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Hematodinium spp. infections in wild and cultured populations of marine crustaceans along the coast of China

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ABSTRACT: The parasitic dinoflagellate *Hematodinium* spp. infects a broad range of marine crustaceans. Its epidemics have impacted wild populations of various commercial fishery species around the world and the sustainability of mariculture in China. To study the epidemiology of *Hematodinium* spp. in marine crustaceans along the coast of China, we conducted a broad survey of wild and cultured stocks of major crustacean species in 2013 to 2015. *Hematodinium* sp. infections were identified in wild stocks of *Portunus trituberculatus* from Huludao, Laizhou, Qingdao, Yangtze River Estuary and Zhoushan, and *Scylla paramamosain* from Shantou; and cultured stocks of *Portunus trituberculatus* and *Penaeus monodon* from a polyculture pond in Qingdao. In the polyculture pond, *Hematodinium* sp. infections were observed in *Portunus trituberculatus* from June until October, with peak prevalence (up to 90%) observed in late July to early August. Furthermore, *Hematodinium* sp. infection was identified for the first time in the giant tiger prawn *Penaeus monodon* in the polyculture system during the disease outbreak. Phylogenetic analysis indicated that the *Hematodinium* isolate infecting *Penaeus monodon* was identical to the isolate infecting the co-cultured *Portunus trituberculatus*, and it was grouped into *H. perezi* genotype II together with the other isolates reported in China. The *Hematodinium* sp. isolated from *Portunus trituberculatus* appeared to have similar life stages as the *H. perezi* genotype III isolated from the American blue crab *Callinectes sapidus*. Our study indicates that outbreaks of *Hematodinium* disease can be a significant threat to the widely used polyculture system for decapods in China that may be particularly vulnerable to such generalist pathogens.

KEY WORDS: Crab · Parasite · Epidemics · Prevalence · Life cycle · Transmission · Aquaculture

INTRODUCTION

The parasitic dinoflagellates in the genus *Hematodinium* are emerging infectious pathogens in many decapod crustaceans worldwide, resulting in serious commercial losses to fisheries and the aquaculture of various economically important crustaceans (Xu et al. 2007a, Y. Li et al. 2008, Small 2012). In recent years, frequent outbreaks of *Hematodinium* spp. have been observed not only in a broad range of wild crustacean hosts, including American blue crab *Callinectes sapidus* (Messick & Shields 2000, Shields &

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Squyars 2000), snow crab *Chionoecetes opilio* (Meyers et al. 1990, Taylor & Khan 1995, Shields et al. 2005), Alaskan Tanner crab *Chionoecetes bairdi* (Meyers et al. 1987, 1990), Norway lobster *Nephrops norvegicus* (Field et al. 1992), red king crab *Paralithodes camtschaticus* and blue king crab *Paralithodes platypus* (Ryazanova et al. 2010), but also in several cultured crustacean species, including Chinese swimming crab *Portunus trituberculatus* (Xu et al. 2007a, Li et al. 2013), mud crab *Scylla paramamosain* (formerly misidentified as *S. serrata*) (Xu et al. 2007b, Li et al. 2008) and ridgetail white prawn *Exopalaemon carinicauda* (Xu et al. 2010). A high prevalence of infection (up to 100%) has been reported in *Chionoecetes bairdi* (Meyers et al. 1987, 1990, Messick & Shields 2000) and *Callinectes sapidus* (Butler et al. 2014, Gandy et al. 2015). *Hematodinium* parasites mainly live and proliferate in the hemolymph and the hemal spaces of major tissues or organs (e.g. hepatopancreas, heart, gills, muscle) (Stentiford & Shields 2005), leading to dysfunction of these major organs and the subsequent mortality of the infected host (Field & Appleton 1995, Taylor et al. 1996, Wheeler et al. 2007).

The typical diagnostic stages, ameboid and filamentous trophonts, are commonly observed in naturally and experimentally infected crabs (Shields & Squyars 2000, Wheeler et al. 2007, Wang et al. 2015). Although limited studies have been conducted to elucidate the complete life cycle of *Hematodinium* spp., the developmental cycles of only *Hematodinium* sp. isolated from *N. norvegicus* and *H. perezi* isolated from *Callinectes sapidus* have been observed in *in vitro* cultures (Appleton & Vickerman 1998, Li et al. 2011). More recently, the developmental stages of the *Hematodinium* sp. isolated from *Chionoecetes opilio* were also partially documented in *in vitro* cultures (Gaudet et al. 2015). Even with advances in the understanding of the life cycles of these parasites, only 2 species of *Hematodinium* have been fully described, the type species *H. perezi* identified from multiple hosts (Chatton & Poisson 1931) and *H. australis* from the Australian sand crab *Portunus armatus* and mud crab *S. serrata* (Hudson & Shields 1994, Hudson & Lester 1994). The type species *H. perezi* has been classified into 3 genotypes (Small 2012), including *H. perezi* genotype I from harbor crabs *Liocarcinus depurator*, *H. perezi* genotype II from *Portunus trituberculatus* and *S. paramamosain*, and *H. perezi* genotype III from blue crab *Callinectes sapidus*. The remaining reported isolates are currently identified as *Hematodinium* spp. or *Hematodinium*-like spp., due to the lack of comparative studies with the type species and limited understanding of their typical life stages.

