Effects of extensive and intensive shrimp farming on the genetic composition of white spot syndrome virus populations

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ABSTRACT

White spot syndrome virus (WSSV) has a major negative impact on shrimp farming and industry. There are many different shrimp farming practices, and these may affect the genotypic composition of WSSV populations and possibly the virulence of the virus. Here we investigated whether extensive and intensive farming practices (1) result in selection of WSSV genotypes, and (2) affect genotypic composition over time in WSSV populations. WSSV samples were collected from Vietnamese farms on various sites over a period of several years and the samples were then genotyped. We found no significant effect of farm practice on the genotypic composition of WSSV populations. On the other hand, we did find an effect of farm practice on change over time in the ORF23/24 variable region: this region was significantly more stable in extensive farming systems. This result is a first observation suggesting that farm practice may affect the evolutionary dynamics of WSSV. Moreover, these data also suggest that for retrospectively studying the spread of WSSV, it is better to sample from extensive farms than from intensive farms because WSSV populations in extensive farms will be more stable over longer period of time.

Keywords: White spot syndrome virus, shrimp farming, shrimp aquaculture, genetic marker, epidemiology

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INTRODUCTION

White spot syndrome virus (WSSV) has been a scourge on shrimp farming since the early 1990s, causing massive mortality and major damage to many types of shrimp farming operations, from extensive to high-intensity practices (Escobedo-Bonilla *et al.*, 2008). During this period the virus has undergone distinct genotypic change resulting in the occurrence of variants world-wide and in variants with increased fitness and virulence (Marks *et al.*, 2004, 2005). This suggests an adaptive evolution to the novel environment and ecological niche provided by shrimp ponds. In shrimp ponds host density will typically be much higher than in natural habitats. The between- and within-species variation in hosts is also likely to be lower in intensive ponds than in natural environments, and hosts are likely to be stressed due to pond conditions. All of these factors probably contribute to generating a novel environment in which WSSV can thrive and further optimize its fitness.

Shrimp-farming operations are, however, highly varied. Extensive and improved extensive shrimp farms (i) stock shrimp larvae directly from the sea, in part or entirely, (ii) have a relatively low density of shrimp, and (iii) have overlapping generations of shrimp. On the other hand, intensive shrimp farms (i) stock shrimp post-larvae (PL) from hatcheries, and the broodstock can originate from geographic locations far from the farm, (ii) have a relatively high density of shrimp, and (iii) have non-overlapping generations of shrimp. Typically a pond is seeded with postlarvae (PL) and they are subsequently harvested together, after which the pond is drained and cleaned. Because extensive and intensive shrimp farms provide such differing environments, farming practice may lead to differential selection of WSSV genotypes. Moreover, in intensive farming operations, virus populations present in ponds may be largely discontinuous. This discontinuity will arise because (1) regular drainage and cleaning of the pond removes infectious host cadavers or debris, and (2) non-overlapping shrimp generations preclude between-cohort transmission of the virus by infectious shrimp. This may have implications for the genetic composition, evolutionary dynamics and epidemiology of WSSV populations.

Here, we hypothesize that different farming practices will have an effect on WSSV genotype composition and population structure. WSSV isolates were collected from Vietnamese improved extensive and intensive shrimp farms at different geographic locations and different time points. These isolates from each type of farm were genetically characterized, allowing us to test whether farming practice affected genotypic composition and change thereof over time.

METHODS

Classification of shrimp farms

WSSV infected shrimp were purposely collected from shrimp farms with different farming practices. We classified the farms based on farm organization and management, in a manner similar to Nhuong *et al.* (2002) using the following three categories:

(1) Intensive shrimp farming: Pond size varies from 0.2 to 0.6 ha, and a stocking density ranging from 15-30 post larvae per m^2. Shrimp are stocked only once for each crop, industrial shrimp food is used, and water oxygen supply is augmented by machinery. PLs are bought from local hatcheries, but the origin of broodstock is typically unknown. Shrimp crops are harvested after about four months. If there are disease outbreaks, shrimp are quickly harvested and the pond is chemically treated and drained for cleaning prior to new stocking.

