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Prevalence, diversity and co-occurrence of the white spot syndrome virus, monodon baculovirus and *Penaeus stylirostris* densovirus in wild populations of *Penaeus monodon* in the Philippines

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ABSTRACT: The farming of the black tiger shrimp *Penaeus monodon* in the Philippines relies on wild broodstock. PCR was thus used to determine the prevalence of white spot syndrome virus (WSSV), monodon baculovirus (MBV) and *Penaeus stylirostris* densovirus (PstDV) in a total of 178 shrimp from 6 geographically disparate locations where broodstock are captured for use in hatcheries. PCR amplicons were also sequenced to identify phylogenetic relationships of the virus haplotypes detected. Shrimp from southeastern Luzon (Camarines Norte) had the highest prevalence of each of the 3 viruses and were frequently co-infected with 2 or more viruses. No viruses were detected in shrimp from northwestern Luzon (Pangasinan). MBV was most prevalent and PstDV strains displayed the most genetic diversity. WSSV was detected at 3 sites, and a VP28 gene sequence examined was invariant and consistent with strains found in many countries, including Thailand, China, Japan, Korea, Indonesia, Iran, Brazil and Mexico. WSSV open reading frame 94 gene sequence analysis identified location-specific repeat types. MBV sequences were dissimilar to haplotypes detected in India. PstDV sequences were diverse and included 2 lineages detected either in Australia or in the United States, Ecuador, Taiwan, China and Vietnam. The PCR data confirmed that WSSV, MBV and PstDV are endemic in *P. monodon* in the Philippines but that populations at some locations might remain free of infection.

KEY WORDS: White spot syndrome virus · WSSV · *Penaeus stylirostris* densovirus · PstDV · Monodon baculovirus · MBV · *Penaeus monodon* · Prevalence · Diversity · Philippines

INTRODUCTION

Several viruses are pathogenic for the black tiger shrimp *Penaeus monodon*. Diseases caused by viruses, including white spot syndrome virus (WSSV), monodon baculovirus (MBV) and *P. stylirostris* densovirus (PstDV), have resulted in serious economic impacts for farming of this species (Lightner 1996, Flegel 2006). Since the discovery of white spot disease (WSD) in Taiwan in 1992 (Chou et al. 1995), mass mortality resulting from WSD has devastated shrimp aquaculture industries worldwide. WSSV is classified in the genus *Whispovirus* of the family Nimaviridae and has a genome comprised of ~310 kb circular double-stranded DNA (Fauquet et al. 2005). MBV was first detected in *P. monodon* farmed in Taiwan, is classified in the genus *Nucelopolyhedrovirus* of the family Baculoviridae and has a genome comprised of long circular double-stranded DNA (Rohrmann 1986, Belcher & Young 1998). MBV replication occurs in

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the nucleus of hepatopancreatic and midgut epithelial cells, and mortality due to disease primarily impacts larval life stages in hatcheries (Lightner & Redman 1981, Fegan et al. 1991, Lightner 1996, Bondad-Reantaso & McGladdery 2001). PstDV was first detected in juvenile pacific blue shrimp *P. stylirostris* being cultured in Hawaii in 1981 (Lightner et al. 1983), has subsequently been detected in most penaeid species farmed in different regions of the world (Lightner 1996, Tang et al. 2003), is classified in the genus *Brevidensovirus* of the family *Parvoviridae* (Bonami et al. 1990) and has a genome comprised of ~4.1 kb single-stranded DNA (Bonami et al. 1990, Mari et al. 1993). While PstDV infection can result in mortality, disease is more commonly associated with retarded growth and cuticle and rostrum deformities referred to as runt deformity syndrome (Primavera & Quinitio 2000).

To assist the shrimp farming industry in the Philippines in understanding and managing the risks of utilizing wild-caught *P. monodon* broodstock, the prevalence and genetic makeup of WSSV, MBV and PstDV was assessed at 6 disparate geographical locations where broodstock are captured for use in hatcheries.

