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Ultrastructural and sequence characterization of *Penaeus vannamei* nodavirus (*Pv*NV) from Belize

Kathy F. J. Tang*, Carlos R. Pantoja, Rita M. Redman, Solangel A. Navarro, Donald V. Lightner

Department of Veterinary Science and Microbiology, University of Arizona, Tucson, Arizona 85721, USA

ABSTRACT: The *Penaeus vannamei* nodavirus (*Pv*NV), which causes muscle necrosis in *Penaeus vannamei* from Belize, was identified in 2005. Infected shrimp show clinical signs of white, opaque lesions in the tail muscle. Under transmission electron microscopy, the infected cells exhibit increases in various organelles, including mitochondria, Golgi stacks, and rough endoplasmic reticulum. Cytoplasmic inclusions containing para-crystalline arrays of virions were visualized. The viral particle is spherical in shape and 19 to 27 nm in diameter. A cDNA library was constructed from total RNA extracted from infected shrimp. Through nucleotide sequencing from the cDNA clones and northern blot hybridization, the *Pv*NV genome was shown to consist of 2 segments: RNA1 (3111 bp) and RNA2 (1183 bp). RNA1 contains 2 overlapped open reading frames (ORF A and B), which may encode a RNA-dependent RNA polymerase (RdRp) and a B2 protein, respectively. RNA2 contains a single ORF that may encode the viral capsid protein. Sequence analyses showed the presence of 4 RdRp characteristic motifs and 2 conserved domains (RNA-binding B2 protein and viral coat protein) in the *Pv*NV genome. Phylogenetic analysis based on the translated amino acid sequence of the RdRp reveals that *Pv*NV is a member of the genus *Alphanodavirus* and closely related to *Macrobrachium rosenbergii* nodavirus (*Mr*NV). In a study investigating potential *Pv*NV vectors, we monitored the presence of *Pv*NV by RT-PCR in seabird feces and various aquatic organisms collected around a shrimp farm in Belize. *Pv*NV was detected in mosquitofish, seabird feces, barnacles, and zooplankton, suggesting that *Pv*NV can be spread via these carriers.

KEY WORDS: *Penaeus vannamei* nodavirus · *Pv*NV · Ultrastructure · Genomic sequencing · Mechanical carriers

INTRODUCTION

The *Penaeus vannamei* nodavirus (*Pv*NV) is the causative agent of muscle necrosis observed in *P. vannamei* cultured in Belize (Tang et al. 2007). The infection resulted in 50% production loss in affected shrimp ponds. The infected shrimp showed clinical signs of white, opaque lesions in the tails. Histological examination revealed multifocal necrosis and hemocytic fibrosis in the skeletal muscle. Cytoplasmic inclusions were found in the lymphoid organ, muscle, and connective tissues. We determined *Pv*NV to be a nodavirus by partially sequencing the capsid protein-coding region of the virus genome (Tang et al. 2007). There are 2 genera in the *Nodaviridae* family (Schneemann et al. 2005): *Alphanodavirus*, which primarily infects insects, and *Betanodavirus,* which infects fishes. The *Pv*NV sequence is similar that of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) an alphanodavirus which infects the freshwater prawn *M. rosenbergii* (Bonami et al. 2005), so *Pv*NV is also presumed to be an alphanodovirus.

Nodaviruses are small, non-enveloped, icosahedral viruses approximately 25 to 33 nm in diameter (Munday & Nakai 1997, Schneemann et al. 2005). Nodaviruses have a genome of around 4.5 kb consisting of 2 molecules of positive-sense, single-stranded (ss) RNA: RNA-1 (3.1 kb) and RNA-2 (1.4 kb). Infected cells also contain a third ssRNA (RNA3), which is not packaged within the virion. RNA1 encodes the viral

*Email: fengjyu@u.arizona.edu

RNA-dependent RNA polymerase (RdRp), and RNA2 encodes the capsid protein. RNA3 encodes 1 or 2 small proteins (designated B1 and B2). The function of B1 is not known, but B2 is a double-stranded RNA-binding protein described in other nodaviruses, such as *Flock House virus* (FHV) and *Greasy grouper nervous necrosis virus* (GGNNV), to act as a suppressor against host antiviral RNA-silencing response (Li et al. 2002, Lingel et al. 2005, Fenner et al. 2006). However, whether the B2 of *Pv*NV functions similarly in the shrimp cells is not yet known.

In the present study, we examined the morphogenesis of *Pv*NV under transmission electron microscopy (TEM), sequenced its genome (both RNA1 and RNA2), and determined the taxonomic status of the virus. In addition, we investigated potential carriers of *Pv*NV collected from shrimp ponds.

MATERIALS AND METHODS

***Pv*NV-infected shrimp.** Shrimp taxonomy was according to Holthius (1980). The *Pv*NV-infected *Penaeus vannamei* used in this study were generated by laboratory infection from an isolate obtained from a farm in Belize in 2005. The preparation of inoculum and laboratory infection procedure were described by Tang et al (2007).

