Vol. 114: 11–20, 2015
doi: 10.3354/dao02844

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published May 11

Genotyping WSSV isolates from northwestern Mexican shrimp farms affected by white spot disease outbreaks in 2010–2012

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ABSTRACT: White spot disease (WSD) causes high mortality in cultured shrimp throughout the world. Its etiologic agent is the white spot syndrome virus (WSSV). The genomic repeat regions ORF 75, ORF 94, and ORF 125 have been used to classify WSSV isolates in epidemiological studies using PCR with specific primers and sequencing. The present study investigated the variation in nucleotide sequences from 107, 150, and 143 isolates of WSSV collected from *Litopenaeus vannamei* shrimp ponds with WSD outbreaks in northwestern Mexico during the period 2010–2012, in the genomic repeat regions ORFs 75, 94, and 125, respectively. The haplotypic nomenclature for each isolate was based on the number of repeat units and the position of single nucleotide polymorphisms on each ORF. We report finding 17, 43, and 66 haplotypes of ORFs 75, 94, and 125, respectively. The study found high haplotypic diversity in WSSV using the complete sequences of ORFs 94 and 125 as independent variables, but low haplotypic diversity for ORF 75. Different haplotypes of WSSV were found from region-to-region and year-to-year, though some individual haplotypes were found in different places and in more than one growing cycle. While these results suggest a high rate of mutation of the viral genome at these loci, or perhaps the introduction of new viral strains into the area, they are useful as a tool for epidemiological surveys. Two haplotypes from some of the ORFs in the same shrimp were encountered, suggesting the possibility of multiple infections.

KEY WORDS: White spot syndrome virus · ORF 75 · ORF 94 · ORF 125 · Haplotypes · *Litopenaeus vannamei* · Whiteleg shrimp · Northwestern Mexico · Epidemiological studies

INTRODUCTION

White spot disease (WSD) is a highly contagious viral illness caused by the white spot syndrome virus (WSSV) that causes serious economic losses in shrimp aquaculture worldwide (Lightner 1996, Walker & Mohan 2009, Stentiford & Lightner 2011, Kalaimani et al. 2013). The International Committee on Taxonomy of Viruses (ICTV) places WSSV as the only member of the genus *Whispovirus* in the family *Nimaviridae*. Virions of WSSV are ovoid or ellipsoid to bacilliform in shape, have a regular symmetry, and measure 80 to 120 nm in diameter and 250 to 380 nm in length. Most notable is the thread- or flagella-like extension (appendage) at one end (Lo et al. 2012). This virus contains circular, double-stranded DNA (Wang et al. 1995) of approximately 300 kb, with 184 open reading frames (ORFs) (van Hulten et al. 2001).

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Three complete sequences of WSSV have been reported in isolates from Thailand (WSSV-Th), China (WSSV-Cn), and Taiwan (WSSV-Tw) (Marks et al. 2004). They show variations in size of 292 967, 305 107, and 307 287 kb and have 684, 531, and 507 putative ORFs, respectively. The genomes contain a variable number of tandem repeats associated with 3 mini-satellites in ORFs 75, 94, and 125 (van Hulten et al. 2001, Pradeep et al. 2008). The number of repeat units (RUs) in these genomic regions makes them very useful for epidemiological studies (Marks et al. 2004) and for genotyping strains of the virus (Pradeep et al. 2008, Galaviz-Silva et al. 2009).

The RUs of ORF 94 are of 54 bp; ORF 75 shows 2 different types of RUs, one consisting of 45 bp and the other of 102 bp, sorted differently; while those in ORF 125 are of 69 bp (Dieu et al. 2004, Pradeep et al. 2008). In addition, Dieu et al. (2004) reported single-nucleotide polymorphisms (SNPs) in the 3 ORFs. The SNPs in ORF 94 — thymine (T) or guanine (G) — are located at Position 48; in ORF 75 they are found at Positions 3, 15, 30, 40, 42, and 44 of the 45 bp RU type and at Position 83 of the 102 bp RU type; in ORF 125 they are present at Positions 8, 18, 25, 66, and 69.

Epidemiological studies of WSSV based on genotyping conducted in Asia, Africa, and America have improved our understanding of the characteristics and behavior of the disease (Dieu et al. 2004, Shekar et al. 2005, Muller et al. 2010, Tang et al. 2013, Simrouni et al. 2014).