MATERIALS AND METHODS

Shrimp

A total of 178 wild female *Penaeus monodon* were trawled by fishermen at 6 locations in the northern, central and southern Philippines (Camarines Norte, Quezon, Leyte, Pangasinan, Cagayan, Zamboanga del Sur) representing the primary sources of broodstock used in hatcheries. Shrimp from each location were captured by local fishermen provided with a standard procedure for shrimp handling and storage. This procedure specified that shrimp be frozen immediately after collection and shipped frozen to the laboratory within 24 h. On arrival at the laboratory, shrimp were inspected and only those in visibly good condition were used. Information on the collection sites, dates and sample sizes is summarized in Table 1. WSSV, MBV and PstDV positive control material was obtained from the Fish Health Management and Quality Assurance Laboratory, Bureau of Fisheries and Aquatic Resources, Department of Agriculture (BFAR-DA). Tissue samples were dissected aseptically from each shrimp and refrozen at −20°C until processed.

Table 1. *Penaeus monodon* numbers examined from different locations in the Philippines

Location	Shrimp number	Sample code	Collection date
Daet, Camarines Norte	30	CAM 101–130	December 2014
Tagkawayan, Quezon	30	QUE 101–130	February 2015
Tacloban, Leyte	30	LEY 101–130	March 2015
Alaminos, Pangasinan	30	PAN 101–130	March 2015
Buguey, Cagayan	28	CAG 101–128	April 2015
Pagadian, Zamboanga del Sur	30	ZAM 101–130	May 2015

DNA extraction

Total genomic DNA was extracted from hepatopancreas tissue according to the Bioline® DNA Isolation Kit protocol. DNA was quantified using a Shimadzu UV Biospec-Nano spectrophotometer and diluted to a concentration of 10 ng μl^{-1} in PCR-grade purified water.

PCR detection of WSSV, MBV and PstDV

WSSV, MBV and PstDV were detected by PCR or nested PCR using published primer sequences and thermal cycling conditions (Table 2). Each 20 µl reaction contained Vivantis 1 × Taq polymerase buffer, 0.2 mM dNTPs, 0.5 µM forward and reverse primers, 1 U Taq DNA polymerase and 1–2 µl DNA. The sizes of amplified DNA products were confirmed using agarose gel electrophoresis and amplicons were purified and sequenced at First BASE Laboratories, Selangor, Malaysia.

Amplicon sequence analyses

DNA amplicons were sequenced in forward and reverse directions using primers employed in each PCR and sequence chromatograms were checked and edited using DNA Baser version 3.5 (Heracle Biosoft). ClustalW in MEGA 6 (Tamura et al. 2013) was used to align sequences and infer phylogenetic relationships according to the lowest Bayesian's information criterion and Akaike's information criterion scores. Bootstrap support was estimated with 1000 replicates. Phylograms were edited using Adobe® Illustrator CS5.

Table 2. PCR primer sequences and amplicon lengths

Primer name	Sequence (5′–3′)	Product size (bp)	PCR annealing temperature (°C)	Reference
WSSV VP26F	ATGGAATTTGGCAACCTAACAAACCTG	304	52	Dieu et al. (2004)
WSSV VP26R	GGGCTGTGACGGTAGAGATGAC			
WSSV VP28F	GAAACCCACACAGACAATATCG	245	45	Syed Musthaq et al. (2006)
WSSV VP28R	CTTCCCTCAAAGGTGAGATTC			
WSSV ORF94F	TCTACTCGAGGAGGTGACGAC	404–720	60–69	Wongteerasupaya et al. (2003)
WSSV ORF94R	AGCAGGTGTGTACACATTTCATG			
MBV1.4F	CGATTCCATATCGGCCGAATA	533	45	Belcher & Young (1998)
MBV1.4R	TTGGCATGCACTCCCTGAGAT			
MBV1.4NF	TCCAATCGCGTCTGCGATACT	314	50	
MBV1.4NR	CGCTAATGGGGCACAAGTCTC			
IHHNV648F	GAACGGCTTTCGTATTTTGG	648	45	Rai et al. (2009)
IHHNV648R	AGCGTAGGACTTGCCGATTA			
IHHNV309F	TCCAACACTTAGTCAAAACCAA	309	55	Tang et al. (2007)
IHHNV309R	TGTCTGCTACGATGATTATCCA			

RESULTS

WSSV

A WSSV VP28 PCR primer pair (Syed Musthaq et al. 2006) was used to detect WSSV in hepatopancreas tissue sampled from groups of 28–30 wild *Penaeus monodon* (total = 178) collected from each of 6 disparate locations in the Philippines. WSSV DNA was amplified from 36 shrimp collected at 3 of the 6 locations: Camarines Norte (25/30), Zamboanga del Sur (7/30) and Leyte (4/30) (Table 3). For all 36 WSSV-positive shrimp, sequences determined for a 202 bp region of the VP28 gene were identical (GenBank accession numbers: KY273305 to KY273340).

