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Sequence analysis of ORF94 in different White Spot Syndrome Virus (WSSV) isolates of Iran

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Abstract

White spot syndrome virus (WSSV) is a pathogen that causes high mortality in shrimp culture in the whole world. Sequence analysis of WSSV has shown similarity of *WSSV* isolates in different countries with exception of a few variable genomic loci. This study investigated the sequence variation of some Iranian WSSV isolates and previously identified isolates. Samples were collected during target surveillance and were feed, broodstock, post-larvae, artemia, crabs, and wild and cultured shrimp of northern Persian Gulf (Boushehr and Khuzestan provinces). The open reading frame (ORF) 94 sequence of different Iranian WSSV isolates were amplified using specific primers from positive samples. The ORFs 94 sequence of positive samples were sequenced and registered in the Gene Bank and then compared to other WSSV isolates. The number of repeat units in ORF94 showed that WSSV isolates were varied in number. There are SNPs (G and T) in position 48 of RUs that varies in different Boushehr and Khuzestan isolates. Also these sequences were compared to Gene Bank WSSV isolates and showed a high similarity (>90%) to Southeast Asian countries. To our knowledge this is the first report of sequence analysis in Iranian WSSV isolatesapplications.

Keywords: WSSV, Sequence analysis, ORF94

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Introduction

White Spot Syndrome Virus (WSSV) is one of the most serious pathogens of cultured shrimp, often causing high mortality and significant economic losses to shrimp farming around the world (Lo et al., 1996; Hossain et al., 2004; Leu et al., 2007; Sánchez-Paz, 2010). It has been reported that economic losses approach US$10 billion in production and trade (Stentiford et al., 2009; Lightner, 2011; Tang et al., 2012). The first outbreak of WSSV was in Taiwan in 1992 (Hsin-yiu, 1994) and has spread rapidly to all Asian Countries , the Middle East , North , Central and South America (Escobedo-Bonilla et al., 2007; Lightner, 2011; Sánchez-Paz, 2010). It has been reported in Mozambique recently that the virus has spread to African countries too (Hoa et al., 2011b). The first reported incident in Iran was in 2002 and since then, there have been several outbreaks in southern Iran that have caused economical losses to the Iranian shrimp farming industry (Tokhmafshan et al., 2004; Gholamhoseini et al., 2013). WSSV has been classified as a sole member of Whispovirus genus and Nimaviridae family (Marks et al., 2005). There are complete DNA genome sequences of three WSSV isolates in the Gene Bank, including WSSV-TH (AF6902902), WSSV-CN (AF332093) and WSSV-TW (AF440570) isolated respectively in Thailand, China and Taiwan (van Hulten et al., 2001; Yang et al., 2001; Hoa et al., 2011; Shekar et al., 2012;).Recently the fourth WSSV complete genome from Korea (WSSV-KR) was sequenced(Chai et al., 2013). The genome of WSSV is 292 to 307 kbp long and contains 181-184 major ORFs encoding functional proteins (Tsai et al., 2000; van Hulten et al., 2001; Shekar et al., 2012).

Except for a few variable loci, the three WSSV complete genomes share high degree (>99%) of genetic similarity (Marks et al., 2004). Difference of the length of genome between the viral isolates has been due to deletions in ORFs 23/24 and 14/15 and the presence of variable tandem repeats (VNTRs) in ORFs 75, 94 and 125. These variable genetic loci have been considered as valuable markers for molecular genotyping of *WSSV* isolates. single nucleotide mutations, including deletion, insertion or single nucleotide polymorphisms (SNPs) which have been suggested as genetic markers for the successful study of *WSSV* diversity (John et al., 2010; Hoa et al., 2011b; Shekar et al., 2012; González-Galaviz et al., 2013).

Some studies were taken on genotyping of ORF 94 of WSSV isolates. The first report for differentiating WSSV genotypes based on the VNTRs associated with ORF94 was from shrimp samples collected from WSSV outbreak ponds in Thailand between 2000 and 2002. In this study a wide range of variation from 6 to 20 RUs in the tandem repeat units that classified WSSV to 12 different genotypes was reported. The most frequently encountered 54 bp RU recorded in this study was 8 with an alternating thymine or guanine (Tor G) SNP at position 36 within the repeat unit (Wongteerasupaya et al., 2003). In a similar study in Vietnam, WSSV isolates contain 7–17 RUs and a SNP to occur at the 48th position rather than the 36th position in the RUs(Dieu et al., 2004). The other studies on Genotyping of

Brazilian WSSV isolates showed that they were differed for the number of 54 bp RU in compared to isolates from other countries in the Americas and also the Brazilian WSSV isolates were differed for the nucleotide pattern at position 48 of the RU(Muller et al., 2010). In India, Several studies based on the analysis of ORF94 VNTR were carried out. The SNP recorded in their study was T or G at position 48 of The RU. The results showed the prevalence of variable WSSV genotypes in a geographical region. They reported 8,13 and 31 different genotypes of WSSV in India (Syed Musthaq et al., 2006; Pradeep et al., 2008; Walker et al., 2011b; Shekar et al., 2012).