In China, the swimming crab *Portunus trituberculatus* and the mud crab *S. paramamosain* are 2 ecologically and economically significant crustaceans along the coast of China (Fisheries Bureau of Agriculture Ministry of China 2015). These crabs sustain important commercial fisheries in China, with annual landings in 2014 exceeding approximately 700 000 (119 000 t from aquaculture) and 224 000 t (141 000 t from aquaculture), respectively (Fisheries Bureau of Agriculture Ministry of China 2015). However, wild stocks of the 2 crabs have declined over the past decades and have remained at low levels in recent years (Fisheries Bureau of Agriculture Ministry of China 2015). Similar to other commercially valuable species, overfishing and habitat degradation are speculated to be the main factors leading to the declines of wild populations (Deng & Jin 2001, Yu et al. 2004). However, frequent outbreaks of epidemic diseases, especially *Hematodinium* spp., are considered to be the major factors impacting the sustainable development of cultured marine crabs in China (Xu et al. 2007a,b, Y. Li et al. 2008, C. Li et al. 2013, Liu et al. 2014).

Little is known about epizootics of *H. perezi* genotype II in wild host populations in China, nor is the transmission route or disease progression known in aquaculture systems. Thus, our objectives were (1) to carry out a broad survey on the prevalence of *Hematodinium* infection in the wild stocks of *Portunus trituberculatus*, *S. paramamosain* and the Asian paddle crab *Charybdis japonica*, and (2) to examine possible crossover of the parasite into shrimp aquaculture, because the swimming crabs are cultured together with the giant tiger prawn *Penaeus monodon* in polyculture ponds along the coast of China. In addition, the seasonality of infection was investigated in *Portunus trituberculatus*. As part of this study, the *H. perezi* genotype II infecting *Portunus trituberculatus* and *Penaeus monodon* in the polyculture ponds was identified with molecular markers and the parasite in *Portunus trituberculatus* was characterized using *in vitro* cultures.

MATERIALS AND METHODS

Sampling

To assess the prevalence of *Hematodinium* infection in commercial crabs species along the coast of China (Fig. 1), 644 wild crabs, consisting of Chinese swimming crabs *Portunus trituberculatus* (n = 486),

mud crabs *Scylla paramamosain* (n = 70) and Asian paddle crabs *Charybdis japonica* (n = 88), together with cultured *Portunus trituberculatus* (n = 188), were collected during 2013 to 2015. The status of *Hematodinium* infection in these crabs was diagnosed with the specific PCR assay as described in 'Molecular diagnosis of *Hematodinium* infection'. Approximately 200 µl of hemolymph was withdrawn from the juncture (sterilized with 70% ethanol) between the basis and ischium of the 5th walking leg of each crab, using a sterile syringe and a 27-gauge needle. Hemolymph was loaded into 1.5 ml microcentrifuge tubes preloaded with 800 µl ethanol (100%), and temporally held in a cooler. Hemolymph samples were then transported back to the laboratory, where they were maintained in a −20°C freezer until being further processed for DNA extraction.