Most of the epidemiological studies performed with WSSV and these 3 markers are based only on the size of the mini-satellite and are expressed as the number of RUs (Dieu et al. 2004, Pradeep et al. 2008, Dieu et al. 2010, Tan & Shi 2011, Shekar et al. 2012), and Mexico is no exception (Galaviz-Silva et al. 2009, Gonzalez-Galaviz et al. 2013). However, each RU may contain SNPs that are useful for differentiating haplotypes that have the same number of RUs. The findings of epidemiological studies may differ if they do not consider the variability provided by the SNPs and only take into account the number of RUs.

In Mexico, >90% of shrimp production occurs in the northwestern region (including the states of Nayarit, Sinaloa, and Sonora), where WSSV outbreaks have occurred for over 10 yr (CONAPESCA 2011, SAGARPA 2011). In an effort to understand the epidemiological dynamics of WSD outbreaks, academic institutions, shrimp producers, and State Aquatic Health Committees organized in the Strategic Alliance and Innovation Network in Aquaculture (AERI for its initials in Spanish) have carried out a series of studies in shrimp-farming areas in northwestern Mexico (Pérez-Enríquez et al. 2011).

The present work focuses on the genotyping of WSSV isolates with a distribution analysis in *Litopenaeus vannamei* shrimp ponds that have suffered WSD outbreaks in northwestern Mexico in the years 2010 to 2012, using ORFs 94, 75, and 125 as molecular markers.

MATERIALS AND METHODS

Shrimp sampling

Samples were taken from 134 ponds with WSD outbreaks, located in 22 local boards for aquaculture health (LBAHs; i.e. groups of farms located in well-defined geographical zones with arbitrary limits) in Nayarit, Sinaloa, and Sonora in 2010 to 2012 (Fig. 1, Table 1). Five shrimps with signs of the disease were collected from each pond, frozen at –20°C, cold transported, and maintained at –86°C at the laboratory of the Autonomous University of Nayarit until analysis. Each shrimp was individually tagged.

Fig. 1. Location of local boards for aquaculture health (LBAHs), where the viral strains were isolated

Table 1. Number of isolates from ponds with white spot disease (WSD) outbreaks from which completely sequenced amplicons of open reading frames (ORFs) 94, 75, and 125 were obtained, by state and year

State	Year	No. of isolates		
		ORF 94	0RF 75	ORF 125
Sonora	2010	27	18	11
Sinaloa	2011	22	24	43
	2012	42	29	51
Nayarit	2010	16	12	0
	2011	24	2	15
	2012	19	22	23
Total	2010–2012	150	107	143

DNA extraction and WSSV DNA detection

The DNA from 670 shrimps was extracted individually with the QIAamp® DNA Mini Kit according to the manufacturer's protocol. After elution, the DNA was stored at −20°C until used. WSSV detection was performed by nested PCR following Kimura et al. (1996), with slight modifications (Table 2). The reaction mixture was prepared at a final concentration of 4 mM $MgCl_2$, 0.4 mM dNTPs, 8 ng μl^{-1} of each oligonucleotide, 1 U of *Taq* polymerase (Invitrogen®), 1× buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), and 1 µl of the DNA solution, adjusted to a final volume of 25 µl with ddH_2O. For the second reaction, 1 µl of a 1:10 dilution of the product obtained in the first reaction was processed under the conditions described above.

Table 2. Oligonucleotides and cycling program used in the PCR reaction for detection of white spot syndrome virus (WSSV) (Kimura et al. 1996) and ORFs 94, 75, and 125 (Wongteerasupaya et al. 2003, Pradeep et al. 2008)

Primer	Sequence (5′–3′)
Kim Out 1	ATC ATG GCT GCT TCA CAG AC
Kim Out 2	GGC TGG AGA GGA CAA GAC AT
Kim In 1	TCT TCA TCA GAT GCT ACT GC
Kim In 2	TAA CGC TAT CCA GTA TCA CG
Cycling program	
95°C/4 min→40 × (95°C/60 s→55°C/30 s→72°C/60 s)→72°C/5 min	
ORF 94 F	TCT ACT CGA GGA GGT GAC GAC
ORF 94 R	AGC AGG TGT GTA CAC ATT TCA TG
Cycling program	
94°C/4 min→50 × (94°C/45 s→55°C/45 s→72°C/45 s)→72°C/10 min	
ORF 75 F	GCC AGA TTT CTT CCC CTA CC
ORF 75 R	CTC CAT GTA GAG GCA AAG CA
Cycling program	
95°C/5 min→50 × (95°C/45 s→52°C/45 s→72°C/45 s)→72°C/7 min	
ORF 125 F	TGG AAA CAG AGT GAG GGT CA
ORF 125 R	CAT GTC GAC TAT ACG TTG AAT CC
Cycling program	
95°C/5 min→40 × (95°C/45 s→60°C/30 s→72°C/30 s)→72C°C/7 min	

