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Geographic variations among infectious hypodermal and hematopoietic necrosis virus (IHHNV) isolates and characteristics of their infection

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ABSTRACT: Nucleotide sequence variations of a 2.9 kb fragment of infectious hypodermal and hematopoietic necrosis virus (IHHNV) isolated from samples of *Penaeus monodon* were determined and compared with an isolate from Hawaii. The infection characteristics of these isolates were examined by histology, *in situ* hybridization, and laboratory challenge studies with *P. vannamei*. Isolates of IHHNV were obtained from samples collected from the SE Asia region (the Philippines, Thailand, and Taiwan). Isolates of putative IHHNV were obtained from African samples (Tanzania, Madagascar, and Mauritius). The Philippine isolate had a very high nucleotide sequence identity (99.8%) to Hawaii IHHNV. The Thailand isolate showed a slightly lower identity (96.2%). The putative IHHNV sequences collected from Tanzania and Madagascar showed greater divergence from Hawaii IHHNV, 8.2% difference for Tanzania and 14.1% difference for Madagascar. A phylogenetic analysis showed that the Philippine IHHNV clustered with IHHNV found in the western hemisphere. This supports the theory that the Philippines was the origin of IHHNV that was first detected in Hawaii. In the laboratory infection study, both the Philippine and Thailand IHHNV were passed into *P. vannamei*, and the infected shrimp did not suffer any mortalities. In another laboratory infection, *P. vannamei* injected with a tissue homogenate of *P. monodon* from Madagascar, which tested positive for IHHNV by PCR, did not demonstrate IHHNV infection, suggesting that this putative IHHNV is not infectious to *P. vannamei*.

KEY WORDS: Infectious hypodermal and hematopoietic necrosis virus · IHHNV · *Penaeus monodon* · Genetic variations

INTRODUCTION

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is a virus that can have severe pathogenic effects in penaeid shrimp and presents a risk to commercial shrimp farming. When the virus was first discovered in 1981, it resulted in high mortalities (up to 90%) in infected *Penaeus stylirostris* (taxonomy used is according to Holthuis 1980) cultured in Hawaii (Lightner et al. 1983a,b). The virus has since been detected in a number of other penaeid species and from stocks around the world, including the Americas, Oceania, and Asia (Lightner 1996, Flegel 1997). The effects of the virus vary among penaeid species. For example, IHHNV infection does not cause mortality in stocks of *P. vannamei* and *P. monodon*. However, it results in a disease called runt-deformity syndrome in both species (Bell & Lightner 1984, Kalagayan et al. 1991, Primavera & Quinitio 2000), and this can also cause substantial economic losses (Wyban et al. 1992).

Based on morphological and biochemical characteristics, IHHNV is taxonomically placed within the family Parvoviridae (Bonami et al. 1990). It is an ico-

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sahedral, non-enveloped virus and contains a single-stranded, 4.1 kb DNA genome (Bonami et al. 1990, Mari et al. 1993). Nearly the entire IHHNV genome has been sequenced (Nunan et al. 2000, Shike et al. 2000). It contains 3 large open reading frames (ORF1, 2, and 3). ORF1 comprises approximately 50% of the genome encoding a polypeptide of 666 amino acids, which is predicted to be a non-structural protein 1 (NS1) based upon its degree of homology with 2 mosquito brevidensoviruses (Shike et al. 2000). ORF2, which starts 56 nucleotides upstream and overlaps ORF1, could encode a 343 amino acid putative non-structural protein 2 (NS2). The 5′ end of ORF3 also overlaps ORF 1, and the ORF3 encodes a 329 amino acid polypeptide. At least 4 structural proteins of M_rs (74, 47, 39, and 37.5 kDa) are found in the purified virions (Bonami et al. 1990). According to partial amino acid sequencing, the 47 kDa protein was part of ORF3 (B. T. Paulos and D. V. Lightner unpubl. data). Based on the gene structure and organization of other parvoviruses, it is likely that the ORF3 encodes structural proteins.

There is little information available on sequence variation among IHHINV isolates. However, a recent study on IHHNV from infected *Penaeus stylirostris* and *P. vannamei* from the western hemisphere indicated that the IHHNV genome is very stable (Tang & Lightner 2002); less than 0.5% nucleotide sequence divergence was found among 14 isolates collected from Hawaii and the Americas from 1982 to 1997. In this report, we analyzed sequence variations of IHHNV from *P. monodon* collected from SE Asia and Africa, and characterized infection of these isolates through histology, *in situ* hybridization, and laboratory challenge studies.

MATERIALS AND METHODS

Origin of penaeid shrimp. The samples of *Penaeus monodon*, *P. vannamei*, and *P. stylirostris* were sent to our laboratory as diagnostic materials at various times from 1990 to 2001. The samples were archived and stored frozen at –70°C. We selected isolates to represent the major geographic areas where the shrimp are either farmed or found in the wild. We included one of the earliest samples from Hawaii, which was collected during the first reported outbreak of IHHNV. (Data on the origins, life stages, and dates of collection of the samples are summarized in Table 1.)

