Vol. 118: 65–75, 2016
doi: 10.3354/dao02958

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published February 11

Identification and characterization of ostreid herpesvirus 1 associated with massive mortalities of *Scapharca broughtonii* broodstocks in China

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ABSTRACT: In the early summer of 2012 and 2013, mass mortalities of blood ark shell (*Scapharca* [*Anadara*] *broughtonii*), broodstocks were reported in several hatcheries on the coast of northern China. Clinical signs including slow response, gaping valves and pale visceral mass were observed in diseased individuals. In response to these reported mortalities, 238 samples were collected from hatcheries at 6 sites. Microscopic changes including lysed connective tissue, dilation of the digestive tubules, eosinophilic inclusion bodies, nuclear chromatin margination and pyknosis were found in affected animals. Transmission electron microscopy (TEM) revealed herpes-like viral particles within the connective tissue of the mantle. Quantative PCR (qPCR) and nested PCR (nPCR) analysis using primers specific for ostreid herpesvirus 1 (OsHV-1) indicated significant higher prevalence of OsHV-1 DNA in cases associated with mass mortalities than those without mass mortalities ($p = 0.0012$ for qPCR, $p < 0.0001$ for nPCR). qPCR also indicated that samples associated with mass mortalities carried high viral DNA loads, while the loads in apparently healthy samples were significantly lower ($t = 3.15$, df = 92, $p = 0.002$). Sequence analysis of the C2/C6 region of nPCR products revealed 5 newly described variants, which were closely related to each other. Phylogenetic analysis of the 5 virus variants and 48 virus variants reported in previous studies identified 2 main phylogenetic groups, and the 5 virus variants identified here were allocated to a separate subclade. To our knowledge, this is the first report of mass mortalities of bivalve broodstocks associated with OsHV-1 infection.

KEY WORDS: *Scapharca broughtonii* · Herpes-like virus · Ostreid herpesvirus 1 · Mass mortality · Aquaculture

INTRODUCTION

Scapharca broughtonii, a member of the family Arcidae and the genus *Scapharca*, is distributed widely along the coasts of northern China, the Russian Far East, South Korea, and Japan (An & Park 2005, Sugiura et al. 2014). Frequent and intensive fishing caused an extreme decline in the production of *S. broughtonii* after the early 1990s in China (Tang et al. 1994). To eliminate hunting pressure on the wild *S. broughtonii* population, artificial techniques for mass production of *S. broughtonii* seeds

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were developed (Wang & Li 1986). Additionally, in order to promote stock restoration, *S. broughtonii* broodstocks introduced from South Korea to China have been used for hatchery propagation and seed release in recent years (Liang et al. 2011). During 2011 to 2013, about 700 million seeds with shell lengths of 10–15 mm were released to the wild each year in Shandong Province (Z. Qiu unpubl. data). With the expansion of the *S. broughtonii* aquaculture industry, mass mortalities of *S. broughtonii* have been reported in China (Zhang & Wang 1994). Several studies, which focused mainly on environmental factors, have been carried out to investigate the cause of these mortalities (Li 1996, Wang & Sui 1996). The role of herpes-like viruses, one of the most common viral etiologies in bivalves around the world, has never been investigated in *S. broughtonii* mortality events.

Herpes-like viral infections were firstly described in *Crassostrea virginica* adults in 1972 from the east coast of the USA (Farley et al. 1972). Further mortality outbreaks associated with the detection of herpes-like viruses have been reported from different bivalve species, including various species of oyster, clam, scallop and abalone in China and other countries throughout the world (Hine et al. 1992, 1998, Renault et al. 2001, Wang et al. 2002, Chang et al. 2005, Tan et al. 2008, Ren et al. 2013). Mortality outbreaks associated with ostreid herpes-like viruses were usually reported in larvae and spat during the summer period of each year (Renault et al. 1994a, 2000, Arzul et al. 2002). Viral particles isolated from infected *C. gigas* larvae have been fully characterized on both a morphological and molecular basis (Le Deuff & Renault 1999, Davison et al. 2005) and named ostreid herpesvirus 1 (Minson et al. 2000, Arzul et al. 2002). Genome sequencing and comparison showed ostreid herpesvirus 1 is tenuously related to the 2 classes of vertebrate herpesviruses and represents a novel major class of herpesviruses (Davison et al. 2005). Ostreid herpesvirus 1 was subsequently assigned as the founding member of the species *Ostreid herpesvirus 1* (OsHV-1), genus *Ostreavirus*, family *Malacoherpesviridae* and order *Herpesvirales* (Davison et al. 2009).

Mass mortalities of bivalves associated with herpes-like virus in China were firstly reported in Zhikong scallops *Chlamys farreri* in the late 1990s (Song et al. 2001, Wang et al. 2002), and the herpes-like virus was initially named the acute viral necrosis virus (AVNV) (Song et al. 2001, Wang et al. 2002). The completion of genome sequence and genetic comparison of OsHV-1 and AVNV revealed that they are 2 variants of the same virus (Renault et al. 2012, Ren et al. 2013). Recently, OsHV-1 was also detected in farmed *C. gigas* in the other 2 Asian countries (Japan and South Korea) and associated with mass mortalities of larvae in artificial hatcheries in South Korea (Shimahara et al. 2012, Hwang et al. 2013, Jee et al. 2013).