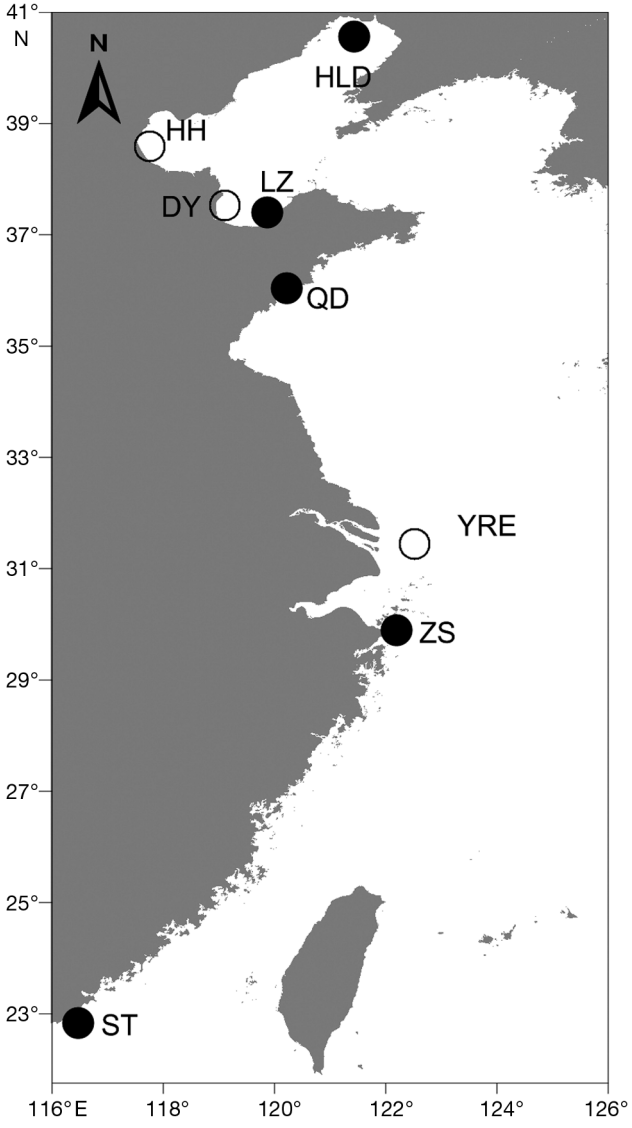

Fig. 1. Marine crustacean sampling sites along the coast of China, indicating (●) presence and (○) absence of *Hematodinium* sp. infections. HLD: Huludao, Liaoning province; HH: Huanghua, Hebei province; DY: Dongying, Shandong province; LZ: Laizhou, Shandong province; QD: Qingdao, Shandong province; YRE: Yangtze River Estuary; ZS: Zhoushan, Zhejiang province; ST: Shantou, Guangdong province

To monitor the outbreak of *Hematodinium* disease in aquaculture facilities, monthly sampling of *Portunus trituberculatus* was conducted in polyculture ponds in Qingdao, Shandong Province (Fig. 1) in 2013 to 2014. Altogether, 391 crabs were sampled from May to September; the sizes (carapace length) of crabs were recorded, and the temperature and salinity in the polyculture ponds were monitored simultaneously. On May 27, 2014, an additional 24 cultured giant tiger prawns *Penaeus monodon* were sampled from a polyculture pond following abnormal mortality in the cultured prawns. The *Hematodinium* infection status of the co-cultured *Portunus trituberculatus* and *Penaeus monodon* was screened using the hemolymph smear assay and further confirmed with the specific PCR assay (as described in the following sections).

Microscopic diagnosis of *Hematodinium* infection

The status of *Hematodinium* infection in cultured *Portunus trituberculatus* was examined using the hemolymph smear assay described by Stentiford & Shields (2005). Briefly, an equal volume of neutral red (0.05% w/v) in 1× phosphate-buffered saline was mixed with a few drops of hemolymph on a glass slide. The mixture was examined with a microscope (Olympus BX 53) to determine the presence of parasites by the distinctive uptake of the vital stain. The intensity of *Hematodinium* infection in each individual was categorized as light, moderate or heavy, based on the number of parasites (<10, 10–100, >100, respectively) observed in blood smears. The hemolymph samples collected from crabs with no overt *Hematodinium* infection (no parasite observed in hemolymph smears) were further processed for molecular diagnosis.

The status of *Hematodinium* infection in cultured *Penaeus monodon* was also pre-examined with the above hemolymph smear assay, using hemolymph squeezed from the sliced tail of prawns. To further confirm the status of *Hematodinium* infection in all cultured prawns, approximately 0.1 to 0.2 g of hepatopancreas tissue was dissected and preserved

with 800 µl ethanol (100%) in 1.5 ml microcentrifuge tubes. The hepatopancreas samples were maintained in a −20°C freezer until processing for DNA extraction and further molecular analysis.

Molecular diagnosis of *Hematodinium* infection

To diagnose *Hematodinium* infections in wild crabs (*Portunus trituberculatus*, *S. paramamosain* and *C. japonica*) and further confirm *Hematodinium* infections in the cultured *Portunus trituberculatus* and *Penaeus monodon* collected from the polyculture ponds, genomic DNA was extracted from the preserved samples using a TIANamp Blood DNA Kit (TIANGEN), according to the manufacturer's protocols. The presence of *Hematodinium* DNA was verified by standard PCR assays performed with a specific primer set (forward primer: 5′-CAT TCA CCG TGA ACC TTA GCC-3′; reverse primer: 5′-CTA GTC ATA CGT TTG AAG AAA GCC-3′) targeting the first internal transcribed spacer (ITS 1) of *Hematodinium* ribosomal DNA (Small et al. 2007). PCR reactions were performed in a LabCycler PCR machine (SensoQuest), with a 25 µl mixture composed of 2 µl of genomic DNA template, 1 µl of each primer (100 pmol μl^{-1}), 12.5 µl of PCR mix (TIANGEN) and 8.5 µl of sterile deionized water. The thermocycling parameters were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C, and a final extension of 5 min at 72°C. The amplified products were visualized by agarose gel electrophoresis (1% w/v), stained with ethidium bromide and viewed under UV light.

Phylogenetic analysis of *Hematodinium* sp. infecting *Penaeus monodon*

The PCR products of the ITS 1 regions amplified from the 3 *Hematodinium*-positive giant tiger prawns were purified with MiniBest DNA fragment purification kits (TaKaRa), according to the manufacturer's instructions. The purified PCR products were inserted into the pMD18-T vector using a TA cloning kit (TaKaRa). Ten independent clones with confirmed recombinant plasmids were sequenced by Invitrogen (Shanghai). For phylogenetic analysis, additional sequences (listed in Table 1) of *H. perezi* isolated from other infected hosts were acquired from the GenBank database. Multiple alignments were performed using the ClustralW algorithm in MacVector 12.6 (MacVector). Then, maximum likelihood and neighbor-joining based phylogenetic trees were constructed using the MEGA5 software (Tamura et al. 2011) with the Kimura 2-parameter (K2) model, as determined by the model test in MEGA5. A total of 1000 bootstrap replicates were performed to assess the robustness of the clades.