Amplification of ORFs 75, 94, and 125

Amplification of the WSSV from the 3 loci was carried out as described by Wongteerasupaya et al. (2003) (for ORF 94) and Pradeep et al. (2008) (for ORFs 75 and 125) (Table 2). Amplifications were prepared in 50 µl volumes with the following final concentrations: 0.2 mM dNTPs, 4 ng μl^{-1} of each oligonucleotide, 2.5 U of *Taq* polymerase enzyme (Invitrogen®), 1× buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl) $MgCl_2$ (1.5 mM for ORF 94; 2.0 mM for ORF 75 and ORF 125), and 1 µl of DNA. Final volume was adjusted with ddH_2O. The cycling programs run in a gradient thermal cycler (Eppendorf®) are listed in Table 2. PCR products were resolved by electrophoresis in 1% agarose gels containing ethidium bromide, and they were visualized by UV transillumination.

Purification and sequencing of amplicons of ORFs 94, 75, and 125

Amplicons from each of the ORFs were amplified again for sequencing. PCR conditions were the same as above, but *Taq* Platinum High-Fidelity (Invitrogen®) was used. Amplicons were purified with the QIAquick Extraction Kit (QIAGEN®) following the manufacturer's instructions.

The purified fragments were sent out for sequencing (Macrogen, Korea). BigDye® terminators were used in an Applied Biosystems® 3730XL automated sequencer. Sequences were analyzed with the BioEdit Sequence Alignment Editor Program Ver. 7.2.5 (www.mbio.ncsu.edu/BioEdit/bioedit.html) to verify that the region was completely sequenced and to quantify the RUs and characterize the SNPs. The RUs were also compared to 3 complete WSSV genomes (GenBank nos. AF332093, AF369029, and AF440570).

Because there were almost no differences among the viral sequences in a sample, the sampling unit was defined as a pond. A haplotype of each ORF was defined as a sequence from an

isolate with a specific number of repeat units (RUs) and SNPs. Haplotypic frequencies were calculated as the ratio of isolates with a specific haplotype to the total number of haplotypes. This analysis was conducted at 3 levels: local (LBAHs), state (Nayarit, Sinaloa, Sonora), and region (northwestern Mexico) (Fig. 1). A temporal analysis was also performed to delineate the dynamics of the presence of viral strains of WSSV in 1 yr and between years (2010 to 2012). Genetic diversity was calculated as haplotype diversity (Hd) = $1 - \Sigma X_i^2$, where X_i is the frequency of the i^{th} haplotype. Hd was obtained by state and for the entire region considering the complete study period (2010 to 2012), though in some cases sampling was not performed in a given year.

RESULTS

Amplicons of approximately 570 bp from the 670 shrimps analyzed were obtained as products of the nested PCR using primers designed by Kimura et al. (1996) that are specific for WSSV (data not shown). A total of 150 were amplified and completely sequenced for ORF 94, 107 for ORF 75, and 143 for ORF 125. These were the numbers of completely sequenced isolates upon which the frequency calculations for each ORF were based. For the remaining shrimps, it was not possible to obtain the complete sequence, or the corresponding region of the ORF was not amplified (Table 1).

Haplotype nomenclature

ORF 94. ORF 94 has a series of tandemly repeated RUs of 54 bp and a polymorphism at Position 48 on each one; therefore, the first criterion for haplotype definition was the number of RUs. However, as several SNPs were present in isolates with the same number of RUs, a composite haplotype for each isolate was built (RU, SNP), where RU is the number of repeat units of the isolate and SNP is the specific SNP pattern (Table 3). Considering only the RUs, 13 different haplotypes were observed during this sampling period in northwestern Mexico, but this number increased to 43 composite haplotypes when the SNP patterns were included (Table 3).