Electron microscopy study. Laboratory-infected shrimp were sampled for electron microscopy study; they were preserved in 6% phosphate-buffered glutaraldehyde and processed for examination as described in Bonami et al. (1992). Electron microscopic observation was performed at 80 kV with a Philips CM12 electron microscope.

RNA extraction, cDNA synthesis and sequence analysis. Hemolymph samples were drawn from the infected shrimp, and subjected to RNA extraction with TRIzol LS (Invitrogen). The RNA was used for construction of a cDNA library (Tang et al. 2007). Each clone was amplified with PCR primers M13F/R (sequences are described in Tang et al. 2005). PCR amplicons were purified and sequenced at the University of Arizona. The nucleotide sequences of RNA1 (larger segment of *Pv*NV genome, GenBank no. HQ259079) and RNA2 (GenBank no. EF137180) were determined by assembling overlapped clones. The determinations of protein molecular mass and isoelectric point (pI) were performed with the European molecular biology open software suite program (EMBOSS) (Rice et al. 2000). The search for open reading frames (ORFs) and conserved domains was performed at the NCBI website (Marchler-Bauer & Bryant 2004, Marchler-Bauer et al. 2009).

The amino acid sequence of the *Pv*NV RNA-dependent RNA polymerase (encoded by ORF A within the RNA1) was used to construct a phylogenetic tree with 17 other nodaviruses (see Table 1). Following multiple sequence alignment, a neighbor-joining phylogenetic tree was constructed (Saitou & Nei 1987). The data were re-sampled by 1000 bootstrap replicates to determine the confidence indices within the tree.

Probe labeling and northern blot hybridization. Two probes were labeled, one probe (*Pv*NV-190) was from a clone containing a 1.7 kb insert (see Fig. 2A for probe target regions within the RNA1). Another probe (*Pv*NV-4) targets the RNA2 region and was described in Tang et al (2007). These 2 gene probes were labeled with digoxigenin-11-dUTP (Roche) by PCR.

Total RNA extracted from hemolymph were separated in a 1% formaldehyde gel. After transferring the RNA to a nylon membrane, the membrane was hybridized with both *Pv*NV-190 and *Pv*NV-4. After washes, the membrane was incubated with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) and then visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Investigation of *Pv*NV carriers by RT-PCR. Aquatic organisms and seabird feces were sampled from a farm in Belize in 2008. The samples included wild shrimp (3 pooled samples, unknown species), barnacles (3 pooled samples, Cirripedia), mangrove crabs (1 pooled sample, unknown species), mosquitofish (1 pooled sample, *Gambusia* spp.), zooplankton (3 pooled samples), and *Penaeus vannamei* broodstock (10 individual shrimp). Total RNA was extracted from each sample using a High-Pure RNA extraction kit (Roche). The rTth DNA polymerase (Applied Biosystems) was used for the first-step RT-PCR detection. The extracted RNA was reverse transcribed at 60°C for 30 min, and the PCR was initiated at 94°C for 2 min, followed by 40 cycles of 94°C for 15 s, 60°C for 1 min, ending with 60°C for 5 min. The sequence of RT-PCR primers (outer primers: *Pv*NV339F/R; inner primers: *Pv*NV246NF/R) and nested PCR procedure are described by Tang et al. (2007).

To determine if the replicative intermediate of *Pv*NV was present in the barnacles, the extracted RNA was denatured at 100°C for 3 min, chilled on ice, then subjected to reverse transcription with one primer, either *Pv*NV339F (will anneal to negative-strand RNA) or *Pv*NV339R (will anneal to positive-strand RNA). The reaction was carried out at 45°C for 30 min with the SuperScript III reverse transcriptase (Invitrogen). The cDNA was then amplified with the inner primers *Pv*NV246F/R. The PCR reaction was initiated at 94°C for 5 min, followed by 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s, ending with 72°C for 7 min. An aliquot of the PCR product was analyzed by 1.5% agarose gel electrophoresis.

RESULTS

Morphogenesis of *Pv*NV in the infected cells

Examination of the *Pv*NV-infected shrimp tissues via TEM revealed the presence of intracytoplasmic inclusions within the cells of the lymphoid organ and of necrotic skeletal muscle. The lymphoid organ tissue sections were used for the ultrastructural characterization. During the early stages of virus infection, cells were observed to have increased numbers of organelles, including mitochondria, Golgi stacks, and rough endoplasmic reticulum. Also, the mitochondria in the proximity of the virogenic stroma were enlarged with either disintegrated or empty cristae (Fig. 1A,B). Membrane invaginations, presumably of endoplasmic reticulum, were observed within the viral inclusions. At the later stages of infection, the nucleocapsids were mature and aggregated in a paracystalline array that was surrounded by a well-defined membrane (Fig. 1C). The viral particle exhibited spherical in shape with a size ranged from 19 to 27 nm in diameter (40 virions were measured) (Fig. 1D).