(2) Improved extensive farming: Pond size greatly varies, ranging from 1 to 15 ha, including ditches and surrounding dikes. Shrimp seed are trapped from wild stock by making use of tides, and sometimes farmers supplement wild stock with PL from hatcheries (approximately once a month, although this varies greatly between farms). This results in a low stocking density of 1-2 shrimp per m^2. No additional feed is required in this system as shrimp use natural feed in pond. Farmers typically harvest shrimp once or twice a month, based on tides. Large shrimp are trapped by nets as marketable harvest, although small shrimp suffering from disease are also often trapped and hereby removed from the pond.

(3) Shrimp-rice farming: similar to improved extensive farming in terms of farm management, although the plots used are somewhat smaller than improved extensive ponds. In the Mekong Delta of Vietnam, farmers use the same plot to cultivate rice in the rainy season, when freshwater is abundant, and shrimp in the dry season, when saline water is used to flood the plot.

Collection of virus isolates

Shrimp showing disease symptoms characteristic for WSSV were selected, cleaned on the outside with 70% ethanol, and stored in 96% ethanol during transportation to Can Tho University (Vietnam). The ethanol was then removed and samples were stored at -20° C until further processing. All shrimp selected were *Penaeus monodon*. Information on the WSSV infected shrimp collected is recorded in Table 1, and geographic locations are given in Fig. 1.

Genetic characterization of virus isolates

The characteristics of WSSV have been described by Vlak *et al.* (2005) and further International Commission on Taxonomy of Virus (ICTV) updates can be found at http://ictvonline.org. Marks *et al.* (2004) identified five variable regions (see Fig. 2): three loci with variable number of tandem repeats (VNTR; ORF75, ORF94 and ORF125; ORF nomenclature according to Van Hulten *et al.*, 2001), and two loci with large deletions (ORF23/24, ORF14/15). These variable loci have been employed as markers in different studies on different spatiotemporal scales (e.g. Wongteerasupaya *et al*., 2003; Dieu *et al.,* 2004; Hoa *et al.,* 2005, Marks *et al.*, 2005; Waikhom *et al.,* 2006; Pradeep *et al.,* 2008a, 2008b). Here we employed these five variable regions as markers.

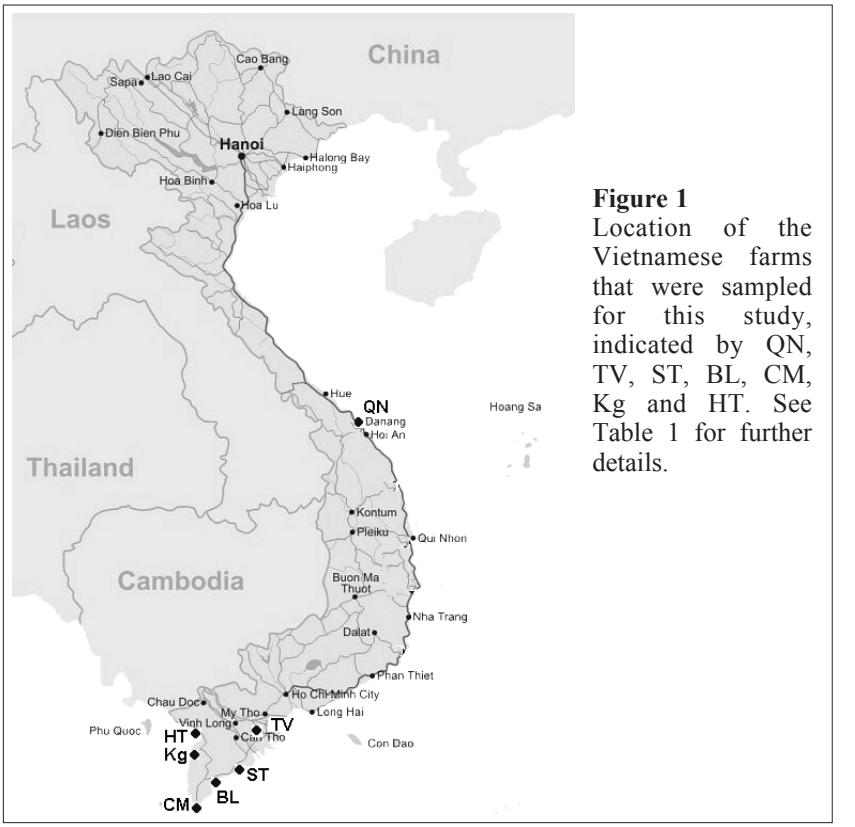

Figure 1
Location of the Vietnamese farms that were sampled for this study, indicated by QN, TV, ST, BL, CM, Kg and HT. See Table 1 for further details.