ORF 75. ORF 75 is characterized by 2 types of RUs (Dieu et al. 2004), one of 45 bp and the other of 102 bp. Since we observed isolates with the same number of RUs but different arrangements of these 2 RU types, each one was considered distinct. Thus, the composite haplotype was named according to the number of RUs and the sequence of the total number of RUs. In this case, the 45 bp RU was defined as alpha (α), and the 102 bp RU, as beta (β) (Table 4). In the case of the haplotype with 7 RUs, we observed an

Table 3. WSSV haplotypes for ORF 94 registered in northwestern Mexico during the period 2010–2012. Classification is based on the number of repeat units (RUs) and the single-nucleotide polymorphism (SNP) patterns at Position 48 on each RU

Haplotype	No. of RUs	Pattern of SNPs	Haplotype	No. of RUs	Pattern of SNPs
3,1	3	G,T,T	8,8	8	G,G,T,G,G,G,G,G
4,1	4	G,G,T,T	9,1	9	T,T,T,G,G,T,G,T,T
4,2	4	T,T,T,T	9,2	9	G,G,T,T,G,G,G,G,G
5,1	5	T,T,G,T,T	9,5	9	G,G,G,G,G,G,G,G,G
5,2	5	G,T,T,G,T	10,1	10	T,T,T,T,G,T,T,G,T,T
5,3	5	G,G,G,G,T	10,2	10	T,T,T,T,G,G,T,G,T,T
5,4	5	T,T,G,T,G	10,3	10	G,G,T,T,T,G,G,G,G,G
6,1	6	T,T,T,G,T,T	10,4	10	G,G,G,G,G,G,G,G,G,G
6,2	6	T,T,G,G,T,T	10,5	10	G,G,T,G,G,G,G,G,G,G
6,3	6	T,T,T,G,T,G	11,1	11	T,T,T,T,T,T,T,T,T,G,G
6,4	6	T,T,T,G,G,T	11,2	11	G,G,T,G,G,G,G,G,G,G,G
6,5	6	G,T,T,T,G,T	11,3	11	T,T,T,T,T,T,T,G,G,G,T
6,6	6	T,T,G,G,G,T	12,1	12	T,T,T,T,T,G,T,T,T,G,G,T
7,1	7	T,T,G,T,G,T,T	12,2	12	T,T,T,T,T,T,G,G,T,G,T,T
7,2	7	T,T,G,G,T,T,T	12,3	12	T,T,T,T,T,T,T,T,T,T,G,G
7,3	7	G,G,G,G,G,G,T	12,4	12	G,G,G,G,T,G,T,T,G,G,G,T
7,4	7	T,G,G,G,G,G,T	13,1	13	T,T,T,T,T,T,G,G,T,T,G,G,T
8,1	8	T,T,T,T,G,G,G,T	13,2	13	T,T,T,T,T,G,G,T,T,G,G,G,G
8,2	8	G,G,G,T,G,G,G,G	13,3	13	T,T,T,T,T,T,T,T,T,G,G,G,T
8,3	8	G,G,G,G,G,G,G,G,G	15,1	15	G,T,T,T,G,G,G,G,G,G,G,G,G,G,G
8,4	8	G,G,G,G,G,G,G,G,T	17,1	17	T,G,G,G,G,G,G,G,G,G,G,G,G,G,G,G
8,7	8	T,T,T,T,T,G,T,T			

SNP at Position 138, so the 2 were considered different isolates. As a result, the number of distinct composite haplotypes at ORF 75 was 17 (Table 4).

ORF 125. ORF 125 has RUs of 69 bp and SNPs at Positions 8 (T/C), 18 (G/T), 25 (G/A), 66 (T/G), and 69 (T/C). The base sequences of the first, second and final RUs are very different to the sequences of the remaining RUs (third to penultimate). The first, second and final RUs were not taken into account when the amplicons of ORF 125 were characterized based on the SNPs, but they were considered when defining the number of RUs (Pradeep et al. 2008). Considering from the third to the penultimate RU, 10 SNP patterns were observed, each of which was assigned a Roman numeral (Table 5). Next, the composite haplotypes for defining ORF 125 were built with 2 numbers separated by a hyphen, where the first digit indicates the number of RUs and the second the SNP pattern. The total number of composite haplotypes of ORF 125 was 66, though that would decrease to 12 if only the number of RUs were used (Table 6).

Frequencies and geographical distribution of WSSV isolates

ORF 94. Table S1 in the Supplement (www.int-res.com/articles/suppl/d114p011_supp.pdf) shows that northwestern Mexico has small haplotypes of 3 RUs in contrast to other haplotypes that can contain up to 17 RUs. Haplotype 3,1 was the most frequent, as it appeared throughout the region in 55 of 150 isolates (36.7%), and in the majority (63.6%) of the LBAHs (Table S1). In this study, 24 of the 43 haplotypes described for ORF 94 were found only once, in each one of 24 isolates, representing 55.8% of the total (Table S1). Table S1 also shows that the variety of haplotypes based on ORF 94 is present both seasonally and spatially.