The VP28 gene sequence detected was consistent with WSSV strains analyzed from Thailand (EF 194079), China (AY249440), Japan (AY249443), Korea (AY324881), Indonesia (AY249441), India (DQ681069), Iran (AB855742), Brazil (HQ130032) and Mexico (FJ756456).

The open reading frame (ORF) 94 PCR designed to amplify across a variable-number tandem repeat (VNTR) region of the WSSV genome (Wongteerasupaya et al. 2003) generated amplicons for 27 of the 36 WSSV-positive shrimp. All shrimp yielded a single amplicon suggesting the presence of a single WSSV strain. Nucleotide sequences determined for the 27 amplicons (GenBank accession numbers KY273341 to KY273366) had 5 to 11 tandem copies of the 54 bp repeat and were designated ORF94-5 to ORF94-11, with repeat frequencies of ORF94-10 (n = 6), ORF94-11 (n = 6), ORF94-7 (n = 5), ORF94-6 (n = 4), ORF94-9 (n = 4), ORF94-5 (n = 1) and ORF94-8 (n = 1). In

Table 3. Numbers of wild *Penaeus monodon* captured at the 6 locations in the Philippines in which white spot syndrome virus (WSSV), monodon baculovirus (MBV) and/or *Penaeus stylirostris* densovirus (PstDV) were detected by PCR

Viruses	Camarines Norte (n)	Quezon (n)	Leyte (n)	Pangasinan (n)	Cagayan (n)	Zamboanga del Sur (n)	Total (n)
None	1	15	17	30	21	14	98
WSSV only	4	0	0	0	0	2	5
MBV only	1	9	5	0	1	1	17
PstDV only	3	2	2	0	6	6	19
WSSV and MBV	11	0	0	0	0	0	11
MBV and PstDV	1	4	2	0	0	2	9
WSSV and PstDV	5	0	2	0	0	5	12
WSSV, MBV and PstDV	5	0	2	0	0	0	7
WSSV total	25	0	4	0	0	7	36
MBV total	18	13	9	0	1	3	44
PstDV total	14	6	8	0	6	13	47

addition, VNTR types detected at each of the 3 locations were unique: Camarines Norte, ORF94-7, -10 and -11; Leyte, ORF94-5 and -8; Zamboanga del Sur, ORF94-6 and -9. A cytosine thymine (C/T) polymorphism occurred at position 48 in each 54 bp repeat, with T being dominant in strains with ≥10 repeats and C dominant in strains with <10 repeats.

MBV

A nested PCR designed to amplify a 314-bp MBV genome region (Belcher & Young 1998) detected MBV DNA in 52 out of 178 hepatopancreas DNA samples examined at variable prevalence depending on shrimp capture location: Camarines Norte, 83.3%; Quezon, 43.4%; Leyte, 30.0%; Zamboanga del Sur, 10.0%; Cagayan, 6.7%; Pangasinan, 0% (Table 3).

For the 52 MBV PCR amplicons, sequence analysis of the 272 bp region internal to the nested PCR primers (GenBank accession numbers KY274526 to KY274571) identified variations at 12 nucleotide positions. Sequence comparisons identified 8 haplotypes (Fig. 1), but only a small number (up to 3) were detected at each site. Haplotype M-PH 2 was detected most commonly (n = 36) among MBV-positive shrimp from the 5 capture locations where MBV was detected, followed by M-PH 1 (n = 4) found only at Camarines Norte. Distinct haplotypes (M-PH 3, 4, 5, 6, 7 and 8) were detected in 6 shrimp from variable locations.

MBV haplotypes inferred using a maximum likelihood tree constructed using the Tamura-3 model with gamma distribution (T92+G) demarcated 2 lineages, highlighting divergence between MBV strains in the Philippines and India, although with low bootstrap support (61%), and indicated that the MBV strains detected at the different locations were closely related (Fig. 2).