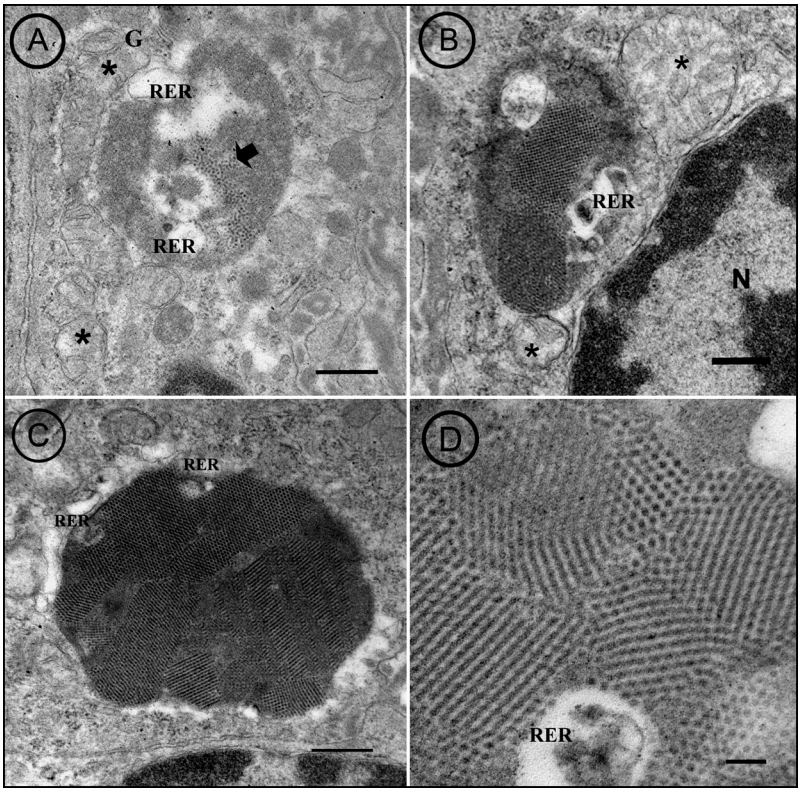

Fig. 1. *Penaeus vannamei* nodavirus (*Pv*NV). Transmission electron micrographs of infected lymphoid organ tissue. (A) Viral inclusion showing early accumulation of viral particles (arrow). Several mitochondria (*) can be observed in close proximity. G: Golgi stacks; RER: rough endoplasmic reticulum. (B) Mature viral particles began to form a paracrystalline array. Mitochondria (*) are enlarged. N: nucleus. (C) Cytoplasmic inclusion in advanced stage of development shows a dense accumulation of viral particles in paracrystalline arrays. (D) High magnification of a viral inclusion shows spherical viral particles. Scale bars = (A–C) 500 nm, (D) 100 nm

*Pv*NV genomic sequence and analysis

A cDNA library was constructed from the RNA extracted from the hemolymph of *Pv*NV-infected shrimp, and approximately 120 clones were obtained. From these, we sequenced and analyzed 20 clones using the basic local assignment search tool (BLASTx) program at the NCBI website. We found that 11 clones contained inserts with sequences similar to *Macrobrachium rosenbergii* nodavirus (*Mr*NV). These sequences were then assembled into 2 genome segments: RNA1 and RNA2 (Fig. 2A).

The larger genome segment (RNA1) consists of 3111 bp based on sequences assembled from 7 overlapped clones. We analyzed this 3111 bp sequence with the BLASTn program (discontiguous megablast algorithm), and this sequence showed 73% identity (E value = 0.00) to the *Mr*NV RNA1 (GenBank no. AY222839). Through an ORF search, RNA1 was found to contain 2 overlapping ORFs. The larger ORF (designated as ORF A, 3102 bp, nucleotide no. 7 to 3108, Fig. 2A) may encode a polypeptide of 1033 amino acids (aa) with a molecular mass of 107 kDa and a pI of 8.3. The BLASTp search of this protein revealed that it had an 83% similarity (E value = 0.00) to the *Mr*NV RdRp gene (GenBank no. AAQ54758) and similarities of 40 to 62% to the RdRp of other nodaviruses (Table 1). The smaller ORF (designated as ORF B, 396 bp, nucleotide no. 2708 to 3103) overlaps with ORF A by a +1 reading frame. ORF B is predicted to encode a polypeptide of 131 aa with a molecular mass of 14 kDa and a pI of 5.2. Through the BLASTp search, the ORF B was found to encode a B2 protein. The *Pv*NV B2 protein is very similar (79%) to that of *Mr*NV, and has less similarity (47 to 58%) to the B2 protein of 5 other alphanodaviruses including *Nodamura virus* (NoV), *Boolarra virus* (BoV), FHV, and *Drosophila melanogaster* American nodavirus (*Dm*ANV).