DNA extraction and PCR amplification. DNA was extracted from either hemolymph or pleopods of shrimp with a high-pure DNA template preparation kit (Roche Molecular Biochemicals), and amplified by PCR. The reaction mixture contained 10 mM Tris-HCl, pH 9.0; 50 mM KCl; 0.1% Triton-X; 200 µM each dNTP: 2 mM $MgCl_2$; 0.3 µM primers, extracted DNA (1 µl), and 0.625 U Taq DNA polymerase (Promega) in a final volume of 50 µl. Amplification was performed for 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 55°C, 3 min at 72°C, with a final extension at 72°C for 7 min. Primers IHHN3065F (5′-GAC GAC GAA GAA TGG ACA GA-3′) and IHHN3065R (5′-TGC CTG GGT AGC TGG TAT GTA TA-3′) were used to amplify a 3.0 kb fragment comprising Nucleotides 792 to 3856. The nucleotide positions refer to the published IHHNV sequence (GenBank AF218266). The amplified DNA was purified with a QIAquick PCR purification kit (Quiagen). The amplified products were directly sequenced with an automated DNA sequencer 377 (Applied Biosystems) at the sequencing facility of the University of Arizona. Sequences obtained from isolates of Thailand, Tanzania, and Madagascar have been assigned GenBank Accession Nos AY102034, AY124937, and AY125423, repectively.

DNA sequence analysis. The nucleotide sequence of 2.9 kb from Nucleotides 816 to 3744 was compared among these IHHNV isolates. Distances among these sequences were calculated using the Jukes-Cantor method, and the un-rooted tree was calculated using the neighbor-joining method (Saitou & Nei 1987) with the GCG package. The un-rooted tree was displayed with the Treeview program (Page 1996). A bootstrapped tree was generated with the PAUP software (Version 4.0 in GCG package); distance analysis was implemented with a heuristic algorithm. The tree was generated with tree-bisection-reconnection. The data were re-sampled by 1000 bootstrap replicates to determine the confidence index within the phylogenetic tree. Bootstrap values greater than 70 (as percentage of 1000 replications) were considered to be related with a significance of 95% (Hillis & Bull 1993).

Digoxigenin-labeled IHHNV probe and *in situ* hybridization. A DNA probe for IHHNV was previously developed in our laboratory (Mari et al. 1993, Lightner 1996). The probe was labeled with digoxigenin-11-dUTP in a PCR reaction as described by Lightner (1996). Postlarval and juvenile *Penaeus monodon* from the Philippines and Taiwan, respectively, were fixed with Davidson's AFA (alcohol-formalin-acetic acid) fixative, processed, embedded in paraffin and sectioned (4 m thick) according to standard methods. The procedures used for routine hematoxylin & eosin (H&E) staining and in situ hybridization were described by Lightner (1996). Tissue sections from IHHNV-infected *P. stylirostris* and specific-pathogen-free (SPF) *P. vannamei* were used, respectively, as positive and negative controls for *in situ* hybridization.

Laboratory infection. Small juveniles of SPF *Penaeus vannamei* (10 shrimp, 0.3 g) were fed minced

tissue from either the Philippine or Thailand IHHNV-infected *P. monodon* at 10% of their body weight once daily for 3 to 10 d in a 90 l glass tank. For the following 2 to 4 wk, the shrimp were maintained on a pelletized ration. After an incubation period of 2 to 4 wk, challenged shrimp were fixed in Davidson's AFA for histological and *in situ* hybridization analyses.

For challenge studies with Madagascar *Penaeus monodon*, injection was used because there was limited shrimp tissue available, and injection is routinely used for laboratory infections (Lightner 1996). These shrimp were found to be IHHNV-positive by PCR; then the tissue was homogenized in a buffer (0.02 M Tris-HCl, pH 7.4, 0.4 M NaCl; 1 g/10 ml), and centrifuged at 5000 × *g* for 30 min. The supernatant was diluted (1:20, v:v) with sterile 2% saline to generate an inoculum. A volume of 50 µl of inoculum was injected into each SPF *P. vannamei* (10 shrimp, 1 g). The shrimp were maintained on pelletized ration for 1 mo, then fixed in Davidson's AFA for *in situ* hybridization analysis.

