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Sequence diversity of the infectious hypodermal and hematopoietic necrosis virus (IHHNV) in cultured shrimp populations in the Philippines

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Abstract. Partial sequence the capsid protein gene of a Philippine isolate of the infectious hypodermal and hematopoietic necrosis virus (IHHNV), a single-stranded DNA virus that infects both wild and cultured shrimps was identified. The obtained sequence shared 96-97% nucleotide identity with the capsid protein gene of the other IHHNV isolates. Phylogenetic analyses showed that the capsid protein gene of the different IHHNV isolates did not cluster in terms of geographical location, indicating the widespread distribution of the virus. Among the Philippine isolates, IHHNV apparently has high genetic diversity. A sequence obtained from an IHHNV-infected shrimp postlarvae, *Penaeus monodon* in Central Philippines did not cluster with the other Philippine isolates and could represent a distinct isolate. The results of this study support the earlier findings that IHHNV has a high evolutionary rate, and given this observed phenomenon, strict monitoring of the movement of shrimps among geographic regions must be observed to prevent the occurrence of future disease outbreaks due to this viral pathogen.
Key Words: infectious hypodermal and hematopoietic necrosis virus, IHHNV, shrimp, *Penaeus monodon*.

Introduction. Viral diseases are serious problems of the shrimp culture industry on a global scale. Among the viruses that infect shrimp, the infectious hypodermal and hematopoietic necrosis virus (IHHNV) is one of the important DNA viruses that cause tremendous mortality in shrimp. This viral pathogen affects two of the most important shrimps species, Litopenaeus stylirostris and Litopenaeus vannamei. During an infection, IHHNV causes up to 90% mortality in *L. stylirostris* juveniles and sub-adults (Lightner et al 1983). This virus is not lethal to *L. vannamei*, but can lead to growth reduction and severe cuticular deformities in the affected shrimp known as the runt deformity syndrome (Kalagayan et al 1991). These deformities can reduce the market value of the shrimp by 10-50%, depending on the severity of the disease (Lightner & Redman 1998).

IHHNV is a single-stranded DNA virus that belongs to the Parvoviridae family (Bonami et al 1990). In addition, its virions are non-enveloped icosahedrons with size of approximately 22 nm in diameter. It has a genome size of 4.1 kb whith three open reading frames (ORFs), coding for a non-structural protein, a capsid protein and a third ORF of unknown function (Shike et al 2000). Moreover, this virus is closely related to the mosquito brevidensoviruses.

The virus is widely distributed in both cultured and wild shrimp populations in the Americas, Asia and Oceania (Nunan et al 2000; Tang & Lightner 2002). The pathogen was first detected in Hawaii in 1981 from *L. stylirostris* stocks imported from Costa Rica and Ecuador (Lightner et al 1983). By 1987, its presence was confirmed in the Gulf of California, Mexico (Lightner et al 1992) and has caused epizootics in both wild and farmed populations of *L. stylirostris* (Pantoja et al 1999). The prevalence of this virus among wild stock of *L. stylirostris* in the Gulf of California reached almost 100% by 2005

(Robles-Sikisaka et al 2010), however, massive mortalities brought about by this pathogen have not been observed since the epizootics in 1990 and an increase in the wild catch of the shrimp has been observed (Morales-Covarrubias et al 1999).

The high prevalence of the virus but with low incidence of shrimp mortalities assocated with the pathogen has led to a presumption that either the shrimp populations of *L. stylirostris* developed resistance against the virus or the pathogen has attained an equilibrium with the host in terms of the activity of virulence-related genes (Morales-Covarrubias et al 1999; Tang & Lightner 2002). It was suggested by Tang & Lightner (2002) that apparently the IHHNV has been stable due to a more balanced host-pathogen relationship, and an evidence of this was the limited nucleotide variation among IHHNV isolates. Another shrimp virus, the white spot syndrome virus (WSSV), also showed the same phenomenon (Zwart et al 2010). However, a recent study done by Robles-Sikisaka et al (2010) using more number of IHHNV isolates showed that IHHNV had high genetic diversity than previously thought and at the same time there was a highly significant subdivision among populations of this virus in Mexican waters. These findings have implications regarding proper management in the movement of shrimp stocks across geographical locations in order to prevent future viral outbreaks.

