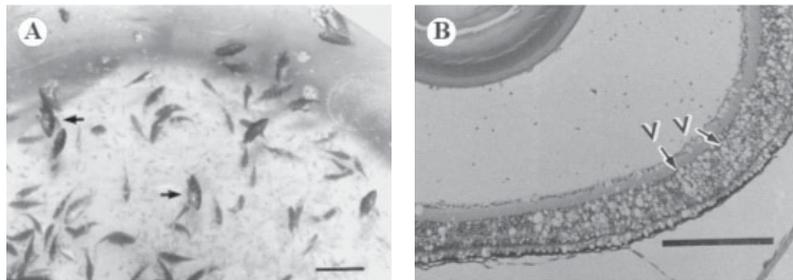


Figure 1. **A.** Diseased fish showing paralytic syndrome; dark body coloration, corkscrew swimming and floating on the water surface. **B.** Severe vacuolation (arrows) in plexiform layer of the retina. H&E, Bar =20 µm.

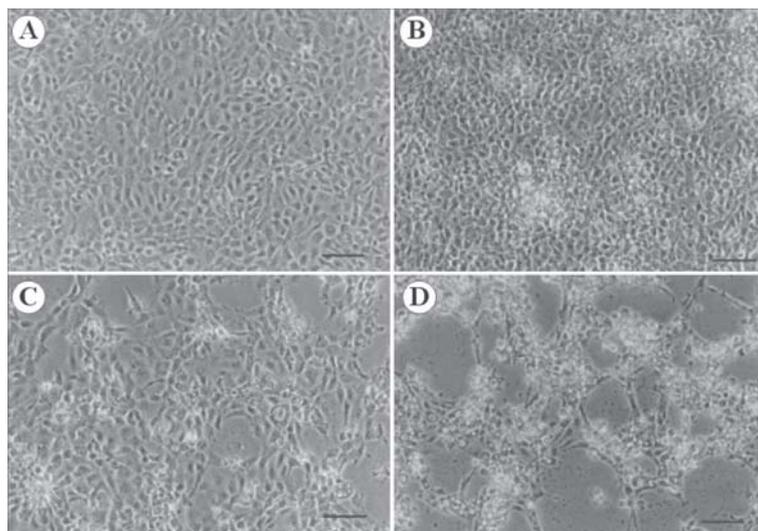


Figure 2. The cytopathic effects (CPE) caused by new virus isolate in SSN-1 cells at different periods post-inoculation (p.i.). **A.** Normal SSN-1 cells. **B.** 3 days p.i. **C.** 5 days p.i. **D.** 6 days p.i. Bars = 20 µm.

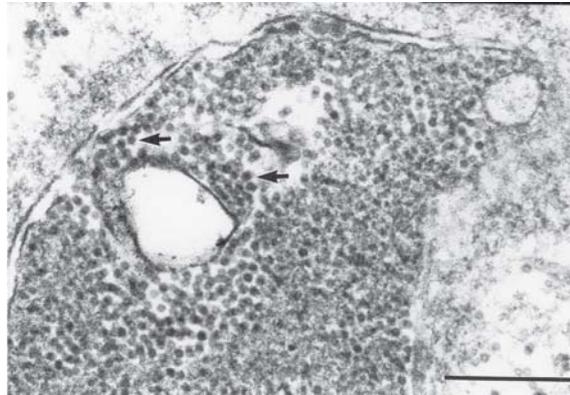


Figure 3. Electron micrographs of SSN-1 cells fixed 24 hr after inoculation with cultivated virus. Aggregates of virion (arrows) are located in membrane-bound structures. Bar = 500 nm.

Virus characterisation

Electron micrographs of the new virus isolate in SSN-1 cells revealed icosahedral to spherical non-enveloped virions 25 nm diameter. Viral assembly was seen in the cytoplasm. Large areas of crystalline arrays were observed in the cytoplasm (Fig. 3). Viral propagations were resistant to chloroform and IUDR treatments, which indicated a naked virions and a RNA genome. These 3 characteristics indicated the isolated virus can be preliminary classified as a member of *Nodaviridae*, *Picornaviridae* or *Caliciviridae*. The virus propagated well in SSN-1 cells and the highest viral titre recorded was 10^8 TCID₅₀ ml⁻¹ in 25 cm² tissue culture flask.