Table 1. *Penaeus* spp. Year, origin and related information on the IHHNV isolates used in the study

Sample	Year	Species	Origin	Stage	GenBank
1	1996	*P. monodon*	Philippines	Postlarvae	
2	2000	*P. monodon*	Thailand	Adult	AY102034
3	2001	*P. monodon*	Taiwan	Juvenile	
4	2000	*P. monodon*	Tanzania	Adult	AY124937
5	2000	*P. monodon*	Madagascar	Adult	AY125423
6	2000	*P. monodon*	Mauritius	Adult	
7	1982	*P. stylirostris*	Hawaii	Juvenile	
8	1990	*P. stylirostris*	Mexico	Juvenile	
9	1991	*P. stylirostris*	Mexico	Adult	
10	1996	*P. stylirostris*	Mexico	Sub-adult	
11	1999	*P. stylirostris*	Mexico	Sub-adult	AF273215
12	1990	*P. vannamei*	Colombia	Juvenile	
13	1992	*P. stylirostris*	Ecuador	Sub-adult	
14	1997	*P. vannamei*	Panama	Juvenile	

RESULTS

Comparisons of IHHNV and putative IHHNV sequences to Hawaii IHHNV

The IHHNV from Hawaii, the best characterized isolate among those studied here, has been completely sequenced (GenBank AF218266). It was used in this study as a reference genotype to assess the variations of IHHNV (and putative IHHNV) sequences of *Penaeus monodon* collected from 6 different regions (Table 1, Samples 1 to 6). The 2.9 kb fragment that is compared contains approximately 70% of the entire viral genome including the full length ORF1 and ORF3. The results showed that the Philippine isolate was most similar to Hawaii IHHNV, with a difference of only 0.2% in the nucleotide sequence (Table 2). The Thailand isolate showed a higher variation from Hawaii IHHNV, with an identity of 96.2%. The Taiwan isolate was very similar (99.7% identity) to the Thailand isolate, differing only in 9 nucleotides. The extracted DNA from *P. monodon* from Tanzania, Madagascar, and Mauritius contained putative IHHNV sequences. Among them, the Tanzania sequence had an identity of 91.8% with the Hawaii IHHNV. The Madagascar sequence had a lower identity, 85.9%. The sequence from Mauritius displayed only 1 nucleotide difference from the Madagascar sequence.

Alignment of these sequences showed that all the nucleotide changes were substitutions and that no new termination codons were introduced. Both ORF1 and ORF3 had the same number of amino acids (aa) in their predicted polypeptides, i.e. 666 aa from ORF1 and 329 aa from ORF3. A length of 352 aa was predicted from the portion of ORF2 that overlaps ORF1. We compared these nucleotide (nt) sequences to that of the Hawaii isolate. The sequence identities ranged from 86 to 100% for ORFl (nt 816 to 2816), and 91 to 99.8% for ORF3 (nt 2758 to 3747) (Table 2). In each case, the Philippine isolate was most similar to the Hawaii isolate and the Madagascar isolate was the least similar. The comparison of amino acid similarity followed the same pattern; and in most cases the amino acid sequence similarities were very close to the corresponding nucleotide sequence identities. However, the ORF3 amino acid sequence similarities for the Tanzania and Madagascar isolates were higher than their corresponding nucleotide sequence identities.

Table 2. Comparison of nucleotide and predicted amino acid sequences of IHHNV (and putative IHHNV) isolated from samples of *Penaeus monodon* in this study with IHHNV isolated from Hawaii

Isolate origin	Nucleotide sequence identity (%)			Amino acid similarity (%)		
	2.9 kb	ORF1	ORF3	ORF1	ORF2	ORF3
Philippines	99.8	100	99.8	100	100	99.1
Thailand	96.2	96.8	98.3	96.4	96.3	97.9
Tanzania	91.8	92.3	94.7	92.8	93.2	98.2
Madagascar	85.9	85.8	91.0	86.0	85.8	95.1

Sequence motifs of ORF1

As shown in Table 2, the putative IHHNV sequences from Africa had more substitutions, particularly in ORF1, than the IHHNV isolates from the western hemisphere (Tang & Lightner 2002). The amino acid sequences predicted for ORF1 were examined to determine whether or not they contained the characteristic motifs found in IHHNV. Although the amino acid differences were distributed throughout the sequence, a higher percentage of substitutions was found among the first 200 amino acids (Fig. 1). Never-

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     1
Seq1 MAKDILHTRQ GEPESLSELL RERTNETHSS QGISPSLLTP GPNQDPKPTT EQLLNMSEEL FQFSDEEDNS QTPPRTSTPE
Seq2 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Seq3 -------s-- ---------- -------d-- ---------- ----e-r--- -------q-- ---------- ----------
Seq4 ---------e -k------m- -r-ad--d-- ---------- ----esrs-- ---------- -k-------- --t-------
Seq5 --en----ce -k-d----m- -rs-es-d-- ---------- ----e-g-s- ---------- -k-------p --------g-