In the early summer of 2012 and 2013, mass mortalities of *S. broughtonii* were reported among batches indigenous to China and those introduced from South Korea. Given the clinical signs and mortality trends and patterns observed in these events, a disease was suspected to be responsible for the outbreaks of these mass mortalities. Samples collected from these hatcheries and the surrounding open sea areas were analyzed for the presence of OsHV-1.

MATERIALS AND METHODS

Scapharca broughtonii introduction and sampling

S. broughtonii broodstocks introduced from South Korea (caught from the wild) were firstly cultivated in pearl nets in the open sea at Site ChangD (see Fig. 1) for at least 2 wk to ease the stress of transportation, and then they were transferred to the hatcheries for further production. Local populations of *S. broughtonii* broodstocks in China were transferred to the hatcheries directly after they were caught in the wild. The water temperatures in the wild were about 10°C and 7°C in 2012 and 2013, respectively, when the broodstocks were transferred to the hatcheries. The water temperature at the hatchery was kept the same as it was in the wild for the first week after the broodstocks were transferred in, and then they were slowly acclimated at a speed of 1°C d^{-1} to 18°C, the temperature at which the mass mortalities occured.

Samples of *S. broughtonii* were taken in June 2012 and during March to July 2013, which was the prime time of mortalities incidences reported by owners of bivalve hatcheries and related organizations. Additionally, *S. broughtonii* juveniles with 5 different sizes (see Table 1) cultivated in the open sea near an affected hatchery at site RiZ were also sampled in May 2013 (about 9°C when sampled). Collected samples were conserved in an ice box and transferred to the laboratory immediately. Upon receipt, each *S. broughtonii* was opened and examined for any signs of abnormality, including shrunken mantle, abnormal coloration, lesions, presence of abscesses and tissue discoloration, etc.

Histopathology

For histopathological examination, gills, mantles and digestive glands dissected from 2 diseased *S. broughtonii* were fixed in Davidson's alcohol, formalin, acetic acid (AFA) fixative for 24 h and then transferred to 70% alcohol for storage. The histological sections were made according to standard protocols including dehydration in ethanol series, clearing, embedding in paraffin and cutting into 5 µm thick sections using a Leica microtome RM2145 (Leica Instruments). The sections were then stained with hematoxylin and eosin (H&E) for standard histopathological evaluation using a light microscope (Nikon Eclipse 80i). In order to compare these with the tissue architecture of normal organs of *S. broughtonii*, tissues collected from one apparently healthy individual were also handled with the same procedure as described above.

Transmission electron microscopy (TEM) examination

For TEM examination, tissues dissected from the same organs as those used for histological examination were fixed directly in cold 2.5% glutaraldehyde (pH 7.2) in 0.2 M sodium cacodylate, post-fixed in 1% osmium tetroxide in the same buffer and dehydrated in ethanol series. Then the tissues were embedded in Epon812 and cut on a Ultracut-E ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a JEOL JEM-1200EX at 80 kV at the Medical College of Qingdao University.

Nucleic acid extraction

Mantle fragments in 30 mg from each individual of all 238 samples (10 batches) were dissected for DNA extraction, which was carried out with a TIANamp™ Marine Animals DNA Kit (Tiangen Biotech) according to the manufacturer's handbook. The extracted nucleic acid were then stored at −40°C until the time of testing. Extraction blank controls were included in the extraction process to detect cross-contamination between samples.

Polymerase chain reaction detection

Molecular detections of OsHV-1 DNA were undertaken using both nested PCR (nPCR) and quantitative PCR (qPCR). The qPCR was adapted from a previously published protocol (Martenot et al. 2010). Briefly, amplification was performed in 25 µl reactions containing 12.5 µl of 2 FastStart Essential DNA Probes Master (Roche Diagnostics), 0.5 µl of each primer (20 µM), 0.5 µl of TaqMan® probes (10 µM), 2 µl of template DNA and 9 µl water. The PCR assay was performed using Bio-Rad CFX Connect Real-Time system (Bio-Rad Laboratories) and run under the following conditions: 1 cycle 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 10 s, 60°C for 30 s. The virus quantitation was carried out by comparison with a standard curve, which was created from a 10-fold dilution series (10^7–10^1 copies μl^{-1}) of plasmid containing the target sequence. A negative control was carried out with 2 µl of deionised water. Each sample was tested in duplicate and the sample was recorded as positive if both replicates were amplified and indicated the presence of OsHV-1 DNA. We estimated the OsHV-1 infection burdens as the mean genomic equivalent (GE) score mg^{-1} of tissue for the 2 replicates.

The nPCR employed here was developed in-house with the purpose of improving the sensitivity of the common PCR assay. A pair of primers targeting an approximately 1100 bp fragment in the C region of the OsHV-1 genome were selected for the first amplification step of the nPCR: the forward primer was named CFor (5′-ATT ACC CAG ATT CCC CTC-3′) and was located at the region flanking open reading frame 4 (ORF 4); the reverse primer was named CRev (5′-CCA CGA ATG TAA ACT GTG AC-3′) and was located in the middle region of ORF 4. We amplified the targeting DNA fragment using CFor/CRev for the first PCR step. We then amplified the first-round PCR products using primer pair C2/C6 (Renault & Arzul 2001), which could generate fragments about 700 bp. Special care was taken to prevent false positive results due to contamination, i.e. frequent changes of disposable gloves and preparation of pre- and post-PCR materials in separate locations.