PstDV

A PCR test designed to amplify a 309 bp region of the PstDV genome (Tang et al. 2007) generated amplicons for 49 of the 178 shrimp tested (Table 3). Detection prevalences varied markedly in shrimp captured at different sites (Camarines Norte, 50.0%; Zamboanga del Sur, 50.0%; Quezon, 23.3%; Cagayan, 23.3%; Leyte, 16.7%; Pangasinan, 0%).

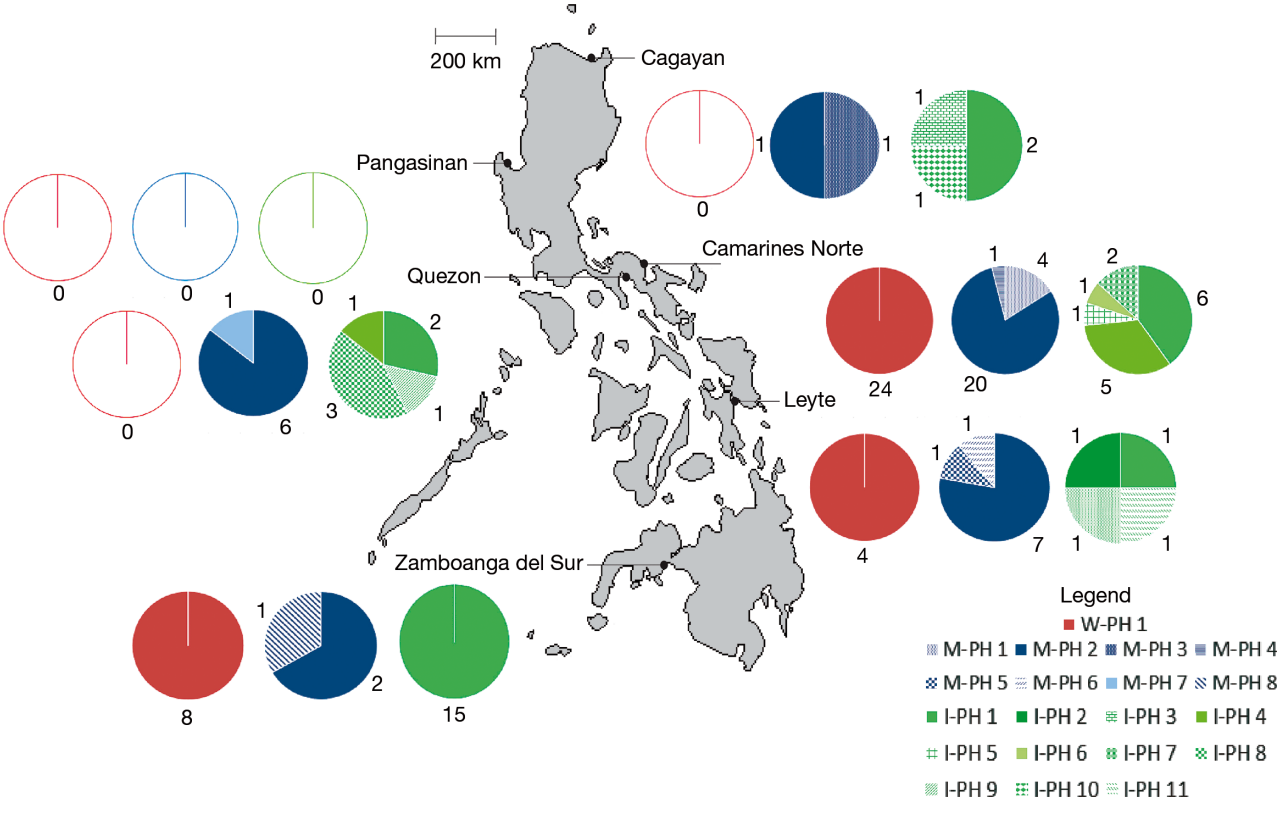

Fig. 1. White spot syndrome virus (WSSV), monodon baculovirus (MBV) and *Penaeus stylirostris* densovirus (PstDV) haplotype distribution in wild *Penaeus monodon* from 6 locations in the Philippines. Numbers outside the graphs are frequency of haplotypes at a given site; W-PH: Philippine WSSV haplotype; M-PH: Philippine MBV haplotyes; I-PH: Philippine PstDV haplotype