The most frequent haplotypes in the 3 LBAHs of the state of Nayarit were 3,1, 10,2 and 8,4 with 22, 13.6, and 10.2%, respectively. The most abundant haplotypes in Sinaloa were 3,1 and 6,3 at 43.8 and 12.5%, respectively; while in Sonora, the 3,1 haplotype accounted for 51.9% and the 7,4 haplotype 44.5% (Table S1). No variation in the pattern of the SNPs in the 3-RU haplotype was observed, though it is widely distributed in the 3 states.

ORF 75. A total of 107 complete sequences were obtained for this ORF 75: 36 in Nayarit, 53 in Sinaloa, and 18 in Sonora (Table 1). The haplotypes summarized in Table S2 in the Supplement were found to be prevalent in each LBAH and state in the year in which the isolates from farms with outbreaks of WSD were obtained. The most common haplotype was 7A, at 38.8, 39.6, and 61% in Nayarit, Sinaloa, and Sonora, respectively, and 45% for the area as a whole. The 7A haplotype was found in 63% of the LBAHs studied, while 6.5% of the haplotypes were found in only 1 isolate (Table S2).

ORF 125. Table S3 in the Supplement shows the large number of haplotypes—66 in total—that appeared when the sequence of ORF 125 was used as the molecular marker. Of these, 19, 48, and 3 were present, respectively, in Nayarit, Sinaloa, and Sonora. Likewise, there were variations between different years in the same LBAH and between different LBAHs in the same year.

Table 4. WSSV haplotypes for ORF 75 registered in northwestern Mexico during the period 2010–2012. Classification is based on the number of RUs, the pattern of their arrangement, and an SNP at Position 138 (only for the haplotype with 7 RUs). α: RU with 45 bp; β: RU with 102 bp

Haplotype	No. of RUs	RU sequence pattern
5A	5	β,4α
6A	6	β,2α,β,2α
7A	7	α,β,2α,β,2α/T_{138}[a]
7B	7	α,β,2α,β,2α/A_{138}[a]
8A	8	β,4α,β,2α
8B	8	α,β,3α,β,2α
9A	9	α,β,4α,β,2α
10A	10	α,β,2α,β,2α,β,2α
11A	11	α,β,3α,β,2α,β,2α
11B	11	α,β,4α,β,α,β,2α
12A	12	α,β,4α,β,2α,β,2α
12B	12	α,β,3α,2β,2α,β,2α
14A	14	α,β,4α,β,α,β,2α,β,2α
15A	15	α,β,4α,β,2α,β,5α
15B	15	α,β,4α,β,2α,β,2α,β,2α
16A	16	α,β,5α,β,2α,β,2α,β,2α
20A	20	α,β,9α,β,2α,β,2α,β,2α

[a]SNP

Table 5. SNP patterns at Positions 8, 18, 25, 66, and 69 observed in the RUs of ORF 125

Pattern	SNP sequence
I	C,G,A,G,T
II	C,G,G,G,T
III	T,G,A,T,C
IV	T,G,A,G,T
V	T,G,G,G,T
VI	T,G,G,T,C
VII	T,G,T,T,C
VIII	T,T,G,G,T
IX	T,T,G,T,C
X	T,G,G,G,C

Table 6. WSSV haplotypes for ORF 125 registered in northwestern Mexico during the period 2010–2012. Classification is based on the number of RUs and the SNP sequence patterns. The first, second, and last RUs are not considered for this nomenclature