In the Philippines, IHHNV has been detected from both *Penaeus monodon* and *L. vannamei* based on the sequences of the IHHNV capsid protein gene that have been deposited in the public database (Genbank, http://www.ncbi.nlm.nih.gov/nucleotide/). Recently, the ban on the importation of *L. vannamei* has been lifted as a means to revive the ailing shrimp industry in the country (Aguiba, 2007). The lifting of the ban means that there must be strict monitoring and screening of the imported shrimp stock for the presence of the virus. Hence, there must be a thorough information on the genetic diversity of IHHNV isolates in the country so that proper monitoring of the presence of the virus can be implemented. This study aimed at profiling the different isolates of IHHNV found in Philippine waters through sequence analysis of its capsid protein gene. It also aimed to characterize the phylogenetic relationship of the Philippine isolates of IHHNV with the other geographical isolates of the virus.

Materials and Methods

Shrimp samples. Shrimp post-larvae (PL 15-20) were obtained from a hatchery in Iloilo, Philippines (Central Philippines). These were immediately placed in microfuge tubes containing 1 ml of DNA extraction buffer (Caipang et al 2004) and kept at room temperature for subsequent extraction of genomic DNA.

Genomic DNA extraction. Genomic DNA from post-larvae was isolated following the modified procedures described by Caipang et al (2011). The genomic DNA was resuspended in 100 µl of 1X TE buffer (pH 7.5) and stored at -20^{o}C for subsequent PCR assays.

PCR amplification and sequence analyses. A 703-bp fragment of the capsid protein gene of IHHNV was amplified using the primers (forward: 5'-TAATGAAGACGAAGAACACGCCGAAGG -3'; reverse:5'- TGGGTAGACTAGGTTTCCAAGGGAT GGTT -3') designed by Yang et al (2007). These primers have been found to be highly specific for the detection of IHHNV.

The reaction mixture consisted of 1.5 µl of both forward and reverse primers (5 pmol), 2 µl of 10x PCR buffer, 1.5 µl of 2 mM dNTP, 1 µl of 15 mM $MgCl_2$, 0.1 µl of Taq DNA polymerase (100 units) (Invitrogen, U.S.A.), 1.5 µl of the DNA template (1 µg ml^{-1}) and scaled up to 20 µl using distilled water. PCR amplification was carried out using the following conditions: initial denaturation at 95^{o}C for 3 min, followed by 40 cycles of denaturation at 95^{o}C for 30 s, annealing at 50^{o}C for 30 sec and elongation at 72^{o}C for 1 min; then a final elongation at 72^{o}C for 5 min. The PCR products (4 µl) were electrophoresed on a 1.5 % agarose gel with 1.0% TBE electrophoresis buffer (pH 8.0) for 30 min, visualized using a hand-held densitometer and photographed.

The PCR products from the amplification of the fragment of the IHHNV capsid protein gene were treated with shrimp alkaline phosphatase to remove unbound phosphates during the PCR reaction. Direct sequencing of the PCR products was done

using Big Dye Terminator ver 3.1 following the procedures of the manufacturer (Applied Biosystems, CA, USA). Comparative sequence analyses were performed by the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/). Multiple alignment of the partial sequences of the IHHNV capsid protein gene was done using CLUSTAL W (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Phylogenetic tree was constructed by the Neighbor Joining Method with 1000 boostrap values of MEGA 4 (Tamura et al 2007).

Results and Discussion. Using a previously published primer set to amplify a fragment of the IHHNV capsid protein, we obtained a partial sequence of the gene consisting of 648 bp in length (Fig 1). This virus was isolated from the post-larvae of *P. monodon* from a hatchery in Central Philippines.

