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PROOF RESEARCH PAPER

Prevalence of *Marteilia* spp. in Thirteen Shellfish Species Collected from China Coast

Liji Xie[1], Zhixun Xie[1,*]

[1] Guangxi Veterinary Research Institute, Department of Biotechnology, Guangxi Key Laboratory of Veterinary Biotechnology, 51 You Ai North Road, Nanning, Guangxi 530001, China.

* Corresponding Author: Tel.: 86.771 3120371;
E-mail: xiezhixun@126.com

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Abstract

Marteilia spp. is listed as a notifiable parasite by the Office International des Epizooties (OIE), and cause physiological disorders and eventually death of the shellfish. Little is known about the prevalence of *Marteilia* spp. in shellfish of China. Therefore we investigated the prevalence of *Marteilia* spp. in 13 species of shellfish collected in the coastal areas of China between 2006 to 2012. A total of 11,581 individual shellfish comprising 13 different species was collected from seven bay areas in China. The shellfish was tested for the prevalence of *Marteilia* spp. following a PCR protocol recommended by OIE. *Marteilia* spp. was found in the shellfish samples (*Crassostrea rivularis*, *Crassostrea gigas* and *Crassostrea angulata*), *Meretrix meretrix*, *Chlamys nobilis*, *Ruditapes philippinarum*, *Perna viridis*, *Tegillarca granosa*, and *Sinonovacula constricta*. The detetion rates of *Marteilia* spp. by PCR method ranged from 0% to 6.67%, with *R. philippinarum* (6.67%) showing the highest detection rate. *Marteilia* spp. sequence of the individual positive shellfish had a high sequence homology (97.4% to 99.6%) with the sequences of other *Marteilia* type "M". Mixed positive of *Marteilia* spp. with other protozoan species (*Haplosporidium nelsoni* and *Perkinsus* spp.) was a common phenomenon (26.28%).

Keywords: Shellfish, Marteilia spp., PCR detection, Chinese coast.

Introduction

In China, the total area of 1,409,000 hectares was used for an annual shellfish production of 12,666,500 tons in the year of 2011, which is up to 60% of the total global output (Ministry of Commerce of the People's Republic of China, http://policy.mofcom.gov.cn/export/shellfish/c1.action). Oysters are the main shellfish product generated in China. A breeding area of more than 116,000 hectares is employed, and more than 3,600,000 tons are produced annually in China, representing approximately 80% of the global oyster output. In recent years, the annual production of shellfish has been declining due to environmental changes and the occurrence of diseases. *Marteilia* spp. infection poses a serious threat to the coastal ecosystem and the supply of shellfish worldwide (Berthe, Le Roux, Adlard, & Figueras, 2004; Villalba, Mourelle, Carballal, & Carmen lopez, 1993).

Marteilia spp. is listed as a notifiable parasite by the Office International des Epizooties (OIE). *Marteilia* spp. is a protozoan parasite resulting in physiological disorders and eventually death of the animal (Feist, Hine, Bateman, Stentiford, & Longshaw, 2009; Berthe *et al*., 2000). This protozoan parasite is distributed over a wide range of areas, including Oceania and Europe around Albania, Croatia, France, Greece, Italy, Morocco, Portugal, Spain, Sweden, Tunisia, China and the United Kingdom (Wang, Lu, & Liang, 2012; Lopez-Flores *et al*., 2008; Audemard *et al*., 2004; Kleeman, Le, Berthe, & Adlard, 2002). *Marteilia* spp. infection has been identified in many types of mollusks throughout the world, including oyster species (Villalba, 1993), mussel species (Wang, Lu, & Liang, 2012; Le Roux *et al*., 2001) and clam species (Lopez-Flores *et al*., 2008).

However, little has been known about the prevalence of *Marteilia* spp. in shellfish in the coastal areas of China. In this present study, the prevalence of *Marteilia* spp. in 13 shellfish species (*Crassostrea rivularis*, *Crassostrea gigas*, *Crassostrea angulata*, *Meretrix meretrix*, *Chlamys nobilis*, *Ruditapes philippinarum*, *Perna viridis*, *Tegillarca granosa*, *Sinonovacula constricta*, *Haliotis discus hannai*, *Paphia undulate*, *Tapes dorsatus* and *Coelomactra antiquate*) cultured in seven bay areas in China were investigated using a PCR protocol described by Le Roux *et al*. (Le Roux *et al*., 2001) and this PCR

method is also recommended by OIE.

C. rivularis is the major oyster species in Zhanjiang, Haikou and Qinzhou, and *C. gigas* is the major oyster species in Dalian and Qingdao, while *C. angulata* is the major oyster species in Xiamen. We therefore analyzed the prevalence of *Marteilia* spp. in these three different oyster species (*C. rivularis*, *C. gigas* and *C. angulata*) sampled from various locations (Dalian, Qingdao, Wenzhou, Xiamen, Zhanjiang, Haikou and Qinzhou) and in different seasons (spring, summer, autumn and winter).