DNA was extracted from tissue behind the heads of collected shrimp, and screened for the presence of WSSV according to published procedures (Dieu *et al*., 2004). PCR on the genomic variable loci of WSSV was performed with 1µl DNA extract (approximately 250 ng DNA), using Taq DNA polymerase (Promega). Specific PCR primers, conditions used and amplicon lengths are shown in Table 2; PCR for VNTRs is described elsewhere (Dieu

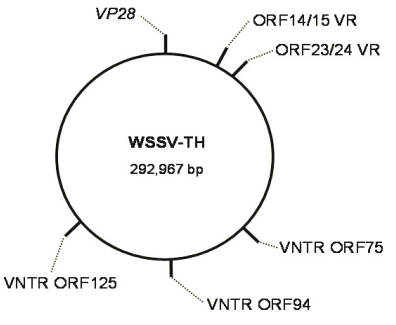

Figure 2: The location of the five variable regions used here to characterize WSSV samples, shown in the WSSV-TH genome (Van Hulten *et al*., 2001; Marks *et al.*, 2004). VR stands for variable region, and VNTR for variable number of tandem repeats. The location of the major WSSV virion envelope protein gene *VP28* is given as a reference point only

et al., 2004). PCR products were analyzed, sequenced and computational analysis was done according to published procedures (Dieu *et al*., 2004). For analysis of the deletion loci (ORF14/15 and ORF23/24), two WSSV infected shrimp from each pond were PCR analyzed.

Statistical analysis

To analyze VNTR data we considered the number of repeat units (ORF94 and ORF125). For ORF75, we considered the total length of the repeat region, since this variable locus contains two types of repeats. To analyze data from genomic deletions (ORF14/15 and ORF23/24) we considered the length of the genomic deletion. We refer to these quantitative data as 'locus trait values'. Because the data set is limited, we grouped improved extensive and shrimp-rice farms together as being 'extensive', and compared them to intensive farms.

As a simple test of whether farming practice had an effect on genotypic composition, we performed a Mann-Whitney *U*-test (SPSS 15.0; SPSS Inc., Chicago, IL, USA), with farming practice as the independent variable and locus trait value (RU number, total length of TRs or deletion size) as the dependent variable. We only included data from the years 2002, 2006 and 2008 from the CM site (Ca Mau, Table 1) to avoid biasing our analysis due to high number of samples from a single site and small time intervals (see Table 3). CM 2006 was chosen as the intermediate sample because for that year the VNTR data are complete.

Table 1. WSSV isolates analysed in this study

Region	Province	Place (district)	Farming practice	Origin of post larvae	Date of collection	Abbreviation
Central VN	Quang Nam	Nui Thanh	Intensive	Central region	2003, 2008	QN
South VN	Tra Vinh	Duyen Hai	Intensive	Unknown	2004, 2006	TV
	Ha Tien	Thuan Yen	Intensive	Unknown	2004, 2005	HT
	Kien Giang	Kien Luong	Intensive	Central region	2003, 2005	Kg
	Soc Trang	My Xuyen	Rice-shrimp	Unknown	2002, 2004, 2008	ST
	Bac Lieu	Vinh Loi	Extensive	Unknown	2004, 2008	BL
	Ca Mau	Tan Thanh	Extensive	Local	2002, 2004, 2005, 2006, 2007, 2008	CM

To test whether farming practice had an effect on changes in genotypic composition over time, we first determined whether there was a change in locus trait value between samples from the same site. For sites at which more than two samples were available, we compared the earliest and the latest available samples only. Thus the genotypic data from two samples from different time points represent one event: locus trait values are either the same or