The aim of this study was to analyze the ORF94 among some Iranian WSSV isolates from the point of view of nucleotide sequence and the number of repeat units (RUs) and SNPs. This information provide a basis for molecular genotyping, molecular epidemiology and source recognizing of the viral isolates in Iran.

Materials and methods

Samples for virus detection were collected during target surveillance in 2012 and involved feed, broodstock, postlarvae, artemia, crabs, and wild and cultured shrimps of Boushehr and Khuzestan provinces, north-west Persian Gulf coasts. All samples were preserved in 70% Ethanol (Manivannan et al., 2002). Specimens were procured from ten sites of shrimp farming and eight active hatcheries in Boushehr and Khozeshtan, wild shrimp and crabs in incoming water channels of grow out ponds and also wild shrimp (*M. affinis, P. semisulcatus, P. stylifera*) and crabs (*Portunus segnis* and *Chiromantes boulengeri*) in the grow out ponds. The samples of hatcheries involved the pleopod of broodstock (*L. vannamei*), post larvae (>pL5), live(Artemia and Rotifer), fresh and dry feed. In totally 4,700 samples were collected and screened for this study.

DNA extraction from the samples was done using a commercial DTAB-CTAB DNA extraction kit (Farming IntelliGene Technology Corporation, Taipei, Taiwan). The extracted DNA was evaluated by biophotometer and its quality and quantity were determined. Also the integrity of DNA was evaluated by agarose gel electrophoresis.

Extracted DNAs were screened for WSSV by a nested PCR using the IQ2000™ WSSV detection and prevention system (Farming IntelliGene Tech. Corp., Taiwan). All positive samples were also confirmed by a nested PCR based on OIE recommendation. In this PCR, Primer pairs 146F1/R1 and 146F2/R2 (OIE, 2013) were used in the first and second reactions, respectively and amplifications were performed as follows: initial denaturation at 94°C for two minutes followed by 30 cycles of 94°C for 30 seconds, 62°C for 30 seconds and 72°C for 30 seconds .The final extension was at 72°C for two minutes. Following this, an aliquot of the PCR mixture was analyzed in a 1.5% agarose gel containing ethidium bromide and was then photographed(OIE, 2013).

The specific primers including ORF 94-F (5□-TCT ACT CGA GGA GGT GAC GAC-

3□) and 94-R (5□-AGC AGG TGT GTA CAC ATT TCA TG-3□) were used in ORF94 PCR reaction. Amplification was performed using a thermal program consisting on five minute at 94°C, followed by 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 72°C for one minute and a final extension at 72°C for seven minutes. The PCR products were electrophoresed on 1.5% agarose gel(Muller et al., 2010; Hoa et al., 2011a; Tang et al., 2012). DNA bands were purified from the gel by the Qiagen gel extraction kit and sequenced (Macrogen, Korea). The sequences were registered in the Gene Bank.

Sequences of *ORF94* of Iranian isolates of WSSV were aligned to determine the similarity of some Iranian WSSV isolates with existing data in the Gene Bank.

Results

The *Litopenaeus vannamei* samples were collected from ten major culture sites and eight hatcheries during shrimp farming in 2011 and 2012 in Boushehr and Khuzestan provinces in southern Iran. By monitoring the presence of *WSSV* using a nested PCR we detected six isolates. Two isolates were from Boushehr (IRWSSVBU1 and IRWSSVBU2) and four isolates were from Khuzestan (IRWSSVKH3 to IRWSSVKH6). All the samples from wild crustaceans were negative. In order to characterize the WSSV isolates, two previous positive samples (IRWSSVKH1 and IRWSSVKH2) from the archives of 2010 and 2011 were also included. PCR products of ORF94 with specific amplicons of 300 to 600 bp (Fig 1) were sequenced and the resulting sequences were registered in the Gene Bank with accession No. KC906274, KC906275, KC906266, KC906276, KC906277, KF157841, KF157840, KF157835. Also these sequences were analyzed for the number of RUs by Mega 5 software (Table1). Consensus of RUs in ORF94 (54bp) was CGC AAA AAG CGT GCC GCA CCT CCA CCT GAG GAT GAA GAA GAG GAT GAT TTC TAC (Tang et al., 2012). The number of RUs are summarized in Table1. The results showed Three RUs in Khuzestan isolates from 2011 to 2012 and six RUs in 2010 isolate. Similar pattern of RUs was also observed in Boushehr isolates; IRWSSVBU1 and IRWSSVBU2 possessed three and six RUs respectively. The number of RUs of Iranian and exotic isolates has compared in Table 2.