Haplotype	No. of RUs	SNP sequence pattern	Haplotype	No. of RUs	SNP sequence pattern
6-1	6	IX(2),VIII	10-8	10	VI(4),VIII,IV(2)
6-2	6	VI,IX,VIII	10-9	10	VI(6),IX
6-3	6	VI,VIII,IV	10-10	10	VI(6),VIII
7-1	7	I(4)	10-11	10	VI(6),IV
7-2	7	VI(3),V	11-1	11	V,VI(4),V,IV(2)
7-3	7	VI(2),V,II	11-2	11	V,VI(5),VIII,IV
7-5	7	VI,VIII,II,I	11-3	11	V,VI(5),VIII,VI
7-6	7	VIII,II,I(2)	11-4	11	V,VI(5),V,IV
7-7	7	IX(2),VIII,I	11-5	11	VI(6),V,IV
7-8	7	IX(2),VIII,IV	11-6	11	X,VI(5),V,IV
8-1	8	VI,VIII,II,I(2)	11-7	11	V,VI(4),IX,VIII,IV
8-2	8	VI(3),V,IV	11-8	11	VI(5),IX,VIII,IV
8-3	8	IX(2),VIII(2),I	11-9	11	VI(6),VIII,IV
8-5	8	V,II(2),I(2)	11-10	11	VI(7),IV
8-6	8	II,I(4)	11-11	11	VI(7),V
9-1	9	V,I(5)	11-12	11	VI(6),IX,IV
9-2	9	III,VI(2),IX,VIII,IV	11-13	11	VI(6),IX,VIII
9-3	9	VI(2),IX(2),VIII,IV	11-14	11	VI(8)
9-4	9	VI(6)	12-1	12	V(2),VI(6),VIII
9-5	9	VI(4),IX,VIII	12-2	12	VI(8),III
9-6	9	VI(4),VIII,IV	12-3	12	VI(9)
9-7	9	VI(5),IV	12-4	12	VI(7),VIII,IV
9-8	9	IX(4),VIII,IV	12-5	12	VI(7),IX,IV
9-9	9	V,II(2),I(3)	12-6	12	VI(7),IX,VIII
9-10	9	IX(4),VIII,I	12-7	12	VI(6),IX,VIII,IV
9-11	9	VI,VIII,II,I(3)	12-8	12	VI(6),IX,IV(2)
10-1	10	V,I(6)	12-9	12	VI(6),VIII,IV(2)
10-2	10	VI,V,I(5)	13-1	13	VI(7),III,VI,VIII
10-3	10	VI(5),VIII,IV	13-2	13	VI(8),IX,IV
10-4	10	V,VI,IX(2),VIII,IV(2)	14-1	14	VI(10),IV
10-5	10	IX(5),VIII,I	15-1	15	V,VI(7),VIII,VI,VIII,IV
10-6	10	VI(5),IX,IV	16-1	16	VI(12),IV
10-7	10	VI(5),V,IV	17-1	17	V,VI(7),VIII,VI,VIII,IV,VIII,IV

The most frequent haplotypes in Nayarit were 6-1 (21%), 11-2 (16%), and 9-1 (13%). The rest were present only in 1 or 2 outbreaks. In Sinaloa, a wide variety of haplotypes (48) was present. The most frequent one was 7-7 at 11%, followed by 11-9 at 7%. In Sonora, only 3 haplotypes were found: 8-6 at 64%, 11-9 at 27%, and 6-3 at 9% (Table S2).

Genetic diversity

Considering the entire area and time period studied, genetic diversity (Hd) was 0.902, 0.228, and 0.969 for ORFs 94, 75, and 125, respectively (Table 7). Genetic diversity among states was high. The lowest number of haplotypes (N_h) and lowest Hd was observed in Sonora for both ORF 94 and 125. In general, genetic diversity in Nayarit was similar to that in

Table 7. Genetic diversity of WSSV haplotypes by state and throughout northwestern Mexico during the period 2010–2012 using ORF 94, 75, or 125 as molecular markers. N_i: number of isolates; N_h: number of haplotypes; Hd: haplotype diversity

State/Region	ORF	N_i	N_h	Hd
Sonora	94	27	3	0.532
	75	18	3	0.463
	125	11	3	0.512
Sinaloa	94	64	20	0.779
	75	53	10	0.239
	125	94	48	0.961
Nayarit	94	59	23	0.896
	75	36	8	0.227
	125	38	19	0.896
Total in northwestern Mexico	94	150	43	0.902
	75	107	17	0.228
	125	143	66	0.969

Table 8. Presence of 2 haplotypes within single shrimps based on each of the 3 molecular markers used. State of origin and year are shown. LBAH: local board of aquaculture health

ORF	State	Year	LBAH	Haplotypes
94	Nayarit	2011	Tecuala	5,1 and 7,1
			San Blas	10,2 and 12,2
75	Sinaloa	2011	Guasave Norte	7A and 10A
125	Sinaloa	2012	Guasave Sur	7-2 and 10-10
			Angostura	11-9 and 13-3
			Elota	7-7 and 9-10
			Elota	7-7 and 10-5

Sinaloa; but Sinaloa showed a higher N_h and Hd in ORF 125, and the number of isolates analyzed was higher. However, since Nayarit is much smaller than Sinaloa (see Fig. 1), it could be considered as the region with the highest genetic variability.

Cases of double infection were also detected, i.e. single shrimps with 2 haplotypes of the same ORF. For ORF 94 in 2011, 2 shrimps from LBAHs in Nayarit showed this condition. Also in Sinaloa, in 2011 and 2012, 1 and 4 shrimps, respectively, had double infections with the molecular markers ORF 75 and 125 of WSSV (Table 8).