Table 2. Primers used in PCR analysis for the variable loci of WSSV

Primer pair name/ (Detected deletion)	Primer	Sequence (5'-3')	Annealing temp. (°C)/ elongation time (s)	WSSV–CN sequence coordinates	Size (bp) of PCR product for VN samples
VR23/24 –HTvar	Forward	GAGTAGTCTTCAATGGCAATGT	49 / 100	275008-275029	~1200
(10970 bp)	Reverse	GTAAGTTTATTGCTGAGAAG		286105-286086	
VR23/24 – CM	Forward	CAGATAATGCAAACACGAGACAC	49 / 75	275794-275816	~500
(11045 bp)	Reverse	GTAAGTTTATTGCTGAGAAG		286105-286086	
VR23/24 –screen	Forward	CACACTTGAAAAATACACCAG	49 / 75	278179-278199	~550
(8539 bp)	Reverse	GTAAGTTTATTGCTGAGAAG		286105-286086	
VR23/24 –south	Forward	GTAGTGCATGTTTCTCTAAC	49 / 100	275032-275051	~400
(11866 bp)	Reverse	GTAAGTTTATTGCTGAGAAG		286105-286086	
VR23/24 –TV	Forward	CTACAACGGCCAAGTCAT	49 / 100	30701-30718*	~1600
(11450 bp)	Reverse	ATGATTGTATTCGTCGAAGG		286706-286687	
VR23/24 –Kg	Forward	CTACAACGGCCAAGTCAT	49 / 100	30701-30718*	~2600
(12166 bp)	Reverse	CGCAATTCTCCTCGCAGTT		32255-32237*	
VR14/15-screen (6031bp and 5950 bp)	Forward	GAGATGCGAACCACTAAAAG	49 / 75	22904-22923*	~500/ 600
	Reverse	ATGGAGGCGAGACTTGC		24157-24141*	
VR14/15-HT	Forward	GAGATGCGAACCACTAAAAG	49 / 80	22904-22923*	~900
(5138 bp)	Reverse	GAAAAATAAATCACGGGCTAATC		23646-23624*	

* According to WSSV-TH sequence

they are not. We recorded the total number of locus trait value changes for each locus, and then tested whether intensive farms had more changes in locus trait value than extensive farms using a one-sided test of equal proportions (R 2.7.0; The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Genotyping of WSSV isolates

All shrimp samples tested positive for the presence of WSSV using a single step PCR. VNTRs and variable regions were analyzed by PCR for all the studied isolates (Table 3). In order to map deletions in the ORF14/15 and ORF23/24 variable regions, we first performed PCR with the "VR14/15-screen" and "VR23/24-screen" primers on all samples, respectively (Table 2). These primer sets were previously used to detect deletions in six VN-central WSSV isolates (Dieu *et al*., 2004). Those samples that failed to give a PCR product were then analyzed by means of a 'walking PCR' with different primer sets, starting from two ends of variable regions. Genotypes were detected with the corresponding primer set (Table 2). All PCR products were cloned and sequenced to confirm their identity and

Table 3. Genotyping of five variable loci in the WSSV isolates. An asterisk (*) indicates the PCR reaction failed. A double dagger (‡) indicates that the data were excluded from the Mann-Whitney *U*-test (Table 3) to avoid biases. For ORF75 (a) stands for an RU with 102 bp, and (b) for RU with 45 bp.

Farming Practice	Location	Year	ORF75		ORF94	ORF125	ORF14/15	ORF23/24
			RUs (total)	TR Length				
Extensive	ST	2002	abbabb (6)	384	14	8	6031	11,866
		2004	abbab (5)	339	4	5	6031	11,866
		2008	babb (4)	237	*	4	5950	11,866
	BL	2004	babb (4)	237	*	*	6031	11,866
		2008	babb (4)	237	10	7	5950	11,866
	CM	2002	ababb (5)	339	3	4	6031	11,045
		2004‡	*	*	*	*	6031	11,045
		2005‡	abbabb (6)	384	9	*	6031	11,045
		2006	abbab (5)	339	7	5	6031	11,045
		2007‡	ababb (5)	339	*	7	6031	11,045
		2008	babb (4)	237	6	8	5950	11,045
Intensive	QN	2003	ababb (5)	339	10	6	6031	8,539
		2008	abbabb (6)	384	6	7	6031	11,866
	HT	2004	babbb (5)	282	11	6	5138	11,866
		2005	abbab (5)	339	10	5	5950	10,970
	Kg	2003	abbab (5)	339	15	6	6031	12,166
		2005	abbab (5)	339	12	9	6031	11,866
	TV	2004	abbab (5)	384	10	9	6031	11,450
		2006	abab (4)	294	6	7	5950	10,970