Characterization of *Hematodinium* sp. life stages in *in vitro* cultures

To characterize the morphology and life stages of *Hematodinium* sp. identified in the polyculture ponds in Qingdao, Shandong Province, *in vitro* cultures were established in the laboratory according to the methods described by Li et al. (2011). Approximately 0.3 to 1 ml (depending on the intensity of *Hematodinium* infection) of hemolymph was drawn into a 1 ml syringe equipped with a 27-gauge needle, then added to 10 ml of culture medium. The culture medium was modified from basic *Nephrops* saline, as described by Appleton & Vickerman (1998), with the addition of crab serum (5% v/v) to boost the growth of *Hematodinium* parasites and penicillin (100 IU ml^{-1}) and streptomycin (100 µg ml^{-1}) to inhibit potential bacterial contamination. The cell suspension was gently mixed in a sterile 25 cm^2 tissue culture flask, and incubated for 20 min at room temperature (23 °C). The suspension was

Table 1. List of GenBank sequences used in this study

Taxon	GenBank accession no.	Reference
Hematodinium sp. ex *Callinectes sapidus*	DQ925227–DQ925230	Small et al. (2007)
Hematodinium sp. ex *Callinectes sapidus*	FJ844430, FJ844431	Jensen et al. (2010)
Hematodinium sp. ex *Exopalaemon carinicauda*	KX758127	Xu et al. (2010)
Hematodinium sp. ex *Liocarcinus depurator*	EF153725–EF153728, EF065712–EF065715	Small et al. (2012)
Hematodinium sp. ex *Portunus trituberculatus*	EF173452–EF173454	Small et al. (2012)
Hematodinium sp. ex *Penaeus monodon*	KX758131–KX758138	Present study
Hematodinium sp. ex *Portunus trituberculatus*	KX758128–KX7581130	Li et al. (2013)
Hematodinium sp. ex *Scylla paramamosain*	EF173451	Small et al. (2012)

then transferred carefully to a new sterile culture flask and incubated for another 20 min in order to remove crab hemocytes adhering to the plastic surfaces of culture flasks. The *Hematodinium* suspensions were then transferred into new culture flasks pre-loaded with medium to make a final volume of 12 ml. Additional incubation steps were occasionally needed to remove remaining hemocytes from the suspension. Cultures were then incubated in a biosafety II cabinet at room temperature (23°C) in the dark and monitored every 2 to 3 d with an inverted phase contrast microscope (Olympus IX 71) with Hoffman modulation contrast filters. Photographs of the developmental stages of *Hematodinium* in *in vitro* cultures were acquired with an Olympus DP 73 digital camera. After the initial isolation, approximately 50% of the media in the resulting cultures was refreshed weekly.

RESULTS

Prevalence of *Hematodinium* infections in marine crustaceans

Hematodinium infections were detected in *Portunus trituberculatus* and *Scylla paramamosain* from 5 sampling sites with respective prevalence, including Laizhou (LZ, 68.2%, n = 22), Huludao (HLD, 29.4%, n = 34), Qingdao (QD, 33.3%, n = 48), Zhoushan (1) (ZS1, 53.3%, n= 30), Zhoushan (2) (ZS2, 32.0%, n = 25), Zhoushan (3) (ZS3, 48.3%, n = 29) and Shantou (ST, 8.6%, n = 70) (Table 2). The mean prevalence of *Hematodinium* infections in *P. trituberculatus* was 35%, with the highest prevalence observed in LZ (68.2%). No *Hematodinium* infection was observed in wild *P. trituberculatus* collected from the other 3 sampling sites (Huanghua, HH; Dongying, DY; Yangtze River Estuary, YRE). Meanwhile, no *Hematodinium* sp. infection was identified in the Asian paddle crab *Charybdis japonica*, even though it was sampled from locations overlapping with those of the other host species.

Outbreak of *Hematodinium* infection in cultured *Portunus trituberculatus*

No *Hematodinium* infection was identified in the early juvenile crabs sampled on May 27, 2014, approximately 2 wk after seeding into the polyculture pond. However, *Hematodinium* infections were observed in the juvenile crabs 4 wk after they were added to the system, and infections were persistent in the polyculture pond until the end of the culture period. During the sampling period, the prevalence ranged from 4 to 90%, with a mean prevalence of 34.5% (n = 391), and highest prevalences (75.8%, 90%) observed in July (Table 3). The majority of the infected hosts had light infections (Table 3) with filamentous trophonts observed using light microscopy, although no correlation between the prevalence and the intensity of infection was observed over the sampling period.