DISCUSSION

Only isolates from outbreaks of WSD for which the completed sequence of the amplicon derived from at least 1 of the ORFs was determined were included in this study (Table 1). Also, the criterion of analysis was based on the results for individual ORFs, as it was not possible to obtain the complete sequence of all 3 ORFs for all isolates.

ORF 94

Upon using the complete sequence of each ORF, the variety of haplotypes increased and, as in most such studies, where only the number of RUs was considered, there is no expected sequence. However, we can use the first genome of WSSV completely sequenced from an isolate collected in China as a reference (Yang et al. 2001), namely, the ORF 94 haplotype T,T,G,G,G,GG,G,T,T,T,T, which was not found in any of the isolates in our study area.

Interestingly, the 3,1 haplotype was found in all 3 states but in different years, appearing in Sinaloa in 2011, in Sonora in 2010, and in Nayarit in only 1 LBAH (Tecuala) in 2012 (Table S1). This could indicate that this haplotype was generated in — or entered — the northwestern region of Mexico from the north and spread southward (Table S1). The presence of the 3-RU haplotypes has been reported in India and Sonora, Mexico, previously, but this is the first report of the distribution of SNPs at Position 48 of RUs in America; therefore, it is possible that they have the same haplotype described here, though this was not reported (Pradeep et al. 2008, 2009, Gonzalez-Galaviz et al. 2013). Simrouni et al. (2014) reported 2 variants of ORF 94 with 3 RUs isolated in Iran in 2012 that have different patterns from those reported in this work, as they found SNP patterns G,G,G and T,T,T.

The dynamics of the prevalent haplotypes in the LBAHs in Nayarit continued for 3 consecutive years, but no patterns in the appearance of the different haplotypes were observed, except for 12,1 and 13,1, which were present in 2010 and 2011 in the LBAH at Tuxpan. The same phenomenon was observed in Sinaloa. As in Nayarit, 2 haplotypes (3,1 and 8,2) were found in the same LBAHs (Navolato sur, Guasave norte) in 2011 and 2012, respectively. No data were available from Sonora for comparative purposes (Table S1).

When the haplotypes from different LBAHs in Nayarit were compared, distinct ones were observed in each zone, except for 3,1. In contrast, in Sinaloa and Sonora, several haplotypes were found in different LBAHs.

It is important to note that 6 haplotypes were derived from the 6- and 8-RU variants, the ones that showed the highest degree of variability (Table S1). The genotypic diversity of WSSV using ORF 94 as a molecular marker increased as the study moved from north to south; that is, diversity was lower in Sonora than in Sinaloa and Nayarit. Diversity in Nayarit may have been higher because we included isolates from 3 consecutive years, while for Sonora we only included isolates from 2010. Also, the higher genetic diversity in the southern areas might explain why WSD has persisted year after year in Nayarit and Sinaloa since 1999, while there have been some years in Sonora in which it has not been reported, such as 2007 and 2008 (Aquaculture Health Committee for Sonora State unpubl. data). Nevertheless, if we consider the diversity for the entire study area and over the 3 years analyzed, the conclusion is that this molecular marker has high variability (0.902), which again means either a high rate of mutation of the viral genome in this region, or the constant introduction or mobility of new WSSV varieties (Table 7).

As in other works that have reported double infections with isolates of WSSV and different sizes of the ORF 94 region (Wongteerasupaya et al. 2003, Pradeep et al. 2008), we found 2 cases, indicating a frequency of only 1.9% during the study period (Table 8). Some research groups have sought to associate this finding with virulence, but results are contradictory (Hoa et al. 2011, Walker et al. 2011), so its biological role is still unknown. A haplotype lacking the ORF 94 region has been described for Saudi Arabia (Tang et al. 2012).

ORF 75

Despite the complex nomenclature for the RU haplotypes of ORF 75, this proved to be the molecular marker with the lowest number of haplotypes (17), suggesting greater stability of the virus genome in this region. Greater inter-year variability was observed in Nayarit than in Sinaloa. The most frequently observed variant (7A) was present in Sonora in 2010, in Sinaloa in 2011 and 2012, and in Nayarit until 2012.