    81
     QTDPKVCVDN LGIREGTGNG TIQLGSESET SLGSVGNSND RGKKRQRGIT YISDTSDSSG SDDENLGTPH RNKRTRNSNT
     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
     ---------- ---------- ---------n --------d- --q------- ---------- ------d--- ----------
     -s----f--- ---------- ---------d ----ar---- -e-------- ---------- ----d--a-- t---s-h-d-
     -dy----l-- ---------- ---------d -vrns---tn -rq------- ---------- -----m-as- th--skh-dp

   161
     TKETGGGDNR RHQESDHGSN GNRLEPTDGG ESCSSGTQPD FIEGTPNGPD EMDGRRLEES EIDKQVESTT WYTFVIREKP
     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
     ----se-ngg gd-------- -------n-- --n------- ------d--- ---------- --------a- ----------
     -ek-s---c- -----n---- -------n-- --sg------ ------d--- ---------r ---------- ----------
     se--srr-c- -n---nqr-h -----s---- --sc------ ------s--- ---------g -------n-- --------r-

   241                  I                                                       II
     QPRRLSGRTP NFTITDHGDH WHITYSGHPT NKTRHRATIL AYLGVTFAAR AEAEATTVLV RNIKRWILYL IRYGIERLSY
     ---------- -------|-- ------|--- ---------- ---------- ---------- ------|--- ----|-----
     ---------- -------|-- ------|--- ---------- ---------- ---------- -d----|--- ----|-----
     ---------- -------|-- ------|--- ------s--- ---------- ---------- kd----|--- ----|-----
     ---------- -------|-- ------|--- --s---n--- t--------- ------m--- kd----|--- ----|-----

   321
     FGLGHAIFKR IIKYFQQYRR DEDAVDGPCP YMTTTREDRA EEKPKENSAE YDYLQHLVKT KSARTVQELV NKLDDEEYKQ
     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
     ---------- ------r-k- ---------- ---------- ---------- ---------- ---------- ----------
     --------r- ---------- ---t------ ---------- ---t------ ----r----- ---------- ----------
     -----t--r- ---------- e--t------ --ss--de-g ---s------ ----r----s -a-------- ----------

   401
     LWTRTRGQYK DKLRGILTYY NNKKKSNQSQ LSLITNLQNI SKRKPDYDNM QWIKYMLANN DIRVPEILAW IIIVADKKLD
     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
     ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
     ---------- ---------- ---------- ---------- ---------- ---------- ---------- -l--------
     ---------- ---------- ------s--- -----iyk-- -------e-- --l---f--- ---------- ----------

   481            A                                                   B
     KINTLVLQGP TGTGKSLTIG ALLGKLNTGL VTRTGDSNTF HLQNLIGKSY ALFEEPRISQ ITVDDFKLLF EGSDLEVNIK
     ----|----- ---------- ------|--- ---------- ---------- ---------| ---------- ---------- ----------
     ----|----- ---------- ------|--- ---------- ---------- ---------| ---------- ---------- ----------
     ----|----- ---------- ------|--- ---------- ---------- ---------| ---------- ---------- ----------
     ----|----- ---------- ------|--- ---------- ---------- ---------| ---------- ---------- ----------

   561          C
     HQESEIMGRI PIFISTNKDI DYWVPPADGK ALQTRTKTFH LTRQIKGLSD RMNSQYDINP PPDKITSDDF LGLFQEYEKE
     ----------| --------|-- ---------- ---------- ---------- ---------- ---------- ----------
     ----------| --------|-- ---------- ---------- ---------- ---------- ---------- ----------
     ----------| --------|-- ---------- ---------- --s------- ---------- ---------- ----------
     ----------| --------|-- -----t---- ---------- ---------- ---------- ---------- ----e-----