RNA2 consists of 1183 bp, determined by assembling nucleotide sequences from 4 clones, and contains a

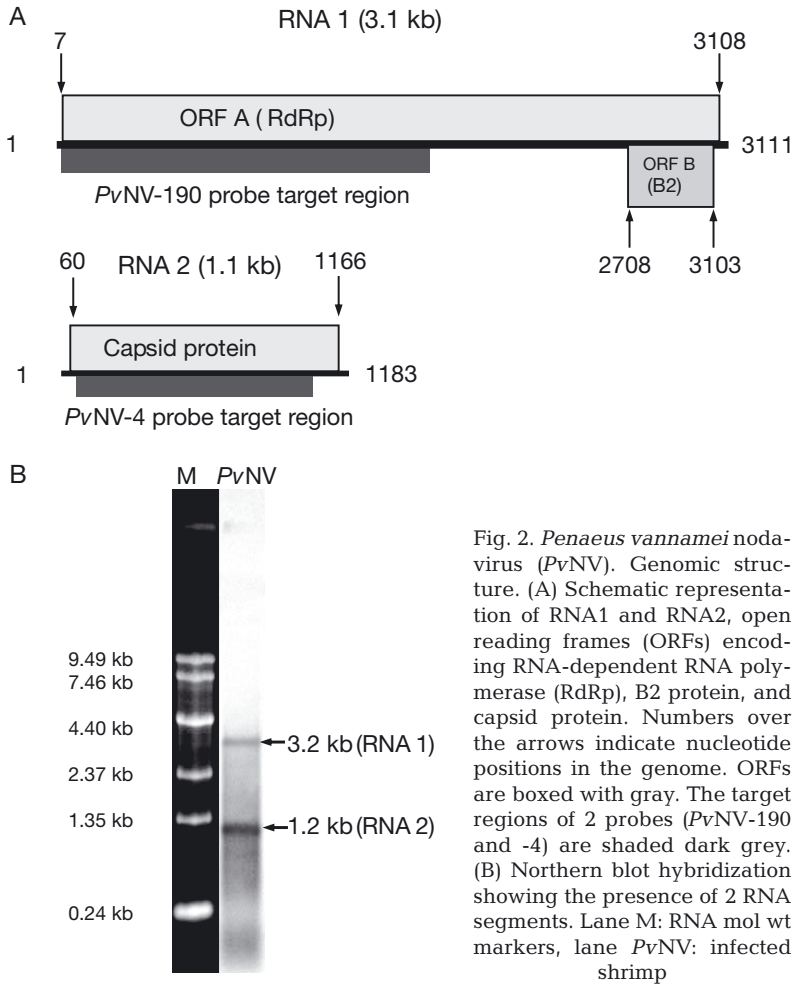

Fig. 2. *Penaeus vannamei* nodavirus (*Pv*NV). Genomic structure. (A) Schematic representation of RNA1 and RNA2, open reading frames (ORFs) encoding RNA-dependent RNA polymerase (RdRp), B2 protein, and capsid protein. Numbers over the arrows indicate nucleotide positions in the genome. ORFs are boxed with gray. The target regions of 2 probes (*Pv*NV-190 and -4) are shaded dark grey. (B) Northern blot hybridization showing the presence of 2 RNA segments. Lane M: RNA mol wt markers, lane *Pv*NV: infected shrimp

single ORF (1107 bp, nucleotide no. 60 to 1166 (Fig. 2A) that encodes a polypeptide of 368 aa with a molecular weight of 40 kDa and a pI of 9.5. The BLASTp search of this protein generated a significant (E value = 2×10^{-7}) match to the capsid protein of *Mr*NV (GenBank no. ABW38169.1).

To determine the size of RNA1 and RNA2, the total RNA extracted from infected shrimp was analyzed in a denaturing gel containing ethidium bromide. However, no RNA segment was visualized under UV after electrophoresis. However, by northern blot hybridization, 2 segments of RNA at sizes of approximately 3.2 and 1.2 kb were revealed (Fig. 2B); these are slightly larger than the sizes determined from genomic sequencing (Fig. 2A).

Phylogenetic analysis with viral RdRp gene

By the phylogenetic analysis based on the amino acid sequence of RdRp, we showed that *Pv*NV clusters with the members of genus *Alphanodavirus,* and, in particular, it is closely related to a nodavirus, *Mr*NV, isolated from the

Table 1. *Penaeus vannamei* nodavirus (*Pv*NV). Percentage similarity of the amino acid sequence of RNA-dependent RNA polymerase compared to other nodaviruses. N/A: not applicable

Virus	Abbreviation	GenBank no.	No. of amino acids	Sequence similarity to *Pv*NV (%)
Alphanodavirus				
Penaeus vannamei nodavirus	*Pv*NV	HQ259079	1033	N/A
Macrobrachium rosenbergii nodavirus	*Mr*NV	AAQ54758	1045	83
Nodamura virus	NoV	NP_077730	1043	62
Drosophila melanogaster American nodavirus	*Dm*ANV	ACU32794	998	57
Flock House virus	FHV	NP_689444	998	57
Black beetle virus	BBV	YP_053043	998	57
Boolarra virus	BoV	NP_689439	998	57
Pariacoto virus	PaV	NP_620109	973	45
Alphanodavirus HB-2007/CHN	ANV	ADF97523	983	44
Betanodavirus				
Striped jack nervous necrosis virus	SJNNV	NP_599247	983	44
Redspotted grouper nervous necrosis virus	RGNNV	ACX69744	982	43
Japanese flounder nervous necrosis virus	JFNNV	ACN58225	982	43
Sea bass Iberian betanodavirus	SBNNV	ACX71275	982	43
Dragon grouper nervous necrosis virus	DGNNV	AAU85148	982	43
Atlantic cod nodavirus	ACNV	ABR23192	981	41
Tiger puffer nervous necrosis virus	TPNNV	YP_003288759	982	41
Barfin flounder nervous necrosis virus	BFNNV	YP_003288756	981	40
Atlantic halibut nodavirus	AHNV	CAC17792	981	40

freshwater prawn (Fig. 3). These 2 nodaviruses are grouped with the NoV with a booststrap value of 88. Comparing the RdRp amino acid sequence, the mean similarities of *Pv*NV to other alphanodaviruses and betanodaviruses are 54 and 42%, respectively.