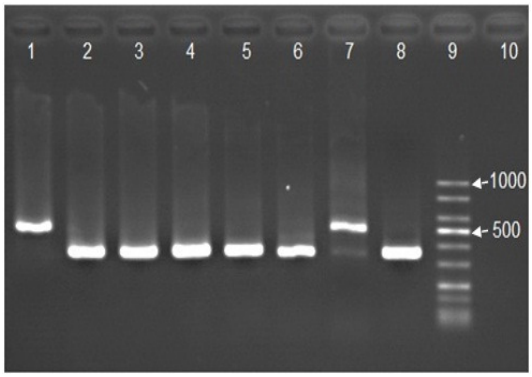

Figure 1: The PCR amplification of ORF94 of different WSSV isolates.

1-IRWSSVKH1 2-IRWSSVKH2

3-IWSSVKH3 4-IRWSSVKH4

5-IRWSSVKH5 6-IRWSSVBU1

7-IRWSSVBU2 8-IRWSSVKH6

9-M 1kb ladder (Jena Bioscience, Germany)

10-Negative Control

Also the Mega5 software was used for sequence alignment and finding SNPs inside ORF94. The results showed that the isolates of Boushehr and Khuzestan provinces have different SNPs. Bushehr has G in position 48 in all of RUs whereas Khuzestan has T in same position in all of RUs. This position include different nucleotides (G or T) in RUs in exotic isolates. The differences among the SNPs in position 48 in different isolates have been shown for two RUs in figure 2. Also these SNPs have been shown for all of RUs in Iranian isolates in Table 1.

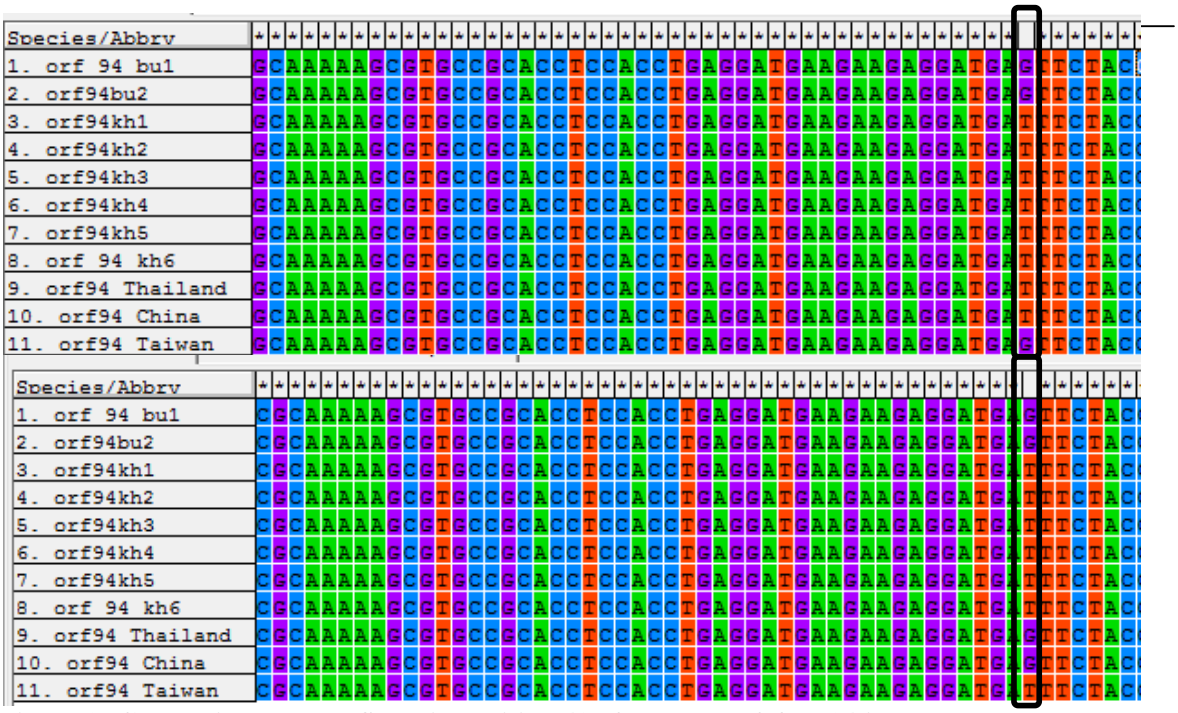

Figure 2: Comparison among SNPs in position 48 of two RUs of ORF94 in Bushehr, Khuzestan and some exotic isolates.