As with ORF 94, data for the 7A haplotype of ORF 75 suggest that some viral variants that appeared in Nayarit in 2012 may have come from the northern reaches of northwestern Mexico and been spread naturally or commercially from north-to-south. However, some other haplotypes appear to be locally generated, or were introduced by some means exclusively into certain micro-regions — in this case into distinct LBAHs (Table S2). Among the LBAHs in the same state, an increased presence of the same haplotypes was observed, especially in neighboring LBAHs, except for Nayarit in 2010 and 2011. These data strongly suggest that ORF 75 tends to be more stable both temporally and geographically.

Also, a 7-RU haplotype previously found in Vietnam by Dieu et al. (2010) had 6 RUs of 45 bp and 1 of 102 bp. In contrast, this study reports a haplotype with 7 RUs, 5 of which are of 45 bp and 2 of which are of 102 bp. Pradeep et al. (2008) have also reported a 7-RU haplotype in India, but the size of each RU was not shown, suggesting that they were generated through multiple passes in local hosts after arriving in Mexico.

The Chinese haplotype reference to ORF 75 (α,β, 4α,β, 2α,β, 2α,β, 2α — here called 15B) was found in this study in 4 LBAHs in Sinaloa in 2011 and 2012 (Table S2). The 15B haplotype was found in several LBAHs in Sinaloa and Sonora. The 14A haplotype found in Cospita, Sinaloa, in 2012, 12A found in Ahome, Sinaloa, in 2012, and 6A found in Guasave, northern Sinaloa, in 2012, all showed the same patterns of RUs as the isolates from China, Vietnam, and Thailand, respectively, suggesting that Asia is the origin of these WSSV variants.

These findings are confirmed by the data in Table 7, which shows the genotypic diversity calculated for WSSV based on the use of ORF 75 as the molecular marker, for both the region as a whole (0.228) and state-by-state. In addition, the first report of a double infection of WSSV is presented in Table 8, using the complete sequence of ORF 75.

ORF 125

Considering the nucleotide sequence in the RUs and not only the RU numbers, high variability for this marker was observed (0.969) throughout northwestern Mexico (Table 7), with only a few exceptions (Guasave Norte haplotypes 9-6 and 10-11). Moreover, the haplotypes found in a specific LBAH one year were not identified in the following year, while most of the haplotypes found in neighboring LBAHs were not shared in the same year (Table S3). Furthermore, the haplotype VI,VIII,VI,VIII(2) (according to our nomenclature) was not found in northwestern Mexico. This could be explained by the high variability in the sequence of this molecular marker.

ORF 125 is the molecular marker for which there are fewer studies based on the nucleotide sequence to define variants of WSSV, yet it is striking that neither Dieu et al. (2004) nor the GenBank reported haplotypes identical to those found in our study. This reinforces the fact that great variability occurs in the genome in this region. It should also be noted that this is the first report which includes cases of double WSSV infections based on the complete nucleotide sequence of ORF 125 (Table 8). This means that for the case of WSSV, the possible occurrence of viral superinfection cannot be excluded, especially since this phenomenon has been described for other viruses (Folimonova 2012, Campbell et al. 2014).

This study reports high genetic diversity in WSSV using the complete sequences of ORFs 94 and 125 as the independent molecular markers in the most important *Litopenaeus vannamei* shrimp-producing regions of Mexico, with low genetic diversity for ORF 75. Different haplotypes of WSSV from region-to-region and year-to-year were seen, although some individual haplotypes were found in different places and in more than one growing cycle. These findings indicate either a high rate of mutation of the viral

genome in these regions or the introduction of new viral strains into the area. Two haplotypes of an ORF can co-exist in the same shrimp, an additional reflection of both high variability and the possibility of multiple infections.

Acknowledgements. This research was funded by CONACyT (Fondo Institucional de Fomento Regional para el Desarrollo Científico, Tecnológico y de Innovación—FORDECYT, Grant No. 143117), by the Aquatic Health Committees of the states of Baja California Sur, Sonora, Sinaloa, and Nayarit, and by the National Association of Shrimp Larvae Producers (ANPLAC), with partial support from the Autonomous University of Nayarit (P/PIFI-2011-18MS40019M-05). For technical support in the laboratory and sampling we thank Alejandro Vázquez, Fabiola Ayón, Jaime Sánchez, Zulia Nieves, Alfonso Sánchez, Alma Castañeda, Emmanuel Frías, Adrián de la Rosa, Luis Sauma, Pablo Parra, Abraham Betancourt, and Armando Delgado.

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Editorial responsibility: Grant Stentiford, Weymouth, UK

Submitted: August 25, 2014; Accepted: December 30, 2014
Proofs received from author(s): March 26, 2015