Sequence motifs and conserved domains in *Pv*NV RdRp, B2 and capsid proteins

Using a conserved domain search, we showed the RdRp protein encoded by the ORF A to contain a domain (NCBI-curated accession no. cd01699) in the region of amino acid nos. 591 to 740, with the presence of 4 characteristic motifs as described by Poch et al (1989) (Fig. 4A).

The B2 protein encoded by the ORF B also contains a conserved domain in the region of amino acid no. 15 to 108. This domain has a significant (E value = 9.53×10^{-5}) match to members of a B2 protein superfamily (pfam 11473). The sequence alignment of the B2 protein conserved domain of *Pv*NV and other alphanodaviruses is indicated (Fig. 4B).

In the capsid protein, an S (shell) domain (pfam-00729: viral coat protein, belongs to the superfamily cl02949) is found in the amino acid nos. 54 to 198. The most conserved region is located at amino acid nos. 54 to 153; the search showed an E value of 5.41×10^{-3} and matches to 10 other viral coat proteins (Fig. 4C).

Studies of the possible vectors for *Pv*NV

Through an RT-PCR method described by Tang et al. (2007), *Pv*NV was detected in 46 out of 81 (57%) *Penaeus vannamei* samples collected during 2006–2008. With this method, a search for reservoir hosts was also carried out in 2008 through the analysis of samples of a number of species collected from, or in the vicinity of, *Pv*NV shrimp ponds. The samples from species living in the ponds included barnacles, a variety of zooplankton, and the mosquitofish. In addition, we analyzed a seabird feces sample found at the pond sites and several species of wild shrimp and mangrove crabs collected from areas near the ponds. We detected *Pv*NV in all samples except for those of the wild shrimp and mangrove crabs (Fig. 5). In addition, 3 out of 10 *P. vannamei* broodstock cultured were positive for *Pv*NV.

The primers *Pv*NV339F/R were used in a RT-PCR reaction to determine if the *Pv*NV was replicating in the barnacles; the results showed that only *Pv*NV339R annealed to the RNA (extracted from samples of Lanes 8 and 17 in Fig. 5) and initiated the cDNA synthesis for the subsequent PCR reaction to generate the

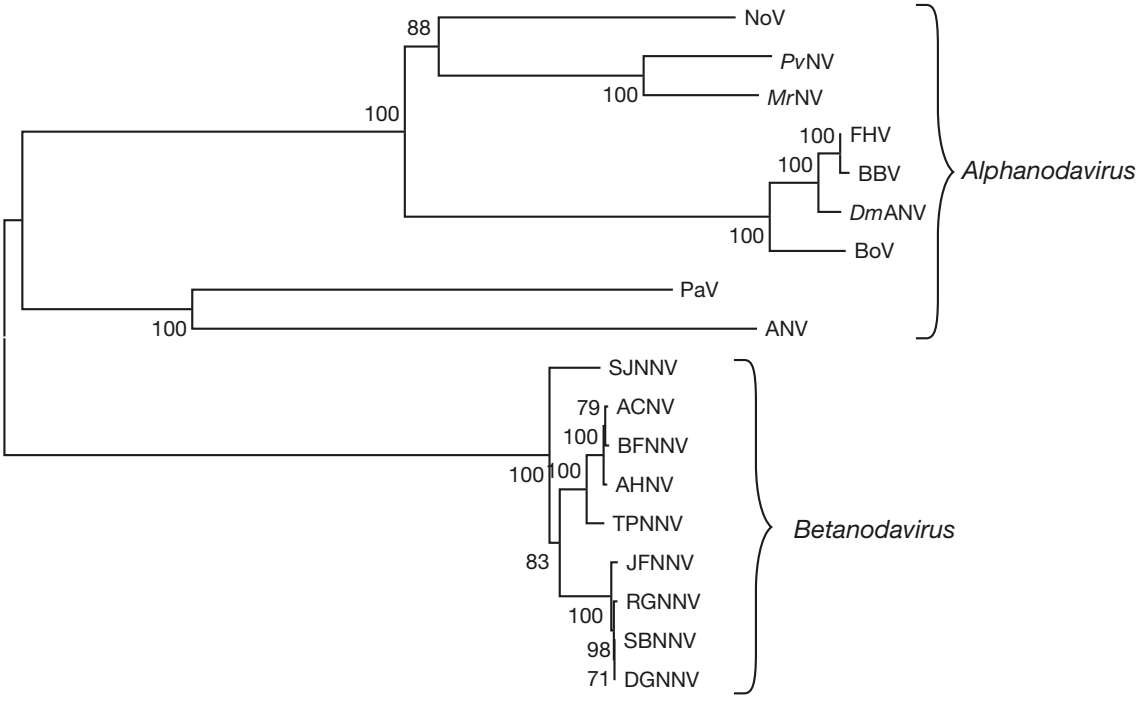

Fig. 3. *Penaeus vannamei* nodavirus (*Pv*NV). Phylogenetic tree based on the RNA-dependent RNA polymerase amino acid sequence, generated by the neighbor-joining method using the MEGA program; numbers indicate percentages of bootstrap support from replicates. For virus abbreviations see Table 1