Table 2. Locations and prevalence (Prev.) of *Hematodinium* infections in marine crustaceans sampled along the coast of China. The status of *Hematodinium* infection in wild crabs was diagnosed with a specific PCR assay; the status of the cultured animals was screened with the hemolymph smear assay and further diagnosed with the specific PCR assay. Dates are given as mo/d/yr

Host species	Sampling site	Sampling date	Source	n	Prev. (%)
Portunus trituberculatus	Laizhou, Shandong	4/25/2014	Wild	22	68.2
	Huludao, Liaoning	4/28/2014	Wild	34	29.4
	Huanghua, Hebei	5/16/2014	Wild	32	0
	Dongying, Shandong	5/17/2014	Wild	30	0
	Qingdao, Shandong	4/21/2014–5/30/2014	Wild	48	33.3
	Zhoushan(1), Zhejiang	4/19/2014	Wild	30	53.3
	Zhoushan(2), Zhejiang	5/21/2014	Wild	25	32.0
	Zhoushan(3), Zhejiang	6/22/2014	Wild	29	48.3
	Yangtze River Estuary	4/30/2015–5/7/2015	Wild	116	0
	Qingdao, Shandong	7/14/2015	Cultured	18	27.8
	Qingdao, Shandong	8/3/2015–8/5/2015	Cultured	170	34.1
	Yangtze River Estuary	11/15/2015–11/22/2015	Wild	120	0
Scylla paramamosain	Shantou, Guangdong	10/29/2013	Wild	70	8.6
Charybdis japonica	Zhoushan, Zhejiang	11/12/2013	Wild	65	0
	Qingdao, Shandong	1/16/2014	Wild	23	0
Penaeus monodon	Qingdao, Shandong	7/31/2014	Cultured	24	29.2

Table 3. Prevalence (Prev.) and intensity of *Hematodinium* sp. infections in cultured Chinese swimming crabs *Portunus trituberculatus* collected from a polyculture pond in Qingdao, Shandong province, along with corresponding temperature (Temp.) and salinity (Sal.) values. Dates are given as mo/d/yr

Sampling date	Size range (mm)	n	Intensity			Prev. (%)	Temp. (°C)	Sal. (‰)
			Light	Moderate	Heavy			
5/27/2014	8–17	36	0	0	0	0	25	29.6
6/13/2014	10–25	40	15	0	0	37.5	27	29
6/27/2014	20–52	11	6	0	0	54.5	28	29.4
7/11/2014	46–89	50	29	9	7	90	28.8	30.2
7/31/2014	75–108	33	21	3	1	75.8	29	29.5
8/21/2014	120–163	49	7	5	2	28.6	29.2	27.4
9/19/2014	136–172	97	17	8	3	28.9	29.4	28.5
9/30/2014	145–178	75	0	1	2	4	29	29.5

Molecular identification and phylogenetic analysis of *Hematodinium* sp. infecting *Penaeus monodon*

The unique ITS 1 amplification products (~298/299 bp) were successfully acquired from DNA samples of the giant tiger prawns *Penaeus monodon* collected from the polyculture pond in Jiaonan, Qingdao. The resulting sequences (GenBank accession nos. KX758131 to KX758138) were 99.2% similar to the ITS 1 genes of *H. perezi* infecting *Portunus trituberculatus* (GenBank accession nos. EF173452 to EF173454), *Scylla paramamosain* (GenBank accession no. EF173451) and *Exopalaemon carinicauda* (GenBank accession no. KX758127) reported in China (Small et al. 2012, Li et al. 2013). Further comparisons revealed a similarity of 98.3% between the *Hematodinium* sp. derived from *Penaeus monodon* and *H. perezi* infecting *Liocarcinus depurator* in Europe (GenBank accession nos. EF153725 to EF153728, EF065712 to EF065715), and 97.6% to *H. perezi* infecting *Callinectes sapidus* in North America (GenBank accession nos. DQ925227 to DQ925230, FJ844430, FJ844431).

Similar to the phylogenetic analysis reported by Small et al. (2012), these ITS 1 sequences formed 3 distinct clades of *H. perezi*. The sequences of *Hematodinium* sp. reported from China (from the present study and previously published studies) formed a monophyletic clade (*H. perezi* genotype II), sister to the other 2 clades representing *H. perezi* genotypes I and III (Fig. 2).

Life stages of *Hematodinium* sp. isolated from *Portunus trituberculatus*

Three isolates of *Hematodinium* sp. from infected *Portunus trituberculatus* were cultured for 40 to 60 d in laboratory. Although not all isolates completed the entire developmental cycle as described by Appleton & Vickerman (1998) and Li et al. (2011), the typical stages, including filamentous trophont, ameboid trophont, arachnoid trophont, arachnoid sporont, schizonts, clump colony, prespore and dinospore, were observed in these cultures or in the hemolymph of infected hosts. Filamentous and ameboid trophonts were commonly observed in the hemolymph of hosts with light to moderate *Hematodinium* infection. Filamentous trophonts (Fig. 3A) had irregular shapes and sizes, and showed slow motility in hemolymph and *in vitro* cultures. Although the ameboid trophonts (Fig. 3B) were spherical or oval in