map the exact position of the deletion. The location and size of the genomic deletion was determined using WSSV-TH-96-II (acc. no. AY753327; Marks *et al.,* 2005) as a reference sequence for ORF14/15, and WSSV-TW (acc. no. AF440570; Wang *et al.*, 1995; see also Marks *et al.*, 2004) as a reference for ORF23/24.

Effects of farm practice on genotypic composition

A Mann-Whitney *U*-test (Table 4) demonstrated that there was no significant effect of farming practice (extensive or intensive) on any locus trait values (RU number, total length of TRs or deletion size) for all geographic locations.

Effects of farm practice on changes in genotypic composition

A test of equal proportions (Table 5) demonstrated that for the ORF75, ORF94, ORF125 and ORF 14/15 variable regions, there was no effect of farming practice on change in locus trait value in consecutive samples from a single geographic location. However, for the ORF23/24 variable region, there was a significant effect of farming practice on change in locus trait value (i.e. deletion size). For the intensive farms a change in locus trait value for ORF23/24

was always observed (4 changes in 4 observed events). For the extensive farms no change in locus trait value was observed (no changes in 3 observed events included in statistical

Table 4. Comparison of locus trait values between extensive and intensive farms. Note that analysis was performed on different measures (Analysis). A Mann-Whitney *U*-test was performed to test for significant differences in trait value between extensive and intensive farms. No significant *P*-values were found.

Locus	Analysis	Samples	Mean ± SE		Mann-Whitney U-test	
			Extensive	Intensive	Z	P
ORF75	Total length TRs (bp)	16	293.6 ± 22.0	337.5 ± 12.9	-1.441	0.149
ORF94	RU number	14	7.33 ± 1.67	10.00 ± 1.05	-1.377	0.169
ORF125	RU number	15	5.86 ± 0.67	6.88 ± 0.52	-1.173	0.241
ORF14/15	Deletion size (kb)	16	6.00 ± 0.00	5.89 ± 0.11	-0.185	0.854
ORF23/24	Deletion size (kb)	16	11.56 ± 0.15	11.19 ± 0.41	-0.507	0.613

analysis, no changes in 8 observed events in total).

Although we analyzed changes in trait locus values over time, the interval between sampling at different locations was irregular (extensive farms: 5.33 ± 0.58 [mean ± SE]; intensive farms: 2.50 ± 0.87). The mean time interval was twice as long on the extensive farms meaning that our analysis of change in viral genotypes over time is probably conservative.

DISCUSSION

We investigated the effect of extensive or intensive shrimp farming on WSSV genotypic composition and on changes in WSSV genotypic composition over time. We found no effect of shrimp-farming practice on WSSV genotypic composition for any of the five variable loci investigated (Table 4). This suggests that the environments associated with extensive and intensive farms are not divergent enough to impose differential selection for WSSV genotypes. On the other hand, the number of samples was relatively small, making it difficult to draw definitive conclusions from these data alone. More intensive sampling in the future followed by genetic analysis should substantiate this claim.

For four out of five variable loci (ORF75, ORF 94, ORF 125, ORF14/15) we found no

Table 5: Comparison of change in locus trait values over time between extensive and intensive farms. A test of equal proportions was performed to test for significant differences in changes in trait value between extensive and intensive farms. Significant *P*-values are marked with an asterisk (*).