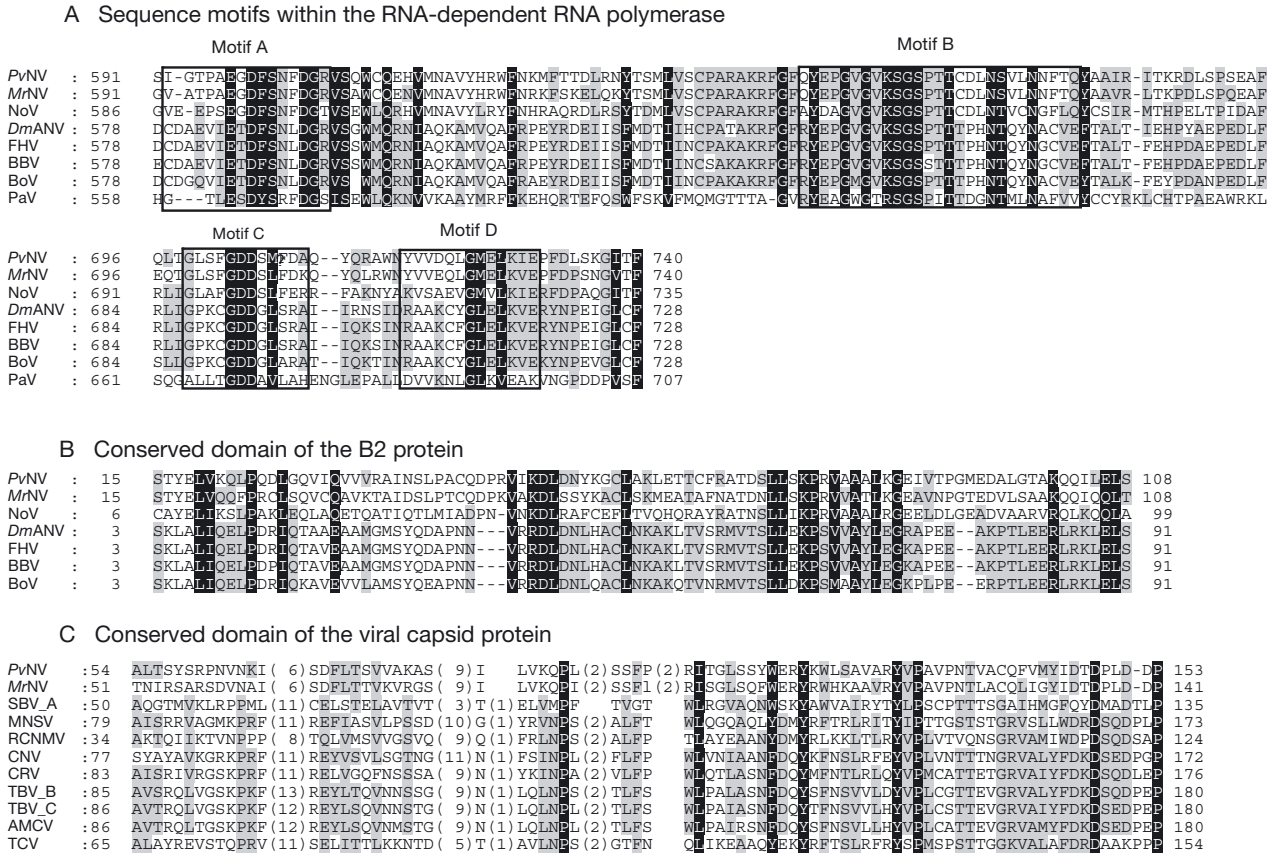

Fig. 4. *Penaeus vannamei* nodavirus (*Pv*NV). Sequence motifs and conserved domains. (A) Sequence motifs of *Pv*NV RdRp aligned with that of *Macrobrachium rosenbergii* nodavirus (*Mr*NV) and 6 alphanodaviruses; viruses and GenBank nos. are listed in Table 1. Letters above the sequences indicate the RdRp motifs. (B) Sequence alignment of a conserved domain in the RNA-binding protein, B2. The viruses and GenBank nos. are *Pv*NV (HQ259079), *Mr*NV (NP_919037.1), NoV (NP_077731.1), *Dm*ANV (ACU32795), FHV (NP_689446.1), BBV (AAA42746.1), BoV (NP_689440.1). (C) Sequence alignment of the conserved domain of viral coat protein. The viruses and GenBank nos. are *Pv*NV (ABO33432.2), *Mr*NV (ABW38169.1), *Southern bean mosaic virus* (SBV_A, P19899), *Melon necrotic spot virus* (MNSV, P22955), *Red clover necrotic mosaic virus* (RCNMV, P22955), *Cucumber necrosis virus* (CNV, P15183), *Cymbidium ringspot virus* (CRV, P17456), *Tomato bushy stunt virus* (TBV-B, 2TBV-B), *Tomato bushy stunt virus*, strain cherry (TBV_C, P11689), *Artichoke mottled crinkle virus* (AMCV, P14836), *Turnip crinkle virus* (TCV, P06663). Numbers in front and at the end of the sequence indicate the position of amino acid in each viral sequence; numbers in parentheses indicate the number of amino acid residues separating the domain. Positions with a 100% similarity are highlighted with black; those with a >60% similarity are highlighted with gray