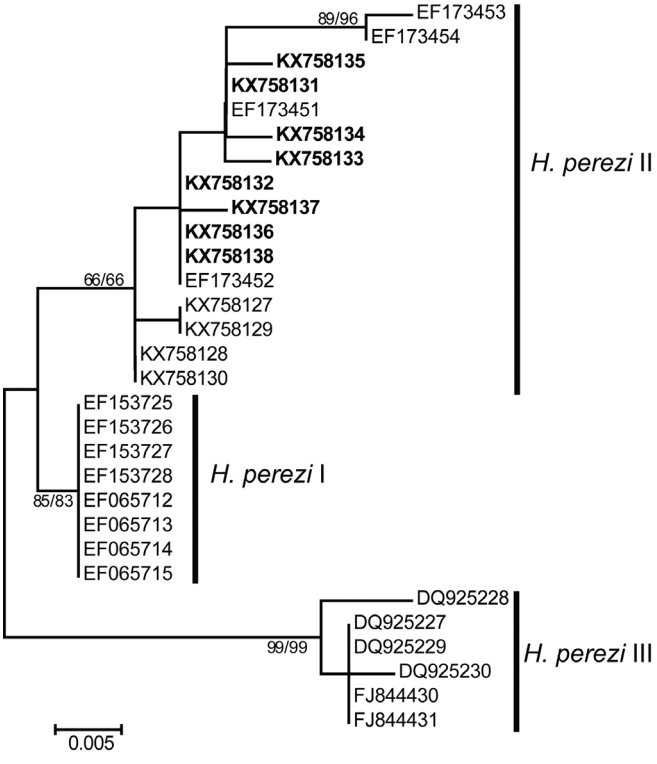

Fig. 2. Molecular phylogenetic analysis of *Hematodinium* sp. infecting *Portunus trituberculatus* and *Penaeus monodon* in the polyculture pond located in Qingdao, Shandong province, based on the sequences of the first internal transcribed spacer (ITS1) of rDNA. I, II and III represent the genotypes I, II and III of *H. perezi*, respectively. The analysis was conducted with the maximum likelihood method in the MEGA5 software. Numbers at nodes represent bootstrap support values calculated with the maximum likelihood (before slash) and neighbor-joining (after slash) methods. Sequences in bold were amplified in the present study, the others were downloaded from GenBank

shape, they were not motile in hemolymph smears or cultures. After filamentous and ameboid trophonts adhered to the bottom of the culture flasks, arachnoid trophonts (Fig. 3C,D) were observed. The arachnoid trophont stage was the primary stage for rapid amplification of the parasite in *in vitro* cultures, but it was not observed in the circulating hemolymph of infected hosts.

The clump colonies (Fig. 3E) were formed by aggregation of trophonts adhering to the bottom of the culture flask. They were also observed in the hemolymph of diseased crabs with moderate to heavy infections and occasionally in histological observation. It is likely the primary stage of proliferation around the hepatopancreas. Schizont-like stages (Fig. 3F) were observed after approximately 35 d in culture, along with the formation of clump colonies. Schizonts are cells with large vacuoles as well as peripheral cytoplasm and a nucleus, ranging from approximately 20 to 40 µm in size. Many mobile coccoid granules were found inside some bigger schizont-like cells. Schizont-like stages were not observed in the hemolymph of infected hosts. Two forms of sporonts, adherent arachnoid sporonts (Fig. 3G) and non-adherent sporoblasts (Fig. 3H), were found in cultures. The non-adherent sporoblasts were similar to the ameboid trophonts, but they were larger and more rounded. The sporoblasts can develop into prespores, the transitional stage before development into dinospores. In late stage *Hematodinium* infections, the hemolymph of infected hosts was overtaken by massive numbers of ameboid cells. These ameboid cells had similar morphology to ameboid trophonts but showed limited uptake of neutral red and were considered to be sporoblasts or prespores as described by Li et al. (2011).

DISCUSSION

Since 2004, sporadic outbreaks of *Hematodinium perezi* genotype II have been reported in several important aquaculture species in China (Xu et al. 2007a,b, 2010, Y. Li et al. 2008, C. Li et al. 2013). The pathogen has gained much attention as an emerging infectious disease agent threatening the sustainabil-