Our initial PCR procedure was optimized to achieve a detection sensitivity of as little as 10 copies of genomic DNA per µl (C. X. Yang et al. unpubl. data). The final protocol was carried on with 1 µl of each template DNA in a 24 µl reaction pre-mixture, containing 2× PCR buffer (20 mM Tris-HCl, 100 mM KCl, 3 mM $MgCl_2$, 0.4 mM of dNTPs, 0.1 U μl^{-1} *Taq* DNA polymerase and PCR enhancer) (Dongsheng Biotech) and 0.4 mM of each primer. The conditions for the first amplification included an initial denaturation for 5 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 52.5°C and 1 min at 72°C; and a final extension for

5 min at 72°C. The conditions for the second amplification were an initial denaturation for 5 min at 94°C; 25 cycles of 30 s at 94°C, 45 s at 50°C and 30 s at 72°C; and a final extension for 5 min at 72°C. Sterilized distilled water and 10 copies μl^{-1} plasmid DNA containing cloned target sequences were used as negative and positive controls.

The PCR products (approximately 700 bp) were then separated by agarose gel electrophoresis (1.5% agarose gels). Finally, PCR products of the positive samples were directly sequenced using primers C2 and C6 (Sangon Biological Engineering Technology & Service Company). For batches where less than 20 positive samples were detected, all PCR products were sequenced. For batches where more than 20 positives samples were detected, only some PCR products (but no less than 10) were selected randomly and sequenced.

Phylogenetic analysis

Sequences were aligned with MAFFT version 7 with the default settings (Katoh et al. 2002, Katoh & Standley 2013), followed by manual adjustments with BioEdit 7.0.0 (Hall 1999). The number of virus variants was calculated with DnaSP 5.10 (Rozas et al. 2003). To compare the phylogenetic relationship between virus variants identified in this study and those reported in other countries, 48 virus variants identified in previous studies were included in the dataset (Table S1 in the Supplement; www.int-res.com/articles/suppl/d118p065_supp.pdf) (Shimahara et al. 2012). Aligned gaps were coded as binary data (sequence present = 1, sequence absent = 0) by the program GapCoder applying the single indel coding method described by Simmons & Ochoterena (2000) and Young & Healy (2003).

RESULTS

Field observations

Scapharca broughtonii broodstocks cultivated in 2 hatcheries, i.e. in LaiZ and ChangD, Shandong Province, China, experienced >75% mortalities in June 2012. Moribund individuals showed clinical signs including slow response, gaping valves and pale visceral mass. Recurrent mass mortalities of *S. broughtonii* broodstocks were observed again in more hatcheries in China (Shandong and Liaoning Province) in April 2013 (Fig. 1). Additional cases of mass mortalities were reported in 2012 (2 cases) and 2013 (4 cases). Mass mortalities broke out when the water temperature reached 18°C in the hatcheries and lasted about 1 wk, while no mass mortality was found in *S. broughtonii* populations cultivated in the open sea near the hatcheries.

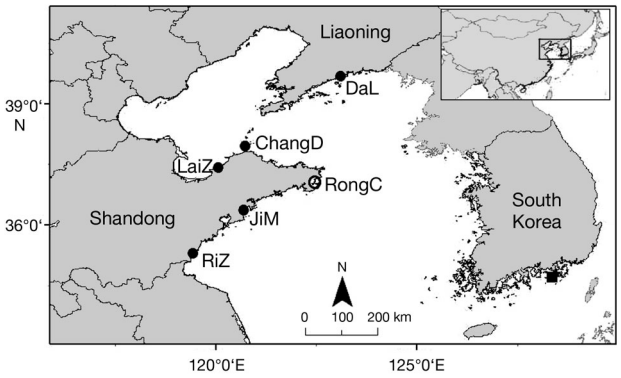

Fig. 1. *Scapharca broughtonii* sampling sites, where OsHV-1 was detected (●) or not detected (○). Site in South Korea (■) from which *S. broughtonii* broodstocks were introduced (to China)

Histopathology

Two *S. broughtonii* confirmed positive for OsHV-1 DNA detected by PCR and 1 healthy *S. broughtonii* were examined by histology. The normal un-infected digestive gland of *S. broughtonii* consists of digestive tubules with various degrees of vacuolization (Fig. 2A). In OsHV-1 PCR positive clams, morphological changes including tubule dilation and lysed connective tissue were observed throughout the sections (Fig. 2B). Eosinophilic inclusion bodies (Fig. 2C) and abnormally shaped nuclei (chromatin margination and pyknosis, Fig. 2D) were also frequently observed in the digestive gland of OsHV-1 PCR positive *S. broughtonii*.