246 bp PCR fragments (Fig. 6). No amplification was obtained when primer *Pv*NV339F was used in the RT step. Thus, the replicate intermediate RNA of *Pv*NV were not detected in barnacles. These intermediate RNA can be detected with the infected *P. vannamei*.

DISCUSSION

We describe here the ultrastructural changes in cells infected with *Pv*NV. These changes showed some similarities with those reported for other nodaviruses, such as mitochondria degeneration, endoplasmic reticulum invagination, and formation of membrane-bound viral inclusions (Grotmol et al. 1999). *Pv*NV viral inclusions were found in close association with mitochondria, suggesting that the replication complexes may also be located in the inner/outer mitochondria membranes, as has been demonstrated in FHV (Miller et al. 2001, Miller & Ahlquist 2002, van Wynsberghe et al. 2007).

We determined the nucleotide sequence of both RNA1 and RNA2 of *Pv*NV through cDNA cloning. By sequence analysis, we propose that *Pv*NV is a species within the genus *Alphanodavirus.* The family *Nodaviridae* is comprised of 2 recognized genera, of which only *Alphanodavirus* is known from arthropod hosts (Schneemann et al. 2005). Therefore, given the taxonomy of the host together with the phylogenetic tree generated from the RdRp sequence, the inclusion of *Pv*NV as a species within this genus is supported.

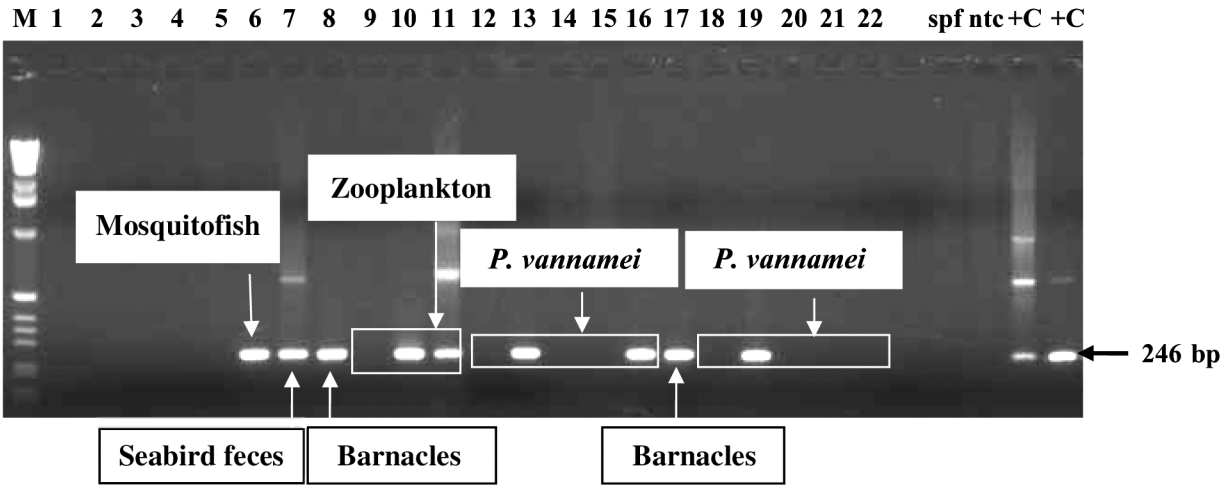

Fig. 5. *Peneaus vannamei* nodavirus (*Pv*NV). RT-PCR detection in samples collected in a Belize farm during 2008. Lanes 1 to 3: wild shrimp; Lanes 4, 8, 17: barnacles; Lane 5: mangrove crab; Lane 6: mosquitofish; Lane 7: seabird feces; Lanes 9 to 11: zooplankton; Lanes 12 to 16 and 18 to 22: *P. vannamei*. +C: positive control. M: 1 kb mol wt markers. The extraneous bands above 246 bp were non-specific products