   641
     IDDIIDNHVR RFNKSKPKEK IQEGCT
     ---------- ---------- ------
     ---------- ----i----- ------
     --e---s--- ----g----t ------
     --e---sy-- ----g----t -e---a
```

Fig. 1. Amino acid sequence alignment of translated ORF1. Seq1: Hawaii IHHNV (GenBank AF218266); Seq2: Philippine IHHNV; Seq3: Thailand IHHNV (GenBank AY102034); Seq4: Tanzania putative IHHNV sequence (GenBank AY124937); Seq5: Madagascar putative-IHHNV sequence (GenBank AY125423). Dashes (---) indicate the same amino acids as those in Hawaii IHHNV. I, II: replication initiator protein motifs. A, B, C: NTP-binding and helicase domains

theless, the replication Initiator Protein Motifs I (aa 258 to 266) and II (aa 307 to 314) (Ilyina & Koonin 1992, Shike et al. 2000) were conserved, as were the NTP-binding and helicase domains A, B, C (aa 460 to 640), which comprise an ATP/GTP-binding site motif (A/G)-X_4-GK(S/T) (aa 489-496) (Walker et al. 1982). Furthermore, BLAST analysis found no matches for these putative IHHNV sequences in the GenBank other than IHHNV.

Phylogenetic analyses

The relationship of these IHHNV (and putative IHHNV) sequences were examined in a phylogenetic analysis with 8 representative IHHNV isolates found in the western hemisphere; 1 Hawaii isolate, 4 isolates collected from the Gulf of California, Mexico, and 3 from Central/South America (Table 1: Samples 7 to 14). The un-rooted tree showed that the distance between the Philippine IHHNV and those from the western hemisphere is only 0.2% (i.e. 0.002 substitutions/site) and they clustered together (Fig. 2A). The Mauritius isolate had the same genotype as the isolate from Madagascar. The Taiwan isolate was grouped with the Thailand isolate. The Madagascar sequence displayed approximately the same distance (14%) from either the Philippine or the Thailand IHHNV. The distance between Thailand and the Philippine IHHNV was 3.8% (0.038 substitutions/site). Two sequences from Africa had a rather large distance between each other, 10% (0.1 substitution/site).

With the brevidensovirus *Aedes densovirus* (GenBank M37899) as an outgroup in a bootstrapped tree, there were 3 main lineages corresponding to the origins of isolates: Thailand/the Philippines/western hemisphere, Tanzania, and Madagascar (Fig. 2B). Philippine and Thailand IHHNV are grouped together with a bootstrap value of 100. The Philippine isolate was closely related to the 4 IHHNV isolates originating from Hawaii and Central/South America, with a bootstrap value of 80. Three isolates from Central/South America were in the same group with a bootstrap value of 86. Four Mexico isolates were grouped together with a bootstrap value of 92 and appeared separate from Hawaii and Central/South America isolates.

In situ detection of IHHNV in Philippine and Thailand *Penaeus monodon*