Table 1. Nucleotide sequence identity comparisons of capsid protein gene among new isolated nodaviruses from Thailand and other selected nodavirus sequences in the GenBank database using the BLAST program and Genetyx program.

GenBank accession codes	Nucleotide sequence identity (%)	Nodaviruses compared	References
Malaba.AF245003.1	718/730 (98%)	Malabaricus nervous necrosis virus	Lin <i>et al.</i> , 2001
Dragon AF245004.1	720/730 (98%)	Dragon nervous necrosis virus	Lin <i>et al.</i> , 2002
Epinep.AF318942	718/730 (98%)	Epinephelus tauvina nervous necrosis virus	Tan <i>et al.</i> , 2002
RGNNV D38636.1	720/730 (98%)	Red-spotted grouper nervous necrosis virus	Nishizawa <i>et al.</i> , 1995
BFNNV D38635.1	606/729 (83%)*	Barfin flounder nervous necrosis virus	Nishizawa <i>et al.</i> , 1995
SJNNV D30814.1	574/735 (78%)*	Striped jack nervous necrosis virus	Nishizawa <i>et al.</i> , 1995
TPNNV D38637.1	568/735 (77%)*	Tiger puffer nervous necrosis virus	Nishizawa <i>et al.</i> , 1995

* Nucleotide homology comparison using Genetyx Program.

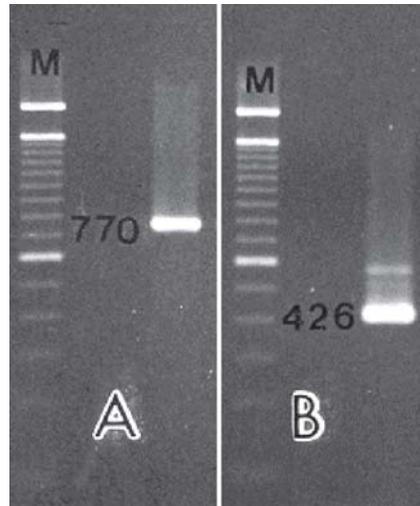


Figure 4. Agarose gel electrophoresis of the products by PCR amplification using specific primers designed from genotype RGNNV (A) and genotype SJNNV (B).