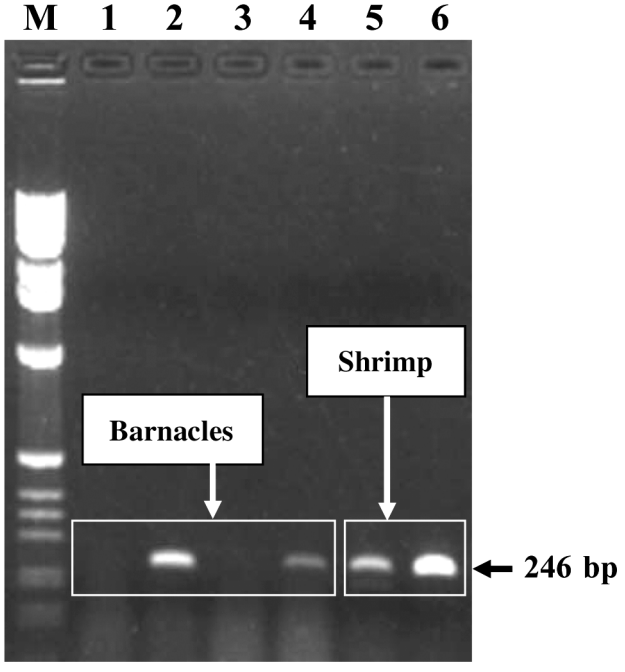

Fig. 6. *Peneaus vannamei* nodavirus (*Pv*NV). RT-PCR detection of replicative intermediate *Pv*NV RNA. Lanes 1 to 4: total RNA extracted from barnacles; Lanes 5 and 6: total RNA extracted from *Pv*NV-infected *Penaeus vannamei*. Lanes 1, 3, and 5: the oligomer *Pv*NV339F was used as the primer for the RT reaction; Lanes 2, 4 and 6 the oligomer *Pv*NV339R was used as the primer for the RT reaction. M: 1 kb mol wt markers

*Pv*NV is most closely related to another freshwater shrimp nodavirus, *Mr*NV, with a high (83%) similarity in the RdRp amino acid sequence. Both *Mr*NV and *Pv*NV were isolated from decapod crustaceans, and neither has been taxonomically classified yet. *Mr*NV contains 2 segments of ssRNA, one of 2.9 kb and the other of 1.3 kb (Bonami et al 2005). *Mr*NV is also associated with an extra small (15 nm in diameter) satellite virus (designated XSV, 796 bp ssRNA) (Qian et al. 2003, Widada & Bonami 2004). It is not known if there is a XSV-like virus associated with *Pv*NV. Our attempts to purify XSV-type and *Pv*NV virions from infected shrimp were not successful, possibly because we have not been able to obtain heavily infected shrimp in laboratory infections. A rigorous search for a *Pv*NV-related XSV-type virus will require further screening of cDNA clones from infected shrimp.

The gross signs of *Mr*NV and *Pv*NV (a white or opaque tail in infected shrimp) are indistinguishable; however, the 2 nodaviruses differ in virulence. *Mr*NV can cause 100% mortality in the infected post-larval and juvenile *Macrobrachium rosenbergii* (Arcier et al. 1999, Sahul Hameed et al. 2004). In contrast, *Pv*NV does not cause mortality in *Penaeus vannamei* in laboratory infections. In addition, *Pv*NV appears to have a limited host range; it did not infect *M. rosenbergii* in a 4 wk injection bioassay determined by a RT-PCR analysis (K. F. J. Tang unpubl. data). For *Mr*NV, several species of marine penaeid shrimp (*Penaeus indicus*, *P. japonicus*, and *P. monodon*) have been identified as reservoir hosts (Sudhakaran et al. 2006). Recently, post-larval *P. monodon* and *P. indicus,* both displaying gross signs of whitish muscle, were found to be infected with *Mr*NV and XSV by RT-PCR (Ravi et al. 2009).

Investigation of the possible vectors for *Pv*NV showed that this virus can be detected by RT-PCR in seabird feces and crustaceans, such as barnacles and

zooplankton — organisms that are often associated with shrimp ponds. Both zooplankton and barnacles were filtering nodaviruses that were present in the pond water. With barnacles, through RT-PCR analysis using strand-specific primers, the negative-strand of *Pv*NV RNA was not detected. It is likely the virus is not replicating in the barnacles, so they may only be acting as mechanical carriers. We also carried out histological examination on 10 individual barnacles and did not observe any lesions of muscle necrosis as seen in the *Pv*NV-infected shrimp (D. V. Lightner unpubl. data). Seabirds are known to act as mechanical vectors for other shrimp viruses, such as *Taura syndrome virus* (TSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV), as the viruses in infected shrimp tissue (that is ingested) are not damaged or inactivated in the digestive tract. Evidence of this is that these viruses detected in seabird feces by PCR (and RT-PCR) were found to be still capable of infecting shrimp in laboratory bioassays (Vanpatten et al. 2004). *Pv*NV is a small, non-enveloped virus with a similar morphology to TSV and IHHNV; therefore, it is likely that *Pv*NV found in the seabird feces in the present study were also infectious. The detection (by RT-PCR) of *Pv*NV in the mosquitofish was unexpected, since there have been no reports of natural infections in vertebrates by alphanodaviruses. It is more likely that the mosquitofish ingested virus-carried zooplankton and thus became a mechanical carrier for *Pv*NV. All of these carrier hosts can contribute to the spread of pathogenic viruses, and it is difficult to completely exclude them from shrimp farms. The prevalence of various vectors may explain why *Pv*NV has become a major threat to local shrimp farming industries.

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