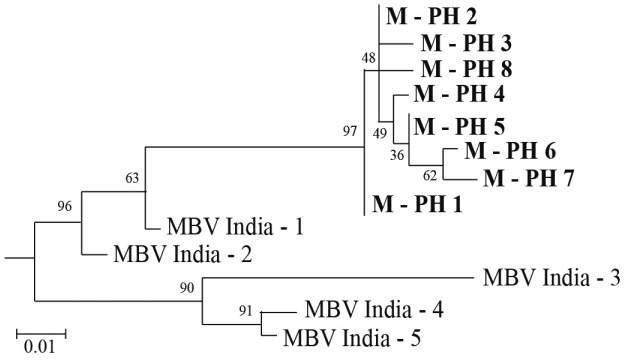

Fig. 2. Phylogenetic relationships of monodon baculovirus (MBV) haplotypes inferred using the maximum likelihood method (see 'Results' for details). Number beside each node represents bootstrap support value. M-PH: Philippine MBV haplotype

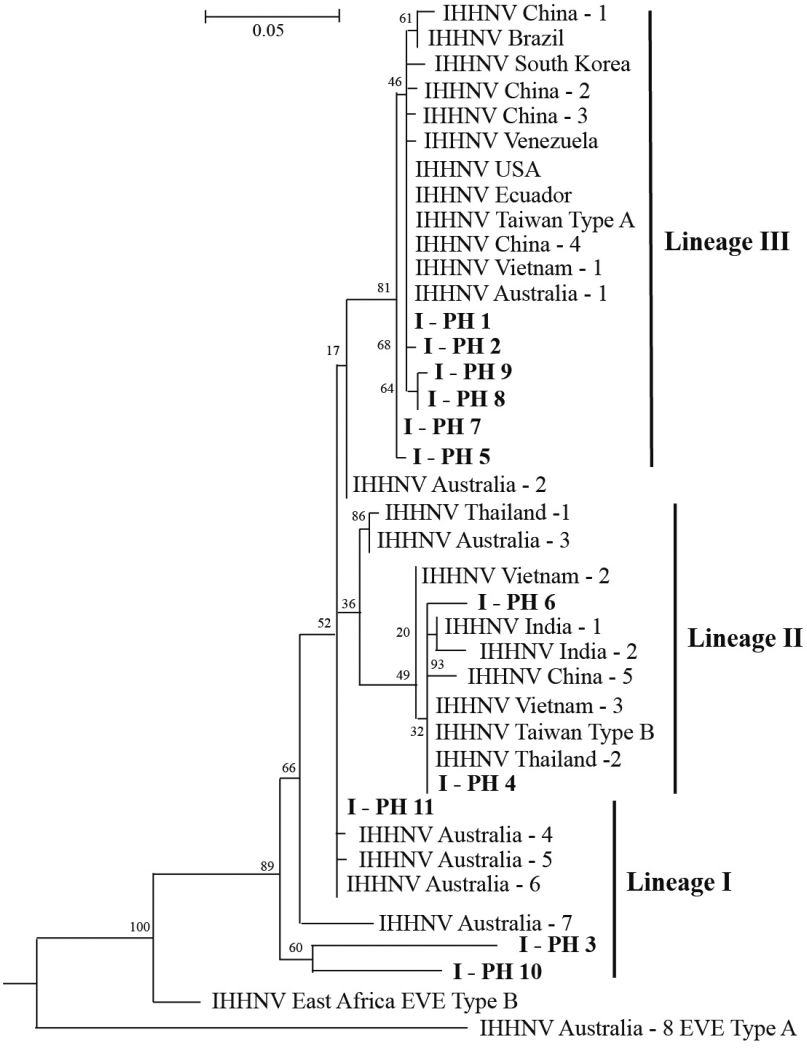

Fig. 3. Phylogenetic relationships of *Penaeus stylirostris* densovirus (PstDV) haplotypes inferred using the maximum likelihood method (see 'Results' for details). Number beside each node represents bootstrap support value. I-PH: Philippine PstDV haplotype

Sequence comparisons of the 265 bp region internal to the PCR primers for the 49 PstDV-positive shrimp (GenBank accession numbers KY273367 to KY273413) identified nucleotide variations at 54 positions. The sequences grouped into 11 haplotypes (Fig. 1) with different haplotype numbers detected at different shrimp capture locations: Camarines Norte, n = 5; Cagayan, n = 3; Quezon and Leyte, n = 4; Zamboanga del Sur, n = 1. The I-PH 2 haplotype (n = 26) occurred most frequently across the 5 capture locations. Distinct PstDV haplotypes (I-PH 3, 5, 6, 9 and 11) were identified in 6 shrimp from different sites. Only 1 PstDV haplotype was identified in shrimp from Zamboanga del Sur.