Observation of viral particles by TEM

TEM revealed herpes-like virus particles in the mantle of moribund *S. broughtonii* (Fig. 3) but not in any tissue of uninfected *S. broughtonii*. Both intranuclear empty capsids and nucleocapsids (Fig. 3A,B) measuring 109.92 ± 1.55 nm (±SE) in diameter were observed within the nucleus of infected cells in the connective tissues of the mantle. Enveloped extracellular viral particles (Fig. 3C,D) measuring 151.16 ± 1.24 nm (±SE) were frequently visualized in the

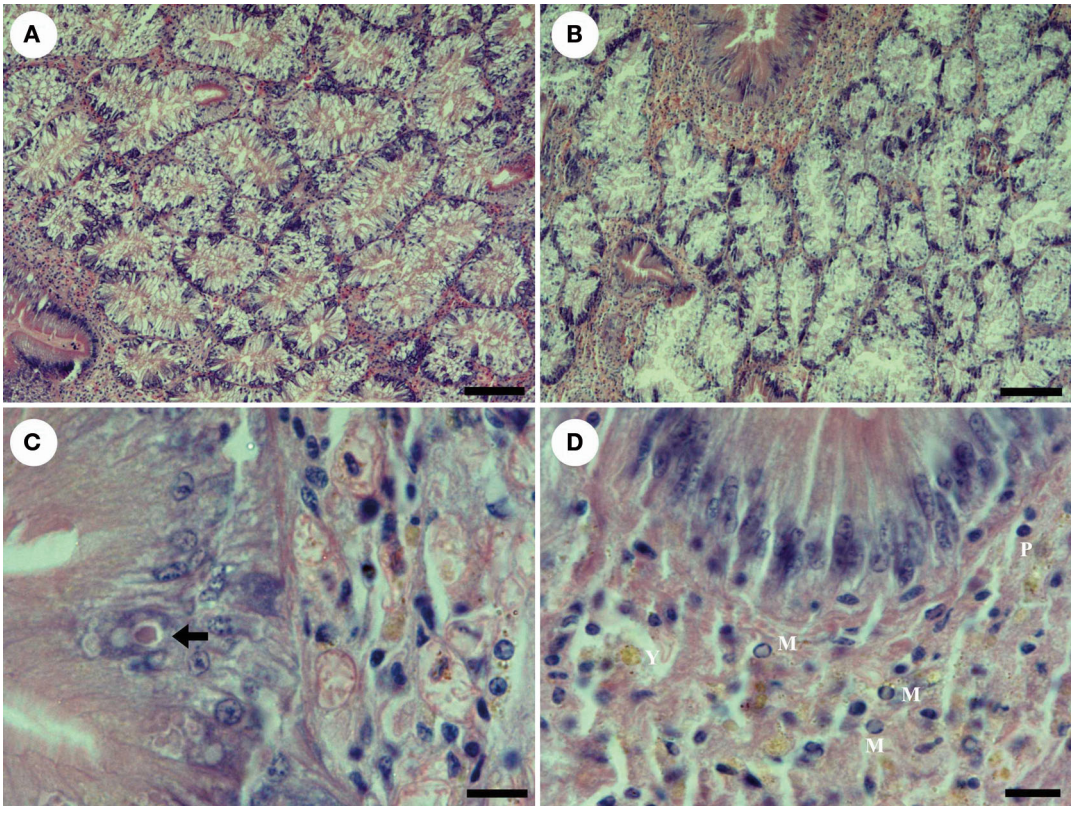

Fig. 2. Photomicrographs of digestive gland and cellular architecture of *Scapharca broughtonii*. (A) Various degrees of vacuolization observed in normal digestive tubules of a *S. broughtonii* tested negative for OsHV-1 DNA by PCR (scale bar = 100 µm). (B) Lysed connective tissue and tubule dilation observed in a *S. broughtonii* collected during a mortality event (scale bar = 100 µm). (C) Inclusion bodies (arrow) in the digestive tubule epithelium of infected *S. broughtonii* (scale bar = 10 µm). (D) Abnormal nuclear features in in the digestive gland of a *S. broughtonii* infected with OsHV-1. M: nuclear margination; P: pycnosis; Y: yellowish material typically associated with OsHV-1 infection (scale bar = 10 µm)

intercellular space under the epithelial cells. Additionally, capsids with pleomorphic cores were also usually found near the chromatin, which may be interpreted as the process of acquisition of nucleoid into capsids (Fig. 3A,B) as reported by Hine & Thorne (1997) and Renault et al. (2000).

Molecular detection of OsHV-1

Out of 238 samples, 133 (55.9%) tested positive for OsHV-1 DNA detection by nPCR assay (Table 1). All samples that were positive by nPCR were also positive by the qPCR assay, while 32 negative samples tested by nPCR were proved positive by qPCR assay. Of the 32 samples, 2 were associated with mass mortalities (GEs = 4.73×10^4 and 1.27×10^3), 30 were apparently healthy (GEs ranged from 6.03 to 420.00, mean = 128.29, median = 61.70). Both qPCR and nPCR assay indicated higher prevalence of OsHV-1 DNA detection in cases with mass mortalities (93/94 = 98.9% for qPCR and 91/94 = 96.8% for nPCR) than those without mass mortalities (72/144 = 50.0% for qPCR and 42/144 = 29.2% for nPCR) (χ^2 = 10.48, df = 1, p = 0.0012 for qPCR and χ^2 = 27.41, df = 1, p < 0.0001 for nPCR). qPCR also indicated that mean GEs for samples associated with mass mortalities (GE mean = 2.22×10^7, SE = 7.04×10^6, n = 93) were significantly higher than those (GE mean = 296.28, SE = 64.90, n = 72) without mass mortalities (t = 3.15, df = 92, p = 0.002). Of the 6 cases of mass mortalities, mean GEs of 3 of them were over 10^6, one of them was near 10^4 and the other 2 were less than 10^3. The mean GEs for all 12 batches with apparently healthy samples ranged from 4.60 to 807.26 (median = 207.33). A high percentage of OsHV-1 DNA was also detected in juveniles with moderate viral loads (which varied from 162.00 to 807.26 GEs, mean = 440.17), and no abnormal mortalities were found among them.