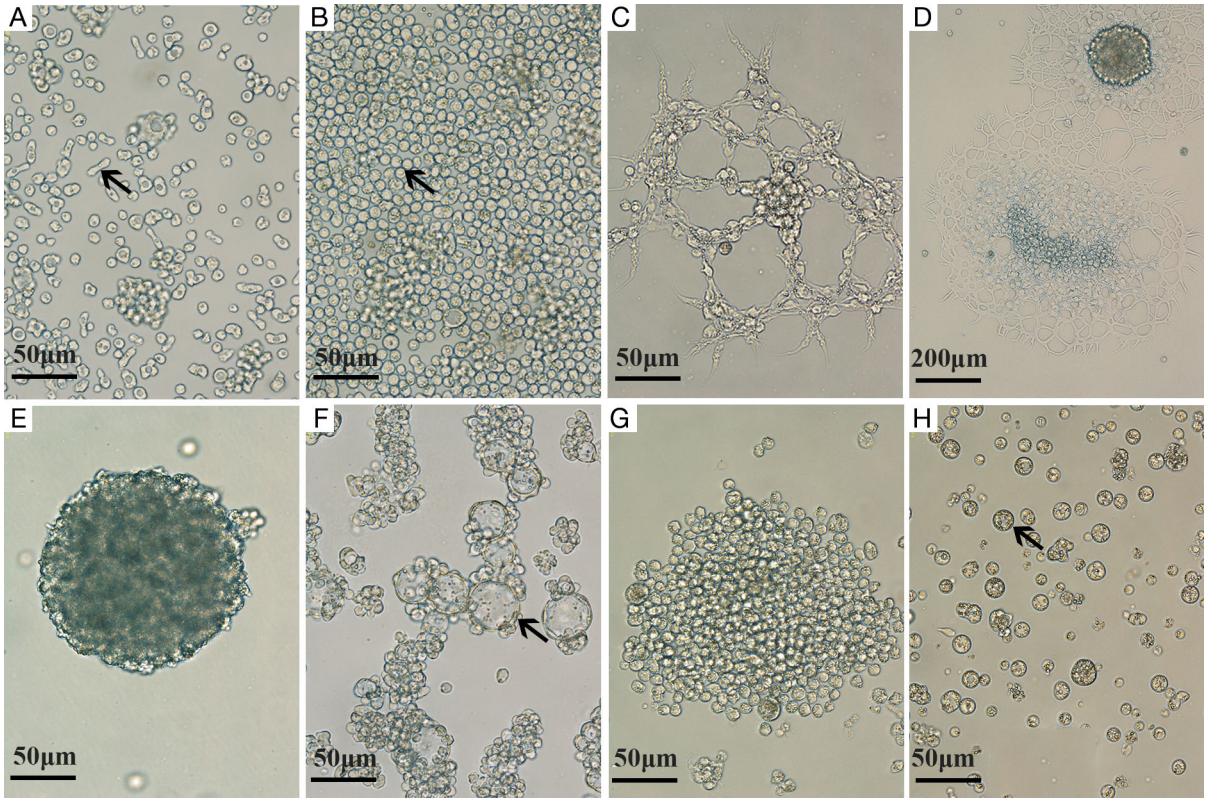

Fig. 3. The *in vitro* life stages of *Hematodinium* sp. isolated from the Chinese swimming crabs *Portunus trituberculatus* in the polyculture pond located in Qingdao, Shandong province. (A) Filamentous trophont (arrow), (B) ameboid trophont (arrow), (C) early stage arachnoid trophont, (D) later stage arachnoid trophont, (E) clump colony, (F) schizont-like stage (arrow), (G) adherent arachnoid sporont, (H) non-adherent sporoblast (arrow)

ity of crustacean aquaculture. In the present study, *H. perezi* genotype II was identified in wild and cultured stocks of crab hosts collected from Huludao, Laizhou, Qingdao, Zhoushan and Shantou, covering the major aquaculture sites of *Portunus trituberculatus* and *Scylla paramamosain* along the coast of China. The prevalence of *Hematodinium* infection ranged from 29.4 to 68.2% in these crab hosts. It is noteworthy that this parasite was also identified for the first time in the giant tiger prawn *Penaeus monodon*, co-cultured with *Portunus trituberculatus* in a polyculture pond in Qingdao, China. The results reveal a broad distribution and further spread of *Hematodinium* infections in marine crustacean species along the coast of China, highlighting its potential threat to the aquaculture and the wild stocks of these commercially valuable crustacean species.

Previous studies have demonstrated that *H. perezi* genotype II infects *Portunus trituberculatus*, *S. paramamosain* and *Exopalaemon carinicauda* in China (Small et al. 2012, Li et al. 2013). In the present study, we observed that a large number of *Penaeus monodon* co-cultured with *Portunus trituberculatus* in a polyculture pond were dead or moribund during the outbreak of *Hematodinium* disease. Even though it is not clear whether *Hematodinium* infections directly caused the death of the penaeid shrimps, the parasite did cause massive mortality of *Portunus trituberculatus* in the polyculture pond, and it kills crab hosts over 40 to 80 d in other systems (Messick & Shields 2000, Shields & Squyars 2000). Our preliminary molecular analysis indicated that the partial ITS 1 sequences of the *Hematodinium* isolated from *Penaeus monodon* clustered with genotype II reported from other crustacean hosts in China. That is, the parasite is the same species and was likely transferred to the shrimp within the polyculture pond. Similar to other geographic strains (Hamilton et al. 2009, Pagenkopp Lohan et al. 2012), the high similarity within the molecular sequences of *Hematodinium* parasites isolated from the 4 species of marine crustaceans in China suggests that the *Hematodinium* sp. is a host generalist, capable of infecting hosts in different families within the Order Decapoda.