Relationships identified among the PstDV haplotypes are shown using a maximum likelihood tree constructed using the Hasegawa-Kishino-Yano model with gamma distribution (HKY+G) (Fig. 3). Haplotypes were separated with low to high bootstrap support. PstDV haplotypes in the Philippines were distributed in the maximum likelihood tree, with several haplotypes (I-PH 3, 10 and 11) clustering with Lineage I and most strains from Australia (KM593909 to KM593913) and I-PH 4 and 6 clustering with Lineage II strains from India (EU552487), Vietnam (KC513422, KF-031144), China (KP733858), Taiwan (AY355307) and Thailand (AY102034). I-PH 1, 2, 5, 7, 8 and 9 clustered with Lineage III strains. I-PH 1, the most frequently detected PstDV haplotype, was identical to strains from the USA (AF 273215), Ecuador (AY362548), Taiwan (AY355306 to AY355308), China (KP733859 to KP733863), Vietnam (JX840067) and Australia (KM272862) (Fig. 3).

Co-occurrence of shrimp viruses

The PCR data provided an opportunity to examine the prevalence at which 2 or more virus types were present in the hepatopancreas of the *P. monodon* examined (Table 3). Virus co-occurrence levels across the 5 capture locations were: WSSV + MBV, 7.3%; WSSV + PstDV, 6.7%; MBV + PstDV, 5.1%; WSSV + MBV + PstDV, 3.4%.

DISCUSSION

WSSV, MBV and PstDV were detected by PCR in wild *Penaeus monodon* captured from 5 of 6 locations in northern, central and southern regions of the Philippines, suggesting that they are endemic and widespread across the country. None of the 3 viruses were detected among any of the 30 shrimp captured at the Pangasinan location in a northwestern region of the Philippines. While these data suggest the these viruses might not occur or occur at low prevalence in *P. monodon* endemic to this region, the frozen shrimp sent for analysis were only assessed visually for signs of inadequate storage; no DNA quality checks or PCR tests for endogenous shrimp genes were performed to assess its integrity. Therefore, it cannot be discounted that the DNA extracted from this group of shrimp had degraded beyond a point where it could be amplified effectively by the PCR tests.

MBV was detected most commonly, WSSV least commonly and sequences determined for PstDV strains were most diverse. Of the 5 out of 6 capture locations in the Philippines at which viruses were detected, all 3 viruses were most prevalent (50 to 83%) among the 30 shrimp captured at Camarines Norte, with many containing various combinations of 2 or all 3 viruses. These findings are consistent with other studies in which wild *P. monodon* have been found to carry multiple viruses (Manivannan et al. 2002, Chayaburakul et al. 2004, Umesha et al. 2006, Prakasha et al. 2007, Tan et al. 2009). The prevalence of WSSV (13 to 80% depending on location) was higher than found in a dry and wet season comparison of wild *P. monodon* sourced from 7 locations (Capiz, Negros Occidental, Bohol, Quezon, Palawan, Misamis Occidental and Surigao del Sur) in the Philippines during 2005 (de la Peña et al. 2007). In that study, WSSV was detected at all locations except Bohol during the dry season (2 to 25% prevalence range) but only at the Palawan location during the wet season.

MBV prevalence levels (10 to 83% depending on location) detected in the groups of *P. monodon* captured at 5 locations were higher than those detected among *P. monodon* sampled in 2005 from the Philippines (de la Peña et al. 2008) using a PCR test employing the MBV1.4NF:1.4NR primer pair known to generate data consistent with the microscopic diagnosis of MBV infection in *P. monodon* postlarvae in hatcheries (Natividad et al. 2006). The widespread detection of MBV was also consistent with data from this study that showed MBV to be present at all evaluated locations except Palawan during the dry season (20% prevalence) but at only 2 of the 7 locations (Negros Occidental and Bohol) during the wet season (9% prevalence) (de la Peña et al. 2008). Taken together with observations of MBV being the most prevalent disease in hatchery and farmed stocks of *P. monodon* in the Philippines in the early 1990s, it is evident that MBV infection is being perpetuated efficiently in wild populations of *P. monodon* (Natividad & Lightner 1992).