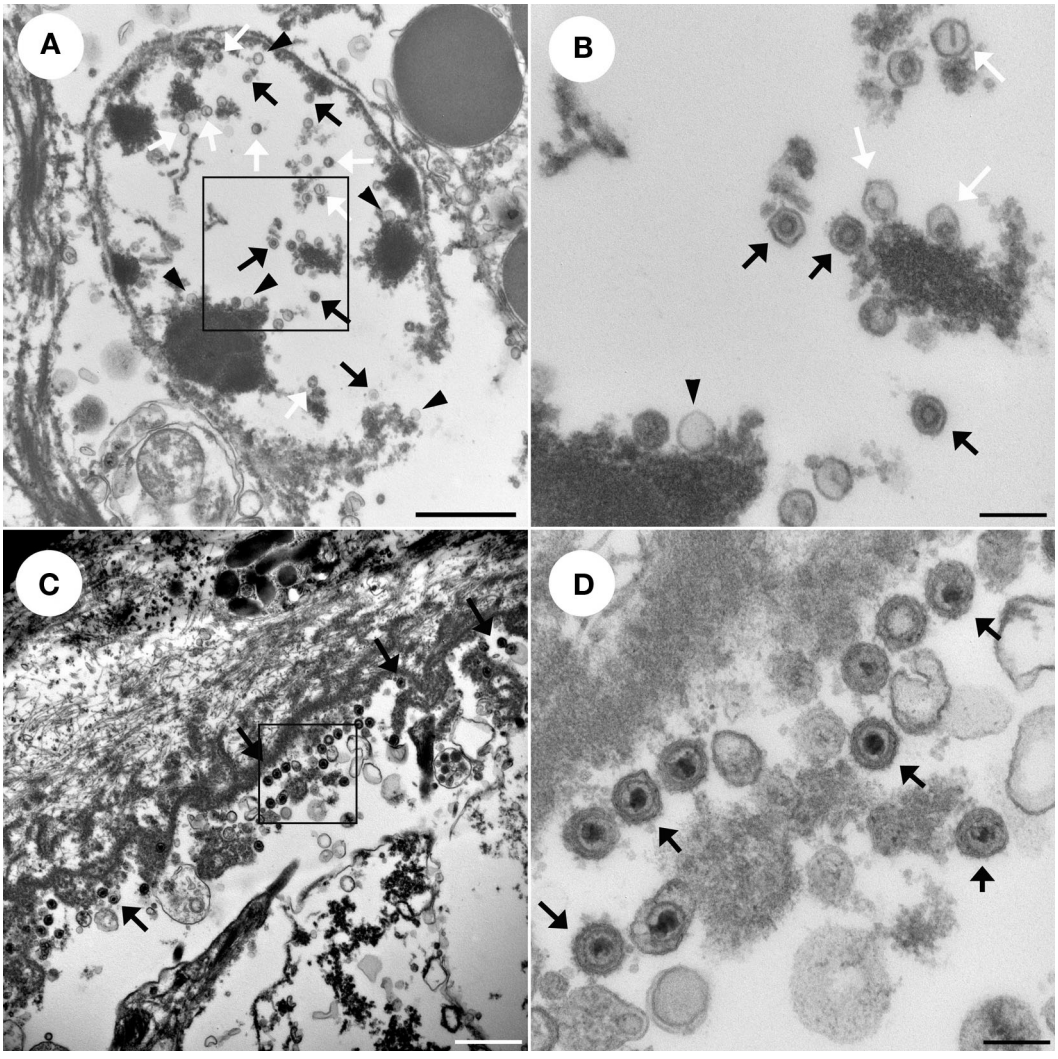

Fig. 3. Herpes-like virus observed in the mantle of infected *Scapharca broughtonii*. (A) Intra-nuclear nucleocapsids (black arrows), empty capsids (arrowheads) and capsids with pleomorphic cores (white arrows) detected in the nuclei of infected cell (scale bar = 1 µm). (B) High magnification graphic of the region indicated by the box in panel (A) (scale bar = 200 nm). (C) Enveloped extracellular virus particles (arrows) observed in the intracellular space under the epithelial cells (scale bar = 1 µm). (D) High magnification graphic of the region indicated by the box in panel (C) (scale bar = 200 nm)

Genetic variation of OsHV-1

Of the 133 positive samples, PCR amplicons of 84 representative samples were sequenced (Table 1). The analysis of these sequences revealed 5 virus variants, which varied from 585 to 593 bp in length. The virus variants were represented as CN1 to CN5 in this study and registered in GenBank with the accession numbers KR075679–KR075683. All of these virus variants were shared by local populations of *S. broughtonii* in China and those introduced from South Korea. The total length was 614 bp after alignment as a result of variation of repeat numbers of the microsatellite locus. Genetic variation was only found in 2 loci with insertion/deletion polymorphism. The main polymorphism was found in a microsatellite region that was characterized by ACT repeats. Compared to 8 ACT repeats in the reference sequence (AY509253), there were 6, 7, 8 and 9 ACT repeats found in CN4, CN2 and CN3, CN5 and CN1, respectively. Another polymorphism locus was characterized by a deletion of G in CN1 and CN2 compared the other 3 virus variants. Of the 84 sequences, 57 (67.9%) belonged to the virus variant CN3, followed by CN2 (15 sequences), CN1 (6 sequences), CN4 (5 sequences) and CN5 (1 sequences) (Table 1).