For the Philippine IHHNV-infected *Penaeus monodon*, histological examination indicated that the hallmark Cowdry A intranuclear inclusion bodies were rare. However, *in situ* hybridization showed that the postlarvae had systemic infections (Fig. 3A), and IHHNV was detected in various tissues including the hemolymph of the coelomic cavity (Fig. 3B). Strong intranuclear staining was found in the epithelial cells of the antennal gland (Fig. 3C). Circulating hemocytes and the epithelial cells of gills were also highly infected (Fig. 3D).

For Thailand IHHNV, the tissue distribution was determined using *Penaeus monodon* from Taiwan

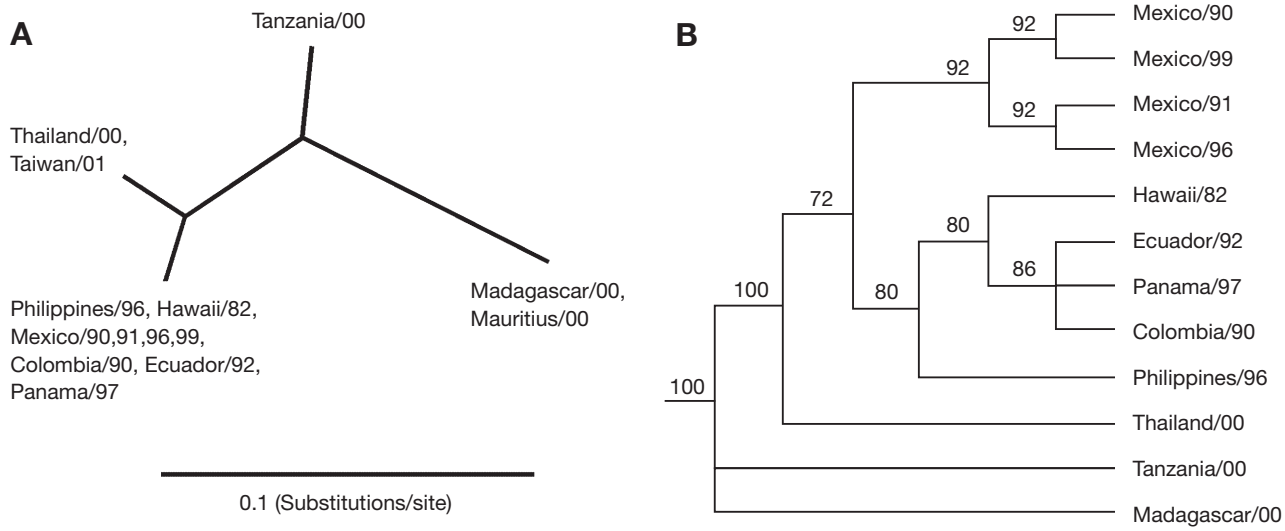

Fig. 2. Phylogenetic analysis of representative IHHNV nucleotide sequences collected from western hemisphere and sequences obtained in the current study. (A) Un-rooted tree generated by neighbor-joining method; bar = 0.1 substitutions per site. (B) Bootstrapped tree generated by PAUP analysis. The tree was rooted using nucleotide sequence of *Aedes densovirus* (GenBank M37899) as an outgroup; numbers indicate the percentages of bootstrap support from 1000 replicates; branches with values of <70 were collapsed. Name of sequence is indicated by the origin and year of isolation in both (A) and (B)

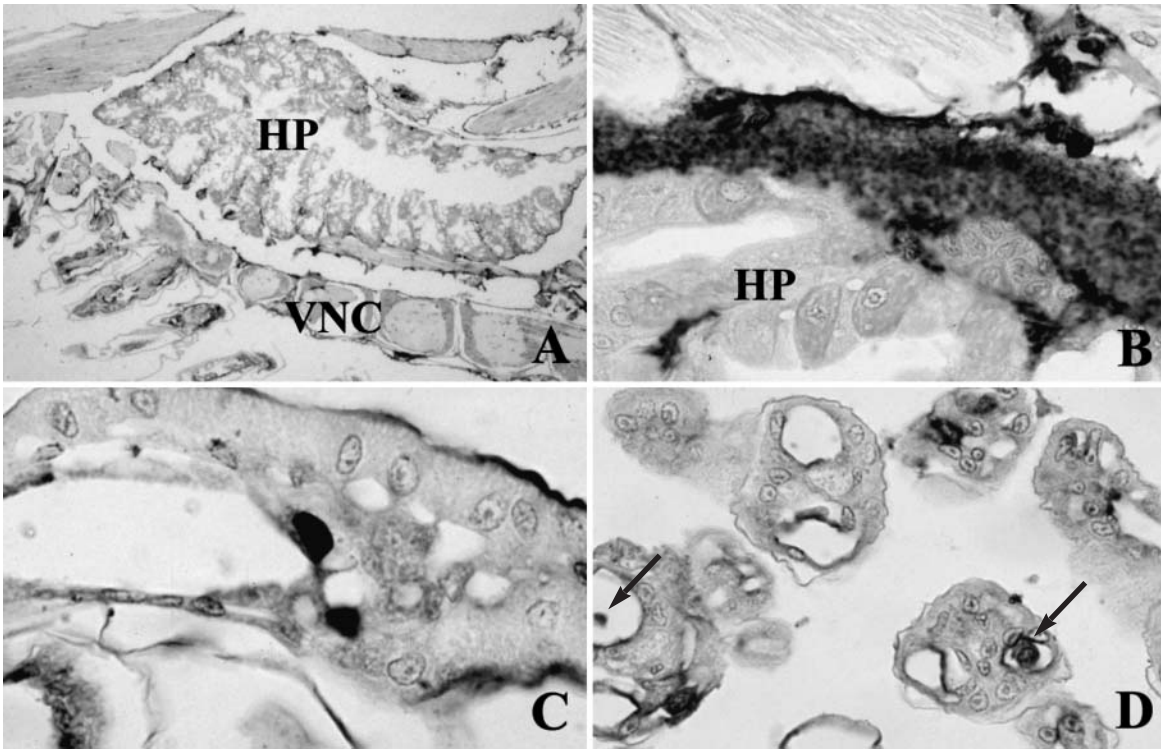

Fig. 3. *Penaeus monodon. In situ* hybridization of Philippine IHHNV-infected postlarvae with a digoxigenin-labeled IHHNV DNA probe. (A) Mid-sagittal section of cephalothorax including tissues of hepatopancreas (HP) and ventral nerve cord (VNC); total magnification 20×. (B) Coelomic cavity and circulating hemocytes; total magnification 200×. (C) Antennal gland; total magnification 300×. (D) Hemocytes (arrows) of the afferent/efferent duct in gills; total magnification 100×

that were naturally infected with Thailand IHHNV (Table 1, Sample 3). H&E staining showed Cowdry A intranuclear inclusion bodies in the antennal gland (Fig. 4A), ganglia, nerve tract, and gills (data not shown). With *in situ* hybridization, IHHNV was found essentially in all the tissues including the antennal gland (Fig. 4B), nerve ganglia (Fig. 4C), heart (Fig. 4D), gills (Fig. 4E), hematopoietic tissue (Fig. 4F), lymphoid organ, and connective tissues (data not shown). Hybridization was found in the cytoplasmic area of infected cells as well as in intranuclear inclusion bodies.

Laboratory infections

To determine if the Philippine IHHNV can infect other species of penaeid shrimp, a laboratory infection was carried out in a bioassay using *Penaeus vannamei* as indicator shrimp: 2 wk after exposure to IHHNV, there were no mortalities among the indicator shrimp. Hemolymph was drawn from the challenged *P. vannamei* for PCR analysis through the use of IHHNV-specific primers (Nunan et al. 2000), and the samples tested positive for IHHNV.