Table 1: Number of Repeat Units (RUs) and variation of SNPs of ORF 94 in Iranian Isolates. The SNPs are located in position 48 of RUs.

Isolate	------ No of RUs------	SNP	Region	Sampling date
IRWSSVBU1	3	GGG	Boushehr	2012
IRWSSVBU2	6	GGGGGG	Boushehr	2012
IRWSSVKH1	6	TTTTTT	Khuzestan	2010
IRWSSVKH2	3	TTT	Khuzestan	2011
IRWSSVKH3	3	TTT	Khuzestan	2012
IRWSSVKH4	3	TTT	Khuzestan	2012
IRWSSVKH5	3	TTT	Khuzestan	2012
IRWSSVKH6	3	TTT	Khuzestan	2012

Table 2: Variations in the number of repeat units (RUs) in ORF94 among WSSV isolates from different geographical regions

Geographical Origin	WSSV-TW	WSSV-TH	WSSV-CN	Brazil	Central America	China	Thailand	Vietnam	USA	Iran	Saudi
Range of RUs in ORF94	6	6	12	4-16	12-19	6-14	6-20	4-17	5-8	3-6	7-13

Discussion

The shrimp industry in Iran is significantly threatened by WSSV infection. It is proposed that the source of WSSV related to first outbreak in 1992 and the trading market in the industry caused the worldwide distribution (Shekar et al., 2012). Several studies were taken on genotyping of ORF94 of WSSV isolates and showed a wide range of variation from 3 to 20 RUs with an alternating Thymine or Guanine (T or G) SNP at position 36 and 48 within the repeat units(Wongteerasupaya et al., 2003; Dieu et al., 2004; Pradeep et al., 2008; Walker et al., 2011a; Shekar et al., 2012; Tang et al., 2012). This study was performed to analyze the VNTR sequences of some Iranian WSSV isolates. All positive samples were detected and confirmed by OIE recommended method and ORF 94 was sequenced to reveal repeat units and SNPs(Shekar et al., 2012; González-Galaviz et al., 2013). Iranian isolates had 3 and 6 RUs in ORF94. The Khuzestan isolates showed a pattern of 6 RUs in 2010 and 3 RUs in 2011 and 2012. The first positive samples with 6 RUs was founded from wild sources in 2010 whereas the second positive samples with 3 RUs was founded in adjacent ponds in 2012 of shrimp farming and originated from a hatchery which its post larvae was used for stocking and also was positive . The isolate from the wild catch *Metapenaeus affinis* (IRWSSV2) in the inlet water channels of Khuzestan's grow out ponds in 2010 had been different from the other isolates in point of number of RUs. Meanwhile the SNPs are same in all of isolates which were collected from Khuzestan.

The Boushehr isolates and its feed (Artemia flake) came from one hatchery's post larvae. These post larvae had used the same feed source (Artemia flake). Both feed and post larvae were positive; meanwhile the feed had 6 RUs whereas the post larvae had 3 RUs. Both samples had same SNPs which was different from Khuzestan SNPs (G in Boushehr and T in Khuzestan).

These results show that the origin of isolates in Khuzestan and Boushehr are different. In the other hand the differences in number of RUs may cause by deletion in ORF 94(Hoa et al., 2011a)

Based on RUs of ORF94, the Iranian isolates are not similar to Americas isolates such as

Brazil, Panama, USA, Mexico, Nicaragua, and Honduras (Muller et al., 2010). In these countries the number of RUs (54 bp) in ORF94 had been including a variation of 4, 5, 8, 12, 13, 14 and 16. In the other hand the number of RUs in ORF94 of South Eastern Asian countries like Taiwan, China, Thailand and Vietnam had been 6, 6-14, 6-20 and 4-17 respectively (Wongteerasupaya et al., 2003; Shekar et al., 2012). Since Iranian isolates show 3 and 6 RUs and shows the Iranian isolates have more similarities with South Eastern Asian countries. Also there was no similarity between the Saudi Arabia and Iranian isolates since the pattern of RUs of Saudi Arabia's isolates are including 7, 13 (Table 2) (Tang et al., 2012).

The SNPs in our samples are too divergent and this showed the different origin of Iranian WSSV isolates. Also the SNPs are too divergent in different countries and even in isolates in same countries which make it too difficult to find the origin of isolates on basis of SNPs variation.

One of the more significant finding to emerge from this study is that the different sources of virus were feed (IRWSSVBU1) and post larvae (IRWSSVBU2). Therefore some considerations can be addressed to avoid significant economic losses in shrimp industry. The identification of the virus sources can help authorities in managing the virus quarantine. Virus specifications could be considered in controlling treatment procedures such as fresh and live feed and wild marine decapods.

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