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        10         20         30         40         50         60
CCACACAGAT GTCTACAATT CAATACTGGA GACTCAATAC ATGTTACTTT CCAAACAAGA

        70         80         90        100        110        120
AGATACTTCG AATTCGACGC TGCCAATGAT GGAAACTTCG ACGGAAAAAA TTTATACTGC

       130        140        150        160        170        180
CTCCCACTAC ATTGGATGAA CTTATATCTC TATGGTCTGA AGAGCAGTGA CAGTTCAGCA

       190        200        210        220        230        240
ACAGAAACAC AGCGATATAA AATGGTAAAA TCAATGATGA AAACCTACGG ATGGAAAGTT

       250        260        270        280        290        300
CACAAAGCAG GCGTCGTAAT GCACTCGATG GTACCCCTTA TGAAAGACTT AAAAGTATCA

       310        320        330        340        350        360
GGAGGAACAT CATTTGAGAC TCTCACATTT ACAGACACCC CATATTTAGA AATATTTAAG

       370        380        390        400        410        420
GATACTACTG GACTACATAA TCAACTATCA ACTAAGGAAG CCGACGTAAC ATTGGCAAAA

       430        440        450        460        470        480
TGGATACAAA ATCCCCAACT TGTGACAGTA CAATCAACAG CAGCAAACTA TGAAGACCCA

       490        500        510        520        530        540
ATCCAACAAT TTGGATTCAT GGAACAAATG CGAACTGGAG ACCGAAAAGC CTATACAATC

       550        560        570        580        590        600
CATGGTGACA CTAGAAATTG GTATGGCGGA GAAATACCAA CAACCGGACC CACCTTCATC

       610        620        630        640        650        660
CCAAAATGGG GTGGCCAAAT AAAATGGGAC AAACCATCCC TTGGAAAC
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Figure 1. Partial nucleotide sequence of the capsid protein gene from a Philippine isolate of IHHNV.

Phylogenetic analysis using the sequences of the capsid protein gene of IHHNV from different geographic isolates showed the presence of different clades (Fig. 2). At least four distinct clades were evident, suggesting that IHHNV has a wide genetic diversity. Interestingly, the IHHNV isolate in the present study seems to have its own clade, which is the transition between the multi-geographic (Mexico and other location) isolates and the Australasian (Australia and Thailand) isolates. Further genetic studies are needed to confirm the exact phylogenetic position of this IHHNV isolate as well as to establish whether this is a distinct haplotype from previously characterized haplotypes of IHHNV (Robles-Sikisaka et al 2010).