Table 1. Prevalence, average genomic equivalents (GE) and variants of OsHV-1 in *Scapharca broughtonii* cultivated in China. qPCR: quantitative PCR; nPCR: nested PCR

Site	Date of sampling	Development stage (shell height, mm)	No. of samples	Mean GE ± SE	Prevalence (%) (95% CI) of qPCR assay	of nPCR assay	Variant (no. of infected mollusks)
ChangD[a]	Jun 2012[b]	Adult	15	$2.58\times10^{7} \pm 2.30\times10^{7}$	100 (75–100)	93 (66–100)	CN2 (10), CN4 (4)
	Mar 2013	Adult	4	252.67 ± 252.67	25 (1–78)	25 (1–78)	CN5 (1)
	Apr 2013[b,c]	Adult	27	$1.11\times10^{7} \pm 2.71\times10^{6}$	100 (85–100)	100 (85–100)	CN1 (4), CN3 (9)
	May 2013	Adult	30	31.84 ± 5.37	63 (44–79)	30 (15–50)	CN1 (2), CN3 (7)
	Jul 2013	Adult	20	4.60 ± 4.60	5 (0–27)	5 (0–27)	CN3 (1)
LaiZ	Jun 2012[b]	Adult	6	345.83 ± 172.48	100 (52–100)	100 (52–100)	CN2 (5), CN4 (1)
JiM	Apr 2013[b,c]	Adult	30	$4.58\times10^{7} \pm 1.79\times10^{7}$	100 (86–100)	97 (81–100)	CN3 (10)
	May 2013	Adult	5	0	0 (0–54)	0 (0–54)	
DaL	Apr 2013[b,c]	Adult	13	38.16 ± 10.86	92 (62–100)	92 (62–100)	CN3 (12)
	May 2013	Adult	5	17.00 ± 5.50	60 (17–93)	0 (0–54)	
RiZ	Apr 2013[b]	Adult	3	$7.06\times10^{3} \pm 1.75\times10^{3}$	100 (31–100)	100 (31–100)	CN3 (3)
	May 2013	Adult	20	32.27 ± 32.27	5 (0–27)	5 (0–27)	CN3 (1)
	May 2013[c]	Juvenile (12.32)	10	292.15 ± 158.65	90 (66–100)	90 (54–99)	CN3 (4)
	May 2013[c]	Juvenile (17.61)	10	417.30 ± 198.46	100 (66–100)	70 (35–92)	CN3 (1)
	May 2013[c]	Juvenile (22.76)	10	807.26 ± 374.41	90 (66–100)	30 (8–65)	CN3 (2)
	May 2013[c]	Juvenile (29.23)	10	162.00 ± 45.30	100 (66–100)	50 (20–80)	CN3 (3)
	May 2013[c]	Juvenile (36.87)	10	552.15 ± 119.88	90 (66–100)	60 (27–86)	CN3 (4)
RongC	Jun 2013	Adult	10	0	0 (0–34)	0 (0–34)	

[a]Hatcheries where *S. broughtonii* introduced from South Korea were cultivated
[b]Abnormal mortalities of *S. broughtonii* found
[c]Batches from which only some samples were selected randomly and sent for sequencing

Among 48 virus variants identified from previous studies, 23 (numbered as JPType1 to JPType23) isolated from Japan (Shimahara et al. 2012), 1 identified in China, 2 (numbered as KRType1 and KRType2) in South Korea, and the other 22 virus variants (numbered as AE1 to AE22) were identified from China (AE 8), USA (AE4 and AE19), Japan (AE9), Ireland (AE13), New Zealand (AE18), Australia (AE20) and France (the other 15 virus variants) (Table S1).

Phylogeny of OsHV-1 virus variants

To estimate the genetic relationship between OsHV-1 virus variants identified in this study and those reported in previous studies, a phylogenetic tree of these virus variants was constructed using the maximum parsimony (MP) method. All the sequences were trimmed from 2 ends to give them equal lengths. Finally, the aligned sequences resulted in a matrix of 614 characters (Fig. S1 in the Supplement). But after being coded by GapCoder, a final data matrix of 642 characters was created, of which 30 were potentially parsimony informative. We obtained 8835 MP trees with 80 steps and recognized 2 major groups in all MP trees (Fig. 4). One group consisted of 33 virus variants (including the variant µVar) that were identified from 6 countries (France, Ireland, Australia, New Zealand, Japan and South Korea). The other group was composed of remaining 20 virus variants (including the OsHV-1 reference virus type and AVNV) identified from 5 countries (France, the USA, Mexico, Japan and China). Virus variants identified in this study were allocated to a separate subclade.