Another laboratory infection performed with Thailand IHHNV was carried out through feeding *Penaeus vannamei* with minced tissue of IHHNV-infected *P. monodon*: 4 wk after exposure to IHHNV, no mortalities were observed. The *P. vannamei* were fixed and analyzed by *in situ* hybridization. The Thailand IHHNV-infected *P. vannamei* showed strong reactions within the antennal gland, hematopoietic nodules, nerve tract, heart, gill filaments, and connective tissues (data not shown). The majority of reaction sites were in cytoplasmic areas with a few intranuclear stainings in the nerve tract. When a 2.9 kb IHHNV fragment from these experimentally infected *P. vannamei* was amplified by PCR and sequenced for comparison with that from *P. monodon*, the results showed that there was no change in nucleotide sequence after passage through *P. vannamei*.

In the challenge study using injection of tissue homogenate from Madagascar *Penaeus monodon*, the indicator shrimp showed no mortality 1 mo after injection. Hemolymph drawn from the challenged shrimp was negative for IHHNV when tested with primers designed from the Madagascar IHHNV sequence. The result of *in situ* hybridization of tissue sections from challenged shrimp was negative.

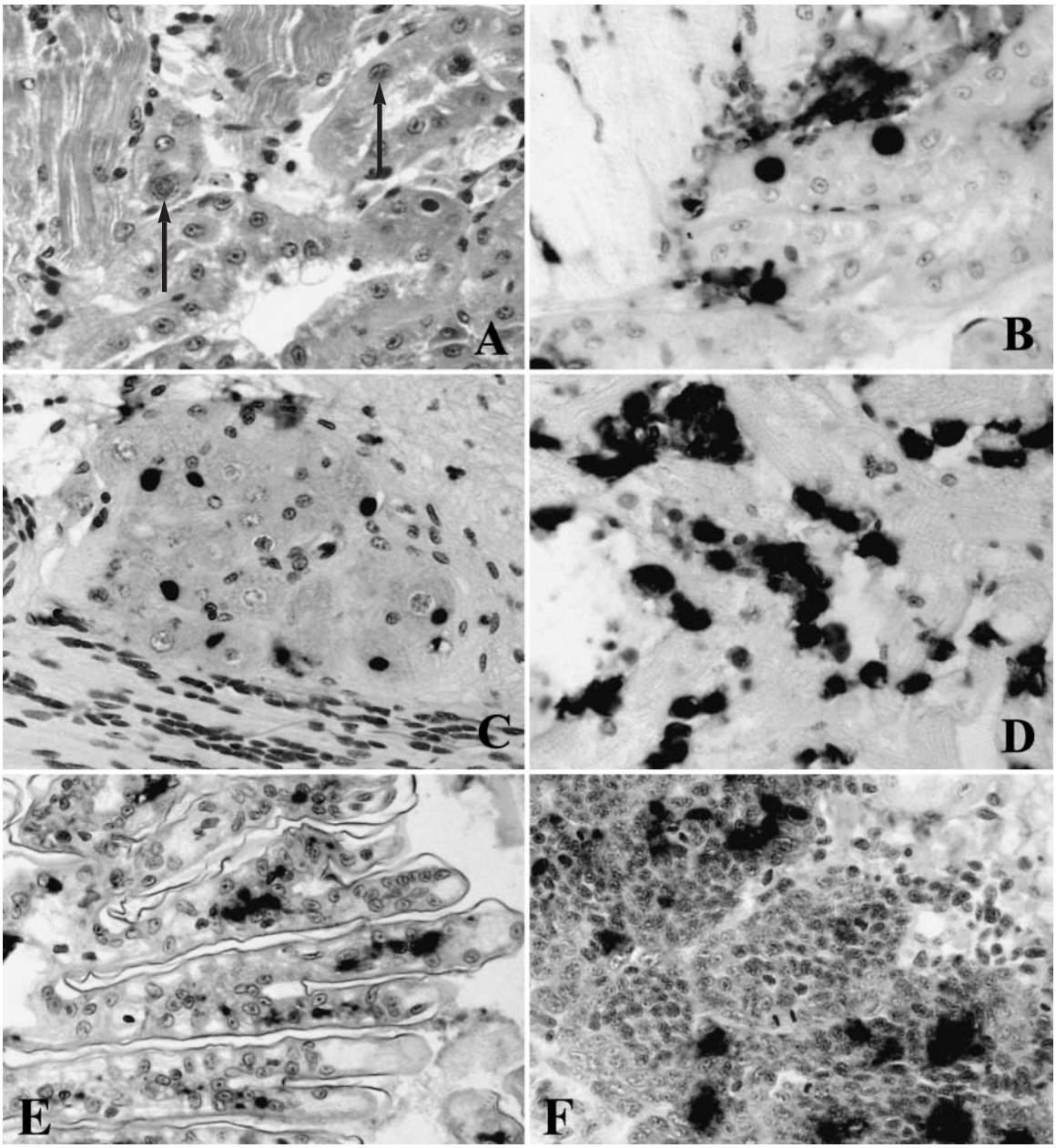

Fig. 4. *Penaeus monodon.* H&E histology and *in situ* hybridization of Thailand IHHNV-infected shrimp. (A) H&E staining of antennal gland (arrows indicate) Cowdry A intranuclear inclusion body; (B) *in situ* hybridization of serial section of antennal gland with a digoxigenin-labeled IHHNV probe; (C), (D), (E), (F) *in situ* detection of IHHNV in nerve ganglia, heart, gills and hematopoietic tissue, respectively. Total magnification 100×

DISCUSSION

Our data support the theory that Philippine stocks could be the origin of IHHNV that is currently found in the western hemisphere, a conclusion reached by Lightner (1999) based on inference from knowledge of the introductions of *Penaeus monodon* stocks into the Americas. We found a high degree of identity of the nucleotide sequences between isolates from the Philippines and Hawaii. Also, in the phylogenetic analysis, the Philippine IHHNV isolate clustered with the isolates found in the western hemisphere.

Based on similar evidence, it is likely that IHHNV may have been introduced to Taiwan from Thailand. Sequence from the Taiwan IHHNV samples had 99.7% identity with those from Thailand, and it is known that both live and frozen shrimp are frequently imported from Thailand to Taiwan.

The 4 Mexico isolates clustered together and were separated from isolates from Hawaii, Central and South America. These 4 Mexico isolates were collected along the northern Gulf of California, Mexico, and may have the same lineage, as it is thought that the spread of IHHNV in this region may have started from 1 farm in 1987 (Lightner et al. 1992).

In general, genetic variation among parvoviruses is only up to 4% (Erdman et al. 1996). We found, however, that IHHNV sequences isolated from *Penaeus monodon* have unexpectedly high variation, up to 14%. This is in contrast to our previous finding of less than 0.5% variation among IHHNV isolates from populations of *P. stylirostris* and *P. vannamei* (Tang & Lightner 2002). The lower variation in *P. stylirostris* and *P. vannamei* may be because of the short time available for variation to develop. These species became infected after exposure to IHHNV from infected stocks of *P. monodon* imported from Asia. Stocks of *P. monodon* were first introduced into the west during the mid to late 1970s, so if this hypothesis is correct, the IHHNV found in the western hemisphere has been circulating in this region for only 2 to 3 decades.