Species of *Hematodinium* have overt seasonality in the wild stocks of other commercially valuable crustacean species. The seasonal prevalence of *Hematodinium* sp. in the American blue crab *Callinectes sapidus* peaks in late autumn, followed by a weak peak in the late spring of the following year (Messick 1994, Messick & Shields 2000, Sheppard et al. 2003, J. D. Shields & C. W. Li unpubl. data). *Hematodinium* sp. infections in the Alaskan Tanner crab, *Chionoecetes bairdi*, peaks in summer, declines in winter and increases again in spring (Eaton et al. 1991, Love et al. 1993), whereas in France, *H. perezi* genotype I causes mortality in the velvet swimming crab *Necora puber* in winter, with prevalence peaks in May and June (Wilhelm & Mialhe 1996). Our field survey did not indicate a clear seasonal pattern of *Hematodinium* infection due to the broad geographical range and the limited samples. However, infections of *H. perezi* genotype II were observed within 4 wk after introduction of juvenile crabs into the polyculture systems, and the prevalence peaked approximately 8 wk (sampled on July 11, 2014) after their exposure (Table 3). The period of *Hematodinium* disease development in the polyculture system was roughly equal to the *in vitro* life cycle of *Hematodinium* sp. isolated from *Callinectes sapidus* (Li et al. 2011). *Hematodinium* infections were consistently diagnosed in sampled crabs during the period of crab culture in the polyculture ponds (approximately 7 mo). Clearly, transmission of *Hematodinium* infections to susceptible hosts occurred in the polyculture system.

Polyculture pond systems, culturing ecologically complementary species together in 1 pond, have been widely applied in the mariculture industry (Wang et al. 2004, Chang & Chen 2008). For example, the Chinese swimming crab *Portunus trituberculatus* has been commonly cultured with penaeid shrimps (e.g. *Penaeus chinensis*, *P. japonica*, *P. monodon*) and even with bivalves (e.g. *Ruditapes philippinarum*, *Argopecten irradians*) in Shandong and Jiangsu provinces (Chang & Chen 2008). Similarly, *Portunus trituberculatus* or *S. paramamosain* have been co-cultured with ridgetail white prawn *E. carinicauda* in Zhejiang province (Xu et al. 2010), and *S. paramamosain* with *Penaeus monodon* and flathead mullet *Mugil cephalus* in Guangdong province (Han 2011). Xu et al. (2010) documented the first occurrence (in 2008) of *Hematodinium* infections in ridgetail white prawns *E. carinicauda* cultured with *Portunus trituberculatus*. Here we report, for the first time, *H. perezi* genotype II from *Penaeus monodon* cultured with *Portunus trituberculatus* in a polyculture pond. Thus, although these polyculture systems can maximize productivity with limited pond space and nutritional load, such intensive systems can be vulnerable when infectious pathogens, especially host generalists such as *H. perezi* genotype II, are introduced into the system. The crowded nature of these systems likely facilitates prompt transmission of the parasite among susceptible hosts.

The transmission route of *Hematodinium* spp. in the polyculture system has not yet been solved. In

Shandong province, the polyculture ponds are generally idle from the end of December until the end of April in the following year, with no crabs or other commercial fishery species being cultured. During the idle season, the ponds are usually renovated by cleaning out sludge, ploughing the subsoil and long-term exposure to the sun, i.e. solarization, and then treated with chlorinated lime or chlorine dioxide to sterilize intake seawater and remaining sludge. After cleaning, early juvenile instars of *Portunus trituberculatus* and *Penaeus monodon* are then introduced into the ponds at the end of May, and cultured until late November or early December when crabs reach market sizes. During the culture season, *R. philippinarum*, artificial baits and small fishes are fed to the crabs and shrimps, and seawater is changed biweekly or monthly, synchronized with the local tidal range. The dinospore, the infectious stage of *H. perezi*, is likely introduced into the polyculture system when the crabs are fed fresh, live baits or via intake of contaminated seawater. The juveniles of susceptible hosts undergo frequent molting, which increases their chance of exposure to the infectious stages of the *Hematodinium* parasite (Shields & Squyars 2000, Stentiford et al. 2001, Stentiford & Shields 2005). The alternate hosts of *Hematodinium* may play important roles in transmission and spread of the disease in wild populations of marine crustaceans (Stentiford & Shields 2005, Small & Pagenkopp 2011, Pagenkopp Lohan et al. 2012). Thus, the role of alternate hosts, particularly amphipods and decapods, during transmission of *Hematodinium* parasites in the polyculture system needs to be further investigated.

The life cycle of *Hematodinium* spp. is not yet fully understood, and only the *in vitro* life cycle of *Hematodinium* sp. infecting Norway lobster *Nephrops norvegicus* and the American blue crab *Callinectes sapidus* has been described comprehensively (Appleton & Vickerman 1998, Li et al. 2011). In the present study, *in vitro* cultures of *Hematodinium* sp. from *Portunus trituberculatus* were established and cultured for up to 2 mo in the laboratory. Most of the typical life stages described in previous studies (Appleton & Vickerman 1998, Li et al. 2011, Gaudet et al. 2015) were observed, including filamentous trophont, ameboid trophont, arachnoid trophont, arachnoid sporont, schizont, clump colony, sporoblast, prespore and dinospore. The dinospores of *Hematodinium* sp. isolated from *C. sapidus* can survive for up to 7 d in seawater with a salinity of 20 to 35, and are speculated to be the major transmission stage of the parasite (Stentiford & Shields 2005, Li et al. 2010). We also observed the presence of motile dinospores in *in vitro* cultures as well as in the affected crabs with late stage *Hematodinium* infections. Although the dinospores are likely the infectious stage, their role in transmission and spread of the disease in polyculture systems still needs to be assessed.

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