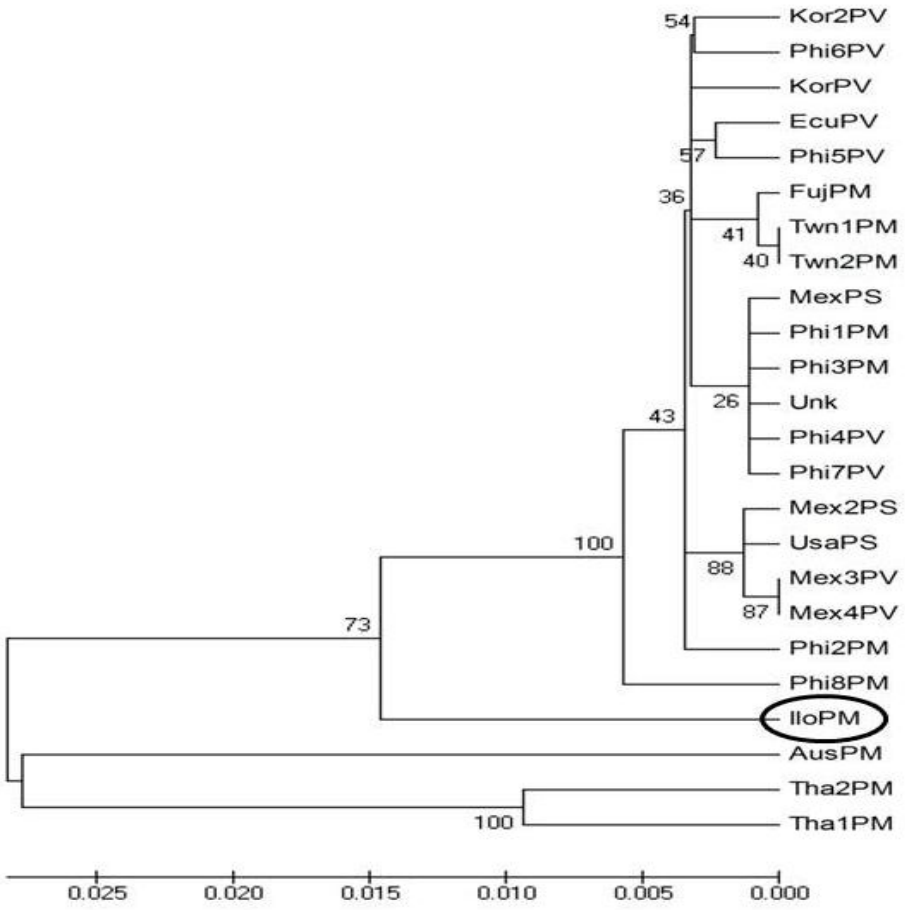

Figure 2. Phylogenetic tree of the capsid protein gene of the different isolates of IHHNV.

Further phylogenetic analysis of the recent isolate of IHHNV showed that it forms a distinct clade from the previously characterized IHHNV isolates from the Philippines (Fig. 3). We could not clearly determine whether this is a result of genetic variation among IHHNV isolates or it could be due to introduction of a new isolate of the virus brought about by the importation of exotic shrimp species such as the white shrimp, *L. vannamei*. Further studies have to be done to establish whether the different IHHNV isolates in the Philippines come from a common ancestor and the occurrence of the different haplotypes is brought about by genetic recombination. Robles-Sikisaka et al (2010) found high levels of diversity of the IHHNV isolates in Mexico. Their findings were in contrast to earlier reports on sequence diversity of IHHNV in which these studies suggested low variation and stability of the virus (Pantoja et al 1999; Tang & Lightner 2002; Tang et al 2003). In addition, Robles-Sikisaka et al (2010) also demonstrated no evidence of recombination of the IHHNV isolates, although this feature is commonly observed in parvoviruses (Shackelton et al 2007).

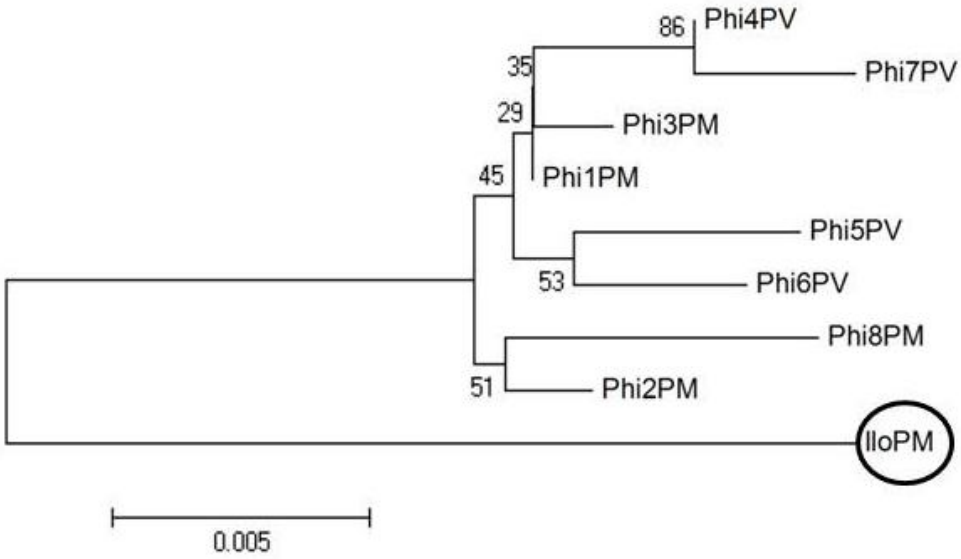

Figure 3. Phylogenetic tree of the capsid protein gene of the different isolates of IHHNV in the Philippines. The virus isolate that was used in the present study is encircled.