Three putative IHHNV sequences were found in the shrimp collected in Tanzania, Madagascar, and Mauritius. The Mauritius nucleotide sequence essentially has the same genotype as that of Madagascar, reflecting the close proximity of these geographic regions. There is a 10% difference between nucleotide sequences from Tanzania and Madagascar. The reason for this rather distinct sequence difference from 2 relatively close areas is not known. Three ORFs that are characteristic of IHHNV can still be found in these sequences. The sequence motifs of replication initiator protein and NTP-binding/helicase in ORF1 are conserved. But there is a higher percentage of variations in ORF1/2 (full length ORF1 and overlapped 95% of ORF2) than in ORF3, which is rather unusual for a small virus with a considerable overlap of ORFs. This is also different from Hawaii IHHNV and most other parvoviruses, in which the structural protein coding region (ORF3) is usually more variable than the nonstructural coding region (ORF1/2) (Truyen et al. 1995, Hemauer et al. 1996, Tang & Lightner 2002). ORF1 is thought to encode NS1, which is thought to be responsible for the majority of enzymatic activities involved in viral transcription and replication. ORF2 could encode NS2, the function of which is less clear but also known to be required for viral multiplication. Greater variation in ORF1 and 2 may indicate weaker selection pressure on these ORFs, suggesting that they do not encode functional NS1 and NS2. If ORF1 and 2 are non-functional, then these putative IHHNV sequences may not be from infectious IHHNV. If so, this could explain the fact that they are not infectious to *Penaeus vannamei*. Further challenge experiments with their natural host, *P. monodon*, are planned to determine if these putative IHHNV sequences are not infectious.

The histopathology of Thailand IHHNV is very similar to that of Philippine IHHNV, indicating that the 3.8% difference in nucleotide sequence does not affect its characteristic pathology. Both Thailand and Philippine IHHNV are infectious to *Penaeus vannamei* in laboratory trials. The amplified 2.9 kb portion of the IHHNV genome was sequenced from these infected *P. vannamei* and was shown to be identical to the original IHHNV sequences, indicating that the IHHNV sequence was not modified by passage through *P. vannamei*.

The emergence of IHHNV in the western hemisphere 2 to 3 decades ago may have been from the movement of IHHNV-infected *Penaeus monodon*. Because IHHNV infection is asymptomatic in *P. monodon*, the presence of the virus was not noticed until stocks of other, more susceptible, species became infected.

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