Locus	Changes / Total Observed (Proportion)		Test of equal proportions	
	Extensive	Intensive	X^2	P
ORF75	2 / 3 (0.67)	2 / 4 (0.50)	0.000	0.500
ORF94	1 / 1 (1.00)	4 / 4 (1.00)	-	-
ORF125	2 / 2 (1.00)	4 / 4 (1.00)	-	-
ORF14/15	3 / 3 (1.00)	2 / 4 (0.50)	0.365	0.727
ORF23/24	0 / 3 (0.00)	4 / 4 (1.00)	5.405	0.030*

effect of farming practice on changes in locus trait value over time (RU number, total length of TRs or deletion size; Table 5). For ORF23/24, however, we did find a significant effect of farming practice on change in locus trait value (deletion size); whereas deletion size never changed over time for an extensive farm, it always changed for intensive farms (Tables 3 and 5). Moreover, the average time between sampling was twice as long on the extensive farms as on intensive farms, meaning that viral populations on extensive farms had twice as much time to undergo genetic change. Overall, these data therefore suggest that our hypothesis that WSSV populations on intensive farms will be more variable is correct. An effect of farm practice on viral genotypic stability is probably due to (i) frequent seeding of PLs infected with different virus strains (Withyachumnarnkul, 1999) and (ii) pond drainage and cleaning regimens.

Why do the data suggest an effect only for ORF23/24, and not for the other loci? First, this is a preliminary study with a limited sample size, the power of the statistical test is low. However, there appears to be an interesting trend for the ORF14/15 data. For samples from four out of five geographic locations in which the deletion size changed over time, there was a shift from the 6031 bp deletion to the 5950 bp deletion. Moreover, the 5950 bp deletion was found only in samples from later years (2005-2008; see Table 3). This suggests that there was selection for a genotype carrying this slightly smaller deletion during that period of time. What could have caused the occurrence of selection at most of the sites? In this period of time, *P. vannamei* was widely introduced in Vietnamese shrimp farms, replacing *P. monodon* (Raux *et al.*, 2003; Corsin, 2005). Others have shown that passaging in different host species can result in differential selection of WSSV genotypes (Waikhom *et al.,* 2006), lending credibility to this explanation. Moreover, the 5950 bp deletion appears to be selected for when WSSV samples obtained from *P. monodon* are passaged in *P. vannamei* (B.T.M. Dieu and J.M. Vlak, unpublished data).

We observed no trends in the VNTR data (Tables 2, 3 and 4). The data of Pradeep *et al.* (2008a, 2008b) suggest that VNTR loci (ORF75, ORF94, ORF125) are more variable than deletion loci (ORF14/15, ORF23/24). We have also found a similar trend for the spread of WSSV in Vietnam (Dieu *et al.*, 2004, ibid. 2010). These observations may explain why we did not find an effect of farming practice on locus trait value for VNTRs: variation is generated too rapidly for VNTRs to be useful markers on larger spatial - and temporal - scales.

Our data suggest that extensive farming leads to fewer changes in deletion size for the ORF23/24 variable region, as compared to intensive farming. This result has important ramifications. First, it suggests that virus populations in extensive farms will be more stable than in intensive farms, a result that we hypothesized based on the way ponds are managed under these farming practices. This will have consequences for WSSV evolutionary dynamics. For intensive farming systems, infection of a pond can be an evolutionary dead end as the pond will eventually be drained and cleaned, leading to the destruction of most virions. On the other hand, if ponds are not carefully cleaned and viruses reach into the surroundings by e.g. marine crabs, fresh water prawn *Macrobrachium rosenbergii* (Hossain

et al., 2001) or polychaetes (Vijayan *et al*., 2005), a disease-free intensively-managed pond is then a resource which can be best exploited by highly virulent genotypes, as there is no cost of virulence (i.e. the pond will be drained irrespective of virus behavior). In extensive farming systems the costs of virulence may be maintained i.e. killing the host at any point in time means that host introduced into the pond at a later time cannot be directly infected. This line of thought may also extend to shrimp-rice farming, because the plots used are never completely drained and cleaned. WSSV is known to cause asymptomatic or avirulent infections (Withyachumnarnkul, 1999; Flegel *et al.*, 2004), which may be important for maintaining the virus in low-density host populations, for example by vertical transmission. Genetic composition of WSSV populations may be one factor which determines virulence, as has been shown by Marks *et al.* (2005) and suggested by Hoa *et al.* (2005). There will, however, be many other factors which will determine WSSV virulence, such as farm management (Corsin *et al.*, 2001), temperature (Rahman *et al.*, 2006), salinity (Liu *et al*., 2006), ammonia-N (Jiang *et al*., 2004) and adaptive shrimp responses (Flegel, 2007).