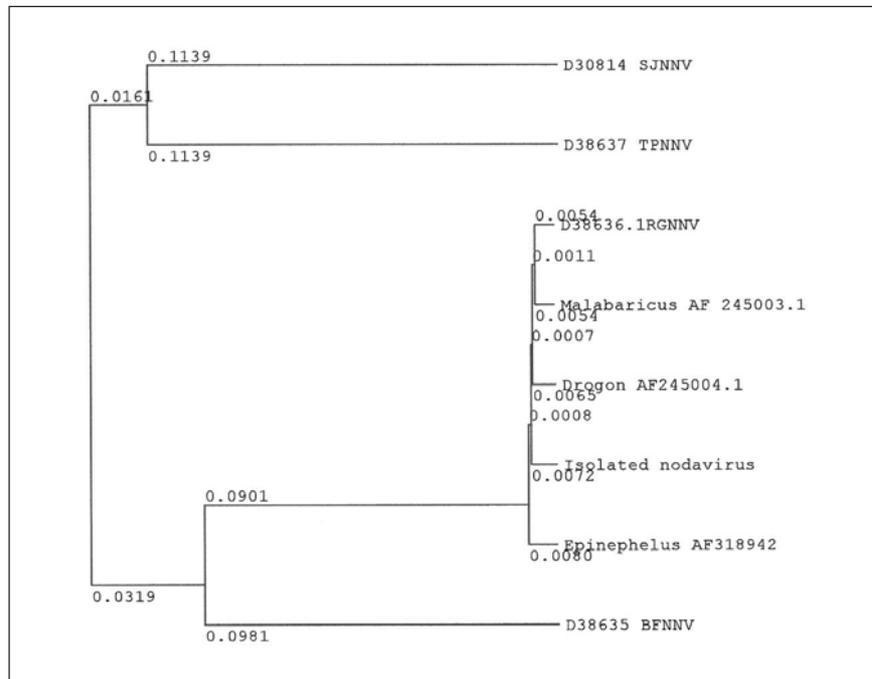


Figure 5. An evolution tree of the new nodavirus isolate from red-spotted grouper and other 4 genotypes, SJNNV, TPNNV, BFNNV and RGNNV of nodaviruses. The tree was created by comparing partial nucleotide sequences of capsid protein gene using UPGMA method of Gentyx program.

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Isolated nodavirus	1:-----AACAGACGGATACGTTGTTGTTGACGCAACCATGGTCCCGACTTCCTGCCACGA	55
RGNNV D38636.1	56: C C C	115
Malaba.AF245003.1	228: T C C	287
Dragon AF245004.1	228: C C C	287
Epinep.AF318942	240: C C C	299
Isolated nodavirus	56:CTGGAACATGCTGCTAGAATCTTCCAGCGATACGCTGTTGAAACACTGGAGTTGAAATT	115
RGNNV D38636.1	116: G C A	175
Malaba.AF245003.1	288: G C G	347
Dragon AF245004.1	288: G C A	347
Epinep.AF318942	300: G C A	359
Isolated nodavirus	116:CAGCCAATGTGCCCGCAAACACGGGCGGTGGTTACGTTGCTGGCTTCCTGCCTGATCCA	175
RGNNV D38636.1	176: C	235
Malaba.AF245003.1	348: C	407
Dragon AF245004.1	348: C	407
Epinep.AF318942	360: T	419
Isolated nodavirus	176:ACTGACAACGATCACACCTTCGACGCGCTTCAAGCAACTCGTGGTGCAGTCGTTGCCAAA	235
RGNNV D38636.1	236: T	295
Malaba.AF245003.1	408: T	467
Dragon AF245004.1	408: C	467
Epinep.AF318942	420: T	479
Isolated nodavirus	236:TGGTGGGAAAGCAGAACAGTCCGACCTCAGTACACCCGCACGCTCCTCTGGACCTCGTCG	295
RGNNV D38636.1	296: T	355
Malaba.AF245003.1	468: A	527
Dragon AF245004.1	468: T	527
Epinep.AF318942	480: T	539
Isolated nodavirus	296:GGAAAGGAGCAGCGTCTCACGTACCTGGTCGGCTGATACTCCTGTGTGTCGGCAACAAC	355
RGNNV D38636.1	356: T	415
Malaba.AF245003.1	528: C	587
Dragon AF245004.1	528: C	587
Epinep.AF318942	540: C	599
Isolated nodavirus	356:ACTGATGTGGTCAACGTGTCACTGCTTGTGCTGGAGTGTTCGACTGAGCGTTCCATCT	415
RGNNV D38636.1	416: G T	475
Malaba.AF245003.1	588: G C	647
Dragon AF245004.1	588: G C	647
Epinep.AF318942	600: G C	659
Isolated nodavirus	416:CTTGAGACACCTGAAGAGACCACCGCTCCCATCATGACACAAGGTTCCCTGTACAACGAT	475
RGNNV D38636.1	476: T	535
Malaba.AF245003.1	648: T	707
Dragon AF245004.1	648: T	707
Epinep.AF318942	660: G	719
Isolated nodavirus	536:CCTGATGGAGCAGTCTTCCAGCTGGATCGTCCGCTGTCCATTGACTACAGCCTTGGAACT	595
RGNNV D38636.1	596: C T	655
Malaba.AF245003.1	768: C T G	827
Dragon AF245004.1	768: T A G	827
Epinep.AF318942	780: C T T	839
Isolated nodavirus	596:GGAGATGTTGACCGTGTGTTTATTGGCACCTCAAGAAGTTGCTGGAAATGCTGGCACA	655
RGNNV D38636.1	656: T A C	715
Malaba.AF245003.1	828: C C C	887
Dragon AF245004.1	828: C C C	887
Epinep.AF318942	840: T C T	899
Isolated nodavirus	656:CCTGCAGGCTGTTTCGCTGGGGCATCTGGGACAACCTTTAAACAAGACGTTACAGATGGC	715
RGNNV D38636.1	716: C C	775
Malaba.AF245003.1	888: C C	947
Dragon AF245004.1	888: C C	947
Epinep.AF318942	900: T T	959
Isolated nodavirus	716:GTTGCCTACTACTCT	730
RGNNV D38636.1	776: C	830
Malaba.AF245003.1	948: C	1007
Dragon AF245004.1	948: C	1007
Epinep.AF318942	960: T	1019