DISCUSSION

The clinical signs and histological observations in infected *Scapharca broughtonii* broodstocks allow investigating abnormalities. The viral particles demonstrated by TEM in this study resemble those previously described from oysters in other countries in morphology (Hine et al. 1992, Renault et al. 1994b, Hine & Thorne 1997, Burge & Friedman 2012), but they were bigger in size than those reported previously (Hine et al. 1998, Renault et al. 1994b). Further evidence by qPCR revealed extremely high viral DNA loads in samples collected in 4 cases of mass mortalities (n = 75), which indicated the association between the detection of OsHV-1 DNA and *S.*

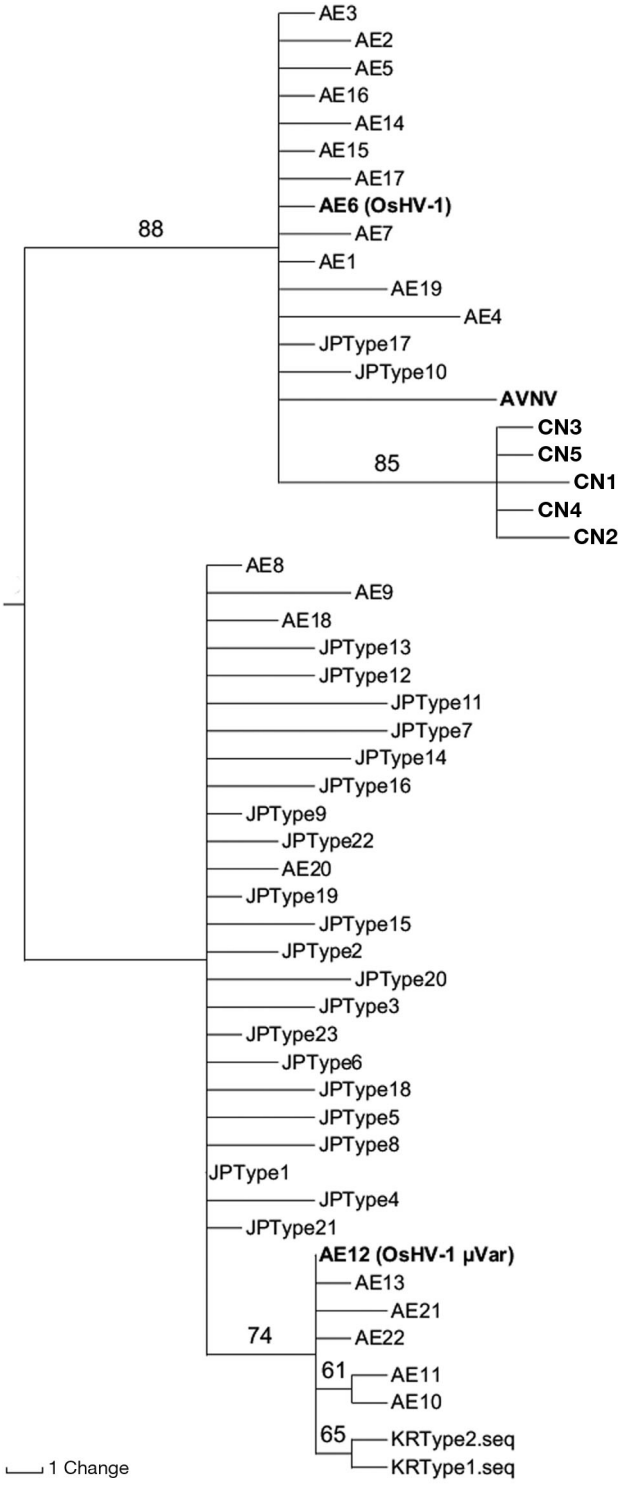

Fig. 4. Strict consensus tree from phylogenetic analyses for the virus variants of OsHV-1 using the maximum parsimony analysis. Numbers above the branches are the parsimony bootstrap support value >50%. CN: virus variants detected in this study. Virus variants identified in Japan (JPType), in South Korea (KRType), in China (AVNV) and in the other countries (AE) (see Table S1 in the Supplement)

broughtonii loss. Additionally, significantly higher prevalence of OsHV-1 DNA was also found by both qPCR and nPCR among the stocks showing mortalities than among apparently healthy stocks. OsHV-1 infection in *S. broughtonii* has not been reported previously. We describe herein for the first time OsHV-1 infection of *S. broughtonii* associated with mass mortality in several commercial hatcheries in China. To our knowledge, this is also the first report of OsHV-1 infection of bivalve broodstocks associated with mass mortalities at lower temperatures.

In comparison to vertebrate herpesvirus, which have a limited number of hosts (Davison 2002), OsHV-1 is capable of infecting a variety of bivalve species belonging to different genera (Hine et al. 1992, Arzul et al. 2001, Renault et al. 2001, Bateman et al. 2012, Ren et al. 2013). Mortalities associated with the OsHV-1 are usually found in larvae and juveniles of susceptible bivalve species (Renault et al. 1995). However, inconsistent with previous studies, the mass mortalities associated with OsHV-1 infection in our study were found in the broodstocks of *S. broughtonii*. Notably, OsHV-1 infection of *Chlamys farreri* associated with mass mortalities was also found in adult individuals in China (Wang et al. 2002). These results may indicate that the OsHV-1 virus variants found in China exhibit different host stage preference compared with those found in the other countries. Further studies should be conducted to determine the underlying reasons for the different life stages susceptible to OsHV-1 variants found in China and in the other countries. It could result from the strain differentiation of OsHV-1, the interaction of the virus and host in a particular environment, the susceptibility of the host, or from other unknown causes (Vásquez-Yeomans et al. 2010).