Finally, effects of shrimp farming practice on changes in locus trait value have implications for studying the spread of WSSV by means of molecular epidemiology (Dieu *et al.*, 2004; Pradeep *et al.*, 2008a, 2008b, Dieu *et al.*, 2010). If WSSV populations in intensive farm systems are more variable, this means that the original genotype which colonized the farm - and the surrounding region - is less likely to be maintained in the virus population than in an extensive farm. Hence, if samples are taken retrospectively to determine virus spread (e.g. Dieu *et al.*, 2004, 2010), then it is best to sample from extensive farms, because the virus population sampled is more likely to be representative of the genotypes that were first introduced - or first became predominant - in that area. The effects of farming practice on the stability of WSSV populations should therefore be given consideration in the design of experiments to study the spread and epidemiology of WSSV.

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REFERENCES

Corsin, F, Turnbull, J.F., Hao, N.V., Mohan, C.V., Phi, T.T., Phuoc, L.H., Tinh, N.T.N. and Morgan, K.L. 2001. Risk factors associated with white spot syndrome virus infection in a Vietnamese rice-shrimp farming system. *Diseases of Aquatic Organisms* 47: 1-12.

Corsin, F. 2005. Supporting Viet Nam to make an informed decision towards *P. vanamei.* Accessed May 25, 2009. Network of Aquaculture Centres in Asia-Pacific news update. URL: http://www.enaca.org/modules/news/article.php?storyid=550

Dieu, B.T.M., Marks, H., Siebenga, J.J., Goldbach, R.W., Zuidema, D., Duong, T.P. and Vlak, J.M. 2004. Molecular epidemiology of white spot syndrome virus within Vietnam.

Journal of General Virology 85: 3607-3618.

Dieu, B.T.M., Marks, H., Zwart, M.P. and Vlak, J.M. 2010. Evaluation of white spot syndrome virus variable DNA loci as molecular markers of virus spread at intermediate spatiotemporal scales. *Journal of General Virology* 91: 1164-1172.

Escobedo-Bonilla, C.M., Alday-Sanz, V., Wille, M., Sorgeloos, P., Pensaert, M.B. and Nauwynck, H.J. 2008. A review on the morphology, molecular characterization, morphogenesis and pathogenesis of white spot syndrome virus. *Journal of Fish Diseases* 31: 1-18.

Flegel, T.W. 2007. Update on viral accommodation, a model for host-viral interaction in shrimp and other arthropods. *Developmental and Comparative Immunology* 31: 217-231.

Flegel, T.W., Nielsen, L., Thamavit, V., Kongtim, S. and Pasharawipas, T. 2004. Presence of multiple viruses in non-diseased, cultivated shrimp at harvest. *Aquaculture* 240: 55-68.

Hoa, T.T., Hodgson, R.A.J., Oanh, D.T., Phoung, N.T., Preston, N.J. and Walker, P.J. 2005. Genotypic variations in tandem repeat DNA segments between ribonucletide reductase subunit genes of White Spot Syndrome Virus (WSSV) isolates from Vietnam, pp. 339-351. In: P.J. Walker, R.G. Lester and M.G. Bondad-Reantaso (eds.), *Diseases in Asian Aquaculture* V, Fish Health Section. Manila, Philippines.

Hossain, M.S., Chakraborty, A., Joseph, B., Otta, S.K., Karunasagar, I., Karunasagar, I. 2001. Detection of new hosts for white spot syndrome virus of shrimp using nested polymerase chain reaction. *Aquaculture* 198: 1–11.

Jiang, G., Yu, R., Zhou, M. 2004. Modulatory effects of ammonia-N on the immune system of *Penaeus japonicus* to virulence of white spot syndrome virus. *Aquaculture* 241: 61–75.

Liu, B., Yu, Z., Song, X., Guan, Y., Jian, X., He, J. 2006. The effect of acute salinity change on white spot syndrome (WSS) outbreaks in *Fenneropenaeus chinensis*. *Aquaculture* 253: 163–170.