Although detected in the Philippines previously (Primavera & Quinitio 2000), the prevalence and genetic makeup of PstDV in disparate wild populations of *P. monodon* has not been examined. The PstDV prevalence (ranging from 16 to 50%) across the 5 locations where it was detected confirmed that it is endemic and remains a threat to *P. monodon* culture in the Philippines.

A WSSV VP28 gene sequence examined displayed no variation among WSSV-positive shrimp detected at 5 of the 6 study locations. The sequence was also identical to WSSV strains examined from Thailand, China, Japan, Korea, Indonesia, Iran and even Brazil and Mexico, thus indicating that strains are related or that the genome region is not particularly useful for epidemiological or genetic diversity studies. In contrast, genotyping using an ORF94 sequence identified VNTR types (ORF94-5, 6, 7, 8, 9, 10 and 11) containing 5 to 11 repeat units (RUs). WSSV strains with similar repeat numbers have been detected in Thailand and Taiwan (6 RUs) and China (12 RUs). ORF94-5 with the least number of RUs has been detected among WSSV strains examined from Mexico (de Jesús Durán-Avelar et al. 2015), Texas and South Carolina (Muller et al. 2010) but not among strains examined from Thailand (Wongteerasupaya et al. 2003), India (Syed Musthaq et al. 2006) or Vietnam (Hoa et al. 2005). Interestingly, distinct ORF94 VNTR types were detected at the 3 locations and a T was always present at position 48 in strains with ≥10 RUs compared with a C in strains with <10 RUs, unlike WSSV strains characterized from other regions. While these findings might be useful for tracing the origin of WSSV strains in wild broodstock within the Philippines and possibly from other countries, until the replication processes that enable increases or decreases in ORF94 RU numbers are delineated, what these variations mean in terms of evolutionary origins of WSSV VNTR types within or across geographically disparate *P. monodon* populations will remain unclear.

Phylogenetic analysis of the PstDV sequences using the HKY+G model revealed that strains detected in *P. monodon* captured at the 5 disparate

locations in the Philippines were genetically diverse and thus distributed across different clades. Most PstDV (I-PH 3, 5, 6, 9 and 11) sequences clustered within Lineage III, similar to PstDV types detected in shrimp from the United States, Ecuador, Taiwan, China and Vietnam. The most common PstDV haplotype in the Philippines, I-PH-1, was found to be identical to strains from the United States, Ecuador, Taiwan, China and Vietnam, suggesting the spread of this haplotype across Asia and the Americas. The possibility that PstDV-infected *P. monodon* from the Philippines might be the origin of PstDV strains now present in the Western Hemisphere (Mexico, Gulf of California, Hawaii, Ecuador, Panama, Colombia) was previously raised based on inference from data on introductions of *P. monodon* broodstock into the Americas (Lightner 1999) and on phylogenetic data (Tang et al. 2003). Moreover, PstDV strains from the Philippines clustering within Lineage I (I-PH 3, 10 and 11) together with Australian PstDV strains suggests that these haplotypes may be derived from a lineage that evolved in Indo-West Pacific *P. monodon*, as suggested previously (Jaroenram et al. 2015).

Overall, the data reported here are consistent with previous reports of WSSV, MBV and PstDV occurring in wild populations of *P. monodon* inhabiting islands throughout the Philippines. They also provide the first information on PstDV prevalence in different populations and on the prevalence of co-infections with these viruses. All shrimp studied appeared healthy and displayed no overt signs of disease, and there is no anecdotal evidence for the *P. monodon* populations from which they were captured being threatened due to disease. This leads to questions around what virus–host interactions and adaptations have evolved in *P. monodon* to accommodate such viral infections and often co-infection by several viruses (Fegan et al. 1991, Flegel et al. 2004). Hypotheses to explain how shrimp survive such assaults include an active viral accommodation concept, in which some physiological mechanism is utilized to tolerate virus infection in an active, but innocuous persistent state that hinders it from progressing to cause disease or from stimulating host-defense responses leading to cell destruction (Flegel & Pasharawipas 1998, Flegel 2007). Populations of wild *P. monodon* in the Philippines might thus provide a useful resource for validating or challenging such hypotheses.

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