Abnormal mortalities of a host caused by an infectious agent have always been associated with complex environmental factors. In the case of OsHV-1 infection, seawater temperature appears to be one of the most important potential factors influencing the incidence and outcome of OsHV-1 infection. High temperatures may enhance the expression of OsHV-1 and thus increase the mortalities associated with the infection (Le Deuff et al. 1996, Petton et al. 2013). In this study, mass mortalities of *S. broughtonii* broodstocks were only observed when the water temperatures had increased to 18°C. On the other hand, no abnormal mortality was observed in juveniles of *S. broughtonii* cultivated in lower temperatures, which carried DNA of the same OsHV-1 variant (CN3). These results highlighted the importance of water temperature as a potential factor influencing

OsHV-1 infection. Although the losses reported here were inevitably associated with elevated seawater temperature in the hatcheries, the temperature threshold was lower than those (23–25°C) previously associated with mass mortalities of *C. farreri* cultivated in the open sea in the 1990s in China (Wang et al. 2002).

Sauvage et al. (2009) reported that an average number of viral copies up to 10^4 mg^{-1} of tissue could be interpreted as evidence of viral infection leading to mass mortality. Furthermore, if the average number of viral copies is less than 1×10^3 mg^{-1} of tissue, this should not be interpreted as an infective status associated with mortality (Pepin et al. 2008). In the present study, viral DNA loads up to or about 10^4 copies mg^{-1} of tissue were detected in 4 batches of samples (n = 75) associated with mass mortalities, while the viral DNA loads in the other 2 batches of samples (n = 19) associated with mortalities were far less than the threshold (10^4 copies mg^{-1} of tissue). These results suggested that apart from OsHV-1 infection other factors also played a role in the onset of mortalities of blood clams in these cases.

Sequencing of 84 out of 133 positive samples generated 5 virus variants of OsHV-1, which allocated into a single subclade. Among the 5 virus variants identified in this study and 48 identified in previous studies, no shared virus variant was found between different countries. In contrast, shared virus variants have been detected between different EU countries (Dundon et al. 2011, Lynch et al. 2012, Roque et al. 2012), which may be due to frequent trade of bivalves in these countries (Pawiro 2010). In this study, 5 virus variants were found in *S. broughtonii* broodstocks both introduced from South Korea and indigenous to China. These variants have also been detected in the other 4 bivalve species (*C. farreri, Patinopecten yessoensis, Ruditapes philippinarum and Crassostrea hongkongensis*) cultivated in the other regions of China recently (Bai et al. 2015) but have not been detected in South Korea (Hwang et al. 2013). Since only *C. gigas* with small sample sizes were tested in South Korea, it is still difficult to deduce whether these variants were introduced from South Korea or not.

There were 32 samples in which OsHV-1 DNA was not detected by nPCR assay but proved to be positive by qPCR assay. The detection failure for 19 of them by nPCR might be explained by small amounts of OsHV-1 DNA contained in these samples, while the other 13 samples containing more than 100 GEs quantified by qPCR also tested negative by nPCR. Amplification failure of OsHV-1 DNA of the same genome region targeted by nPCR was also reported by Friedman et al. (2005). Since this region has been proved to demonstrate a high degree of polymorphism, we suggest that nucleotide variation in nPCR primer-binding sites may be responsible for the amplification failure of some samples with high viral DNA loads. These results indicated that qPCR assay is more sensitive than nPCR assay. Additionally, since nPCR assay is not able to provide quantitative information on the infection intensity of OsHV-1, which is a predictor of lethal infection, qPCR should be employed for routine epidemiologial studies of OsHV-1 infection (Renault et al. 2014).

In conclusion, this study reported findings from 2012 and 2013 for analysis of *S. broughtonii*, which correspond to the first detection of OsHV-1 infection in *S. broughtonii* and were associated with high mortalities in several locations. Our survey also confirmed the diversity of the OsHV-1 genome with the description of 5 newly reported variants associated with the mass mortalities of *S. broughtonii* in China. Since OsHV-1 infection has been detected in bivalves belonging to different genera, it is of importance to conduct surveillance and monitoring programs for OsHV-1 of other sympatric mollusk species in OsHV-1-infected regions. Furthermore, due to the increasing interest in the *S. broughtonii* farming industry, strict quarantine measures should be taken to minimize the risk of introduction of infectious agents along with the introduction of broodstocks.

Acknowledgements. We thank Jing Qian and Junyang Xia for collecting the samples. We are very grateful to Mike Hine and 3 anonymous reviewers who have provided extremely thorough and thoughtful comments on the draft of the manuscript. This research was supported by grants from the China Agriculture Research System (Project No. CARS-48), the National Natural Science Foundation of China under Grant No. 31502208, the Scientific and Technological Innovation Project financially supported by Qingdao National Laboratory for Marine Science and Technology (No. 2015ASKJ01), the special foundation under the Construction Programme for 'Taishan Scholarship' of Shandong Province of China and the National Marine Public Welfare Research Project (No. 201205023).

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Editorial responsibility: Mike Hine, Fouras, France

Submitted: November 3, 2014; Accepted: December 3, 2015
Proofs received from author(s): January 30, 2016