Marks, H., Goldbach, R.W., Vlak, J.M. and van Hulten, M.C.W. 2004. Genetic variation among isolates of white spot syndrome virus. *Archives of Virology* 149: 673-697.

Marks, H., van Duijse, J.J.A., Zuidema, D., van Hulten, M.C.W. and Vlak, J.M. 2005. Fitness and virulence of an ancestral white spot syndrome virus isolate from shrimp. *Virus Research* 110: 9-20.

Nhuong, T.V., Luu, L.T., Tu, T.Q., Tam, P.M. and Nguyet, T.T.A. 2002. Vietnam shrimp farming review. Individual Partner Report for the Project: Policy research for sustainable shrimp farming in Asia., CEMARE, University of Portsmouth, UK and RIA1, Bac Ninh, Vietnam, pp. 19.

Pradeep, B., Shekar, M., Gudkovs, N., Karunasagar, I. and Karunasagar, I. 2008a. Genotyping of white spot syndrome virus prevalent in shrimp farms of India. *Diseases of Aquatic Organisms* 78: 189-198.

Pradeep, B., Shekar, M., Karunasagar, I. and Karunasagar, I. 2008b. Characterization of variable genomic regions of Indian white spot syndrome virus. *Virology* 376: 24-30.

Rahman, M.M., Escobedo-Bonilla, C.M., Corteel, M., Dantas-Lima, J.J., Wille, M., Sanz, V.A., Pensaert, M.B., Sorgeloos, P. and Nauwynck, H.J. 2006. Effect of high water temperature (33 degrees C) on the clinical and virological outcome of experimental infections with white spot syndrome virus (WSSV) in specific pathogen-free (SPF)

Litopenaeus vannamei. Aquaculture 261: 842-849.

Raux, P., Bailly, D. and Tran, V.N. 2006. Vietnamese Shrimp farming at a key point in its development: a review of issues examining whether development is being carried out in a sustainable way. In: *Shrimp culture: Economics, market and trade* (P.S. Leung and C. Engle, eds., Blackwell Publishing, UK), p.107-130.

Sokal, R.R. and Rohlf, F.J. 1995. Biometry: the principles and practice of statistics in biological research. W.H. Freeman and Co, New York.

Van Hulten, M.C.W., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., Sandbrink, H., Lankhorst, R.K. and Vlak, J.M. 2001. The white spot syndrome virus DNA genome sequence, *Virology* 286: 7–22.

Vijayan, K.K., Stalin Raj, V., Balasubramanian, C.P., Alavandi, S. V., Thillai Sekhar, V., Santiago, T.C. 2005. Polychaete worms—a vector for white spot syndrome virus (WSSV). *Disease of Aquatic Organisms* 63: 107-111.

Vlak, J.M., Bonami, J.R., Flegel, T.W., Kou, G.H., Lightner, D.V., Lo, C.F., Loh, P.C. and Walker, P.W. 2005. Family Nimaviridae p.187-192. In: *Virus Taxonomy*, VIIIth ICTV Report.

Waikhom, G., John, K.R., George, M.R. and Jeyaseelan, M.J.P. 2006. Differential host passaging alters pathogenicity and induces genomic variation in white spot syndrome virus. *Aquaculture* 261: 54-63.

Wang, C.H., Lo, C.F., Leu, J.H., Chou, C.M., Yeh, P.Y., Chou, H.Y., Tung, M.C., Chang, C.F., Su, M.S. & Kou, G.H. 1995. Purification and genomic analysis of baculovirus associated with White spot syndrome (WSBV) of *Penaeus monodon. Disease of Aquatic Organisms* 23: 239-242.

Withyachumnarnkul, B. 1999. Results from black tiger shrimp *Penaeus monodon* culture ponds stocked with postlarvae PCR-positive or -negative for white-spot syndrome virus (WSSV). *Diseases of Aquatic Organisms* 39: 21-27.

Wongteerasupaya, C., Pungchai, P., Withyachumnarnkul, B., Boonsaeng, V., Panyim, S., Flegel, T.W. & Walker, P.J. 2003. High variation in repetitive DNA fragment length for white spot syndrome virus (WSSV) isolates in Thailand. *Diseases of Aquatic Organisms* 54: 253-257.