Figure 6. Alignment comparison between an isolated nodavirus and other known nodavirus genotype RGNNV from GenBank. The isolated nodavirus showed minor variation with ~98% nucleotide sequence identity.

RT-PCR amplification and sequence analysis

RT-PCR amplification using specific primers designed from betanodavirus genotype RGNNV (GenBank #D38636.1) gave a single PCR product at ~770 bp in agarose gel while an amplification using specific primers designed from genotype SJNNV gave product at ~ 426 bp and other non-specific product bands (Fig. 4). One 770 bp PCR product was sequenced in both directions and 730 nucleotide bases could be read. Sequence analysis of this product using BLAST program showed 98% (720/730) nucleotide homology to published nucleotide sequences of betanodaviruses, genotype RGNNV (red-spotted grouper nervous necrosis virus), 83% (606/729) nucleotide homology to genotype BFNNV (barfin flounder nervous necrosis virus), 78% (574/735) homology to genotype SJNNV (striped jack nervous necrosis virus) and 77% (568/735) to genotype TPNNV (tiger puffer nervous necrosis virus) (Table 1). An evolution tree comparing partial nucleotide sequences of capsid protein gene of the new nodavirus isolate and other 4 genotypes, SJNNV, TPNNV, BFNNV and RGNNV of nodaviruses indicated showed that the new nodavirus isolate could be grouped as a member of the genotype RGNNV (Fig. 5). Multiple alignments of viruses within genotype RGNNV showed minor variation of nucleotides as indicated in Fig. 6.

DISCUSSION

This is the first report of a betanodavirus isolated from diseased red-spotted grouper fry in Thailand. The viral isolation was achieved using the SSN-1 cell line. The isolated betanodavirus could be grouped as a member of the genotype RGNNV according to RT-PCR amplification and sequence analysis. The cytopathic effect (CPE) was characterised by shrinkage, rounding and refractile cells that developed within 3 days after inoculation at 28°C. The CPE was complete about 7 days post-inoculation.

Viral nervous necrosis (VNN) disease has been found to be a major problem in farms or nurseries that cultured or maintained wild seed of grouper causing high losses in government-owned grouper hatcheries in Thailand. There is a need to develop tools for prevention and control of the disease to help sustainable grouper aquaculture. However, betanodaviruses seem complicated as they have at least 4 different genotypes: tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV), barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV) genotypes (Nishizawa *et al.*, 1997). Findings in this study indicate that this betanodavirus was a member of genotype RGNNV. The RGNNV seemed to spread through east and south coasts of Thailand, as the same genotype could be repeatedly isolated from the diseased grouper fry (Kanchanakhan *et al.*, 2002). The RGNNV may be more prevalent or is more virulent in warm water condition (25-38°C in Thailand), as the virus caused severe mortality in grouper fry during the summer in Chinese Taipei and PR China at temperatures ranging between 25-32°C (Chi *et al.*, 1999a; Lin *et al.*, 2001). In this study, we found the RGNNV propagated well at 28°C, which is in general agreement with other in vitro viral propagation studies (Chi *et al.*, 1999; Iwamoto *et al.*, 2000).

The primer set RGNNV770 has been found useful for PCR detection of VNN disease in grouper fry in Thailand. There is an opportunity to develop specific inner primer set from the 770 bp nucleotide product to develop a more sensitive detection, nested PCR for detection of betanodaviruses in very low amounts that would benefit screening for carriers.

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