Table 1 shows the homology of the capsid protein gene of the IHHNV that was squenced in the present study with the previously characterized sequences of the same gene from different geographical isolates of the virus. It shows that based on the partial sequence obtained in the present isolate, there was a nucleotide homology of 96-97% with the other isolates of IHHNV regardless of the host organism. This indicates that the IHHNV isolates from these different areas could have originated from a single source and the differences in their nucleotide bases might be due to selection pressure and could lead to the emergence of different haplotypes. Further tests particularly extensive sequencing of the genome of the different IHHNV isolates in the Philippines are needed in order to establish the genetic diversity of this virus in the country. Thus, it could provide baseline information on the virulence of this virus in relation to certain mutations in the nucleotide sequences in its genome.

In the present study, we have shown that there are nucleotide differences in the IHHNV capsid protein gene in relation to their geographical location. Such differences could be attributed to the emergence of new strains. This phenomenon was also observed in the genome of the different isolates of the monodon baculovirus (MBV) (Doubrovsky et al 1988). The recently-sequenced capsid protein gene of a Philippine isolate formed a separate sub-clade than the previously-characterized IHHNV isolates from the country. This could mean that there exists at least two strains of IHHNV in the Philippines, and further sequence analyses must be done to support the findings of the present study.

Robles-Sikisaka et al (2010) showed that IHHNV exhibited genetic differentiation among the geographic regions in the Gulf of California. They also asserted that the presence of several quickly evolving lineages of this virus in the region could result in the emergence of virulent strains especially when there are changes in the hist or environmental conditions. Such case was observed in the emergence of the lethal strain of the Taura syndrome virus (TSV) in Mexican shrimp farms (Robles-Sikisaka et al 2010). The lethal TSV strain was not detected by routine diagnostic procedures because of the presence of a mutation in the capsid protein gene (Robles-Sikisaka et al 2002). Hence, to prevent similar outbreaks of potentially lethal strains IHHNV in the future, routine genetic screening of IHHNV isolates must be done to facilitate identification of

undetectable strains of IHHNV. The IHHNV in the Philippines is no exception based on the preliminary findings obtained in the present study, and thus must be strictly monitored.

Table 1

Sequence of homology the capsid protein gene with the other IHHNV isolates

Isolate (Accession No.)	Host	% Homology	Isolate (Accession No.)	Host	% Homology
Taiwan 1 (AY355308.1	*P. monodon*	97	Mex 3 (GU906955.1)	*L. vannamei*	97
Phil 1 (GU138657.1)	*P. monodon*	97	Mex 4 (GU906954.1)	*L. vannamei*	97
Mex 1 (GU906924.1)	*L. stylirostris*	97	Kor 1 (HQ699074.1)	*L. vannamei*	96
Taiwan 2 (AY355306.1)	*P. monodon*	97	Phil 8 (GU138658.1)	*P. monodon*	96
Mex 2 (GU906923.1)	*L. stylirostris*	97	Phil 6 (GU138650.1)	*L. vannamei*	97
Unk (AF218266.2)	*unknown*	97	Ecuador (AY362548.1)	*L. vannamei*	96
Fujian (EF633688.1)	*unknown*	97	Phil 7 (GU138653.1)	*L. vannamei*	96
Phil 2 (GU138660.1)	*P. monodon*	97	Kor 2 (HQ699073.1)	*L. vannamei*	96
Phil 3 (GU138655.1)	*P. monodon*	97	Thai (AY362547.1)	*P. monodon*	97
USA (AF273215.1	*L. stylirostris*	97	Thai 2 (AY102034.1)	*P. monodon*	97
Phil 4 (GU138654.1)	*L. vannamei*	97	Austral (GQ475529.1)	*P. monodon*	97
Phil 5 (GU138649.1)	*L. vannamei*	97			

Phil = Philippines
Mex = Mexico
Kor = Korea
Thai = Thailand
Austral = Australia

Conclusions. In summary, based on partial sequences of the capsid protein gene of IHHNV, there is a possibility of the presence of at least two strains of IHHNV in the Philippines. Routine monitoring of the presence of this virus in the cultured and wild stock must be implemented to prevent the emergence of lethal strains that will result in severe mortalities in the shrimp population. The sequences analysis was done on a particular gene sequence and additional studies involving the coding and non-coding regions of IHHNV are necessary to clearly establish whether the same pattern of strain differences occurs in this virus. Studies are underway to develop and standardize early and rapid detection methods for the different IHHNV isolates.

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