Materials and Methods

Sample Collection

Samples of the 13 shellfish species were collected between 2006 and 2012 from seven bay areas in China: Dalian (Bohai Sea), Qingdao (Yellow Sea), Wenzhou (East China Sea), Xiamen (East China Sea), Zhanjiang (South China Sea), Haikou (South China Sea) and Qinzhou (South China Sea) bays (Figure 1). A total of 11,581 shellfish samples were collected for diagnosis, and their specific details are shown in Table 1. A total of 5,609 oyster samples were collected. To find out the differentiation of the prevalence of *Marteilia* spp. among different oyster species, at different locations and in different seasons, these 5,609 oyster samples was collected from different oyster species at different locations within four seasons. In terms of oyster species, these 5,609 oyster samples include 3,397 *C. rivularis*, 1,351 *C. gigas* and 861 *C. angulata*. Regarding to the locations, these 5,609 oyster samples include 694 oyster (*C. gigas*) of Dalian, 657 oyster (*C. gigas*)of Qingdao, 597 oyster (122 *C. angulata* + 475 *C. rivularis*) of Wenzhou, 739 oyster (*C. angulata*) of Xiamen, 540 oyster (*C. rivularis*) of Zhanjiang, 454 oyster (*C. rivularis*) of Haikou and 1,928 oyster (*C. rivularis*) of Qinzhou. And when counted by different seasons, these 5,609 oyster samples include 1,432 in spring, 1,326 in summer, 1,384 in autumn and 1,467 in winter).

DNA Extraction

Genomic DNA was extracted from the excised tissue samples (gill and digestive gland together) using EasyPure® Marine Animal Genomic DNA Kit as described by Xie (Xie *et al*., 2013). The tissue samples were frozen in liquid nitrogen for 3 min and then ground into powder. The powder was then digested overnight with proteinase K at 56°C, and the genomic DNA was extracted.

PCR, Cloning, and Sequencing

PCR is performed with primers targeting the ITS1 (internal transcribed spacer) region (Pr4:5'-CCG-CAC-ACG-TTC-TTC-ACT-CC-3' and Pr5: 5'-CTC-GCG-AGT-TTC-GAC-AGA-CG-3'), which were also the OIE recommended primers and expected to amplify a 412 base pairs (bp) DNA fragment. Deionized, nuclease-free water was used as a negative control. The PCR reaction solution consisted of 15 µl of 2×PCR mix[a], 0.2 µM of each primer, and 3 µl of the purified genomic template DNA. A denaturation step at 94°C for 10 min was followed by 35 cycles consisting of a denaturation step at 94°C for 1 min, an annealing step at 55°C for 1 min and an extension step at 72 °C for 1 min. After the final extension at 72°C for 10 min, PCR products were analyzed by 1% agarose gel electrophoresis followed by ethidium bromide staining. The results were visualized under UV light.

Individual positive samples from *C. rivulari*, *C. nobilis*, *R. philippinarum*, *P. viridis*, *T.granosa* and *S. constricta* were selected and again amplified under the conditions similar to those of the screening PCR, except for the composition of the PCR mixture. The total volume of each reaction was increased to 50µl, including 25µl of 2×PCR mix , 0.3 µM of each primer, and 5 µl of the purified genomic DNA as a template. The amplified PCR products were gel-extracted using a Gel Extraction Kit, and were ligated into pMD18-T cloning vector, respectively. Plasmids with the inserted DNA fragment were then transformed into competent *E. coli*. DH5α cells. After an overnight incubation, single colonies were inoculated in liquid culture and incubated at 37°C overnight with shaking. The recombinant plasmids for the individual positive sample were analyzed and verified by the PCR method described above.

Phylogenetic Analysis

The recombinant plasmid DNA for the individual positive sample was sequenced, and the sequence data were analyzed using DNAStar software. The sequences were subjected to phylogenetic analysis against the known *Marteilia* spp. sequences deposited in GenBank.

Mixed Positive of Marteilia spp. with Other Protozoans

To investigate if *Marteilia* spp.-positive shellfish were also mixed positive with *Haplosporidium nelsoni*, *Perkinsus* spp., and *Bonamia ostreae*, 156 *Marteilia* spp.-positive shellfish samples were also tested for these three parasites by PCR as previously described (Casas, Villalba, & Reece, 2002; Carnegie, Barber, Culloty, Figueras, & Distel, 2000; Day, Franklin, & Brown, 2000). The primers used in the PCR amplifications are listed in Table 2.

Results

Prevalence of Marteilia spp. in 13 Shellfish Species

A total of 11,581 shellfish was analyzed in this

Table 1. Prevalence of *Marteilia spp.* in 13 shellfish species in China

Shellfish species	No. of shellfish tested	No. of positive sample	Detection rate	No. of mixed positive	Mixed positive rate*
Oyster (*C. rivulari*)	3,397	14	0.42%	5	35.71%
Oyster (*C. gigas*)	1,351	2	0.15%	0	0
Oyster (*C. angulata*)	861	17	1.97%	5	29.41%
Oyster (Total)	5,609	33	0.59%	10	30.30%
M. meretrix	1,026	2	0.19%	0	0
C. nobilis	1,012	9	0.89%	0	0
R. philippinarum	1,005	67	6.67%	23	34.33%
P. viridis	513	3	0.58%	0	0
T. granosa	506	11	2.17%	2	18.18%
S. constricta	603	31	5.14%	6	19.35%
Haliotis discus hannai	521	0	0	0	0
P. undulata	569	0	0	0	0
T. dorsatus	107	0	0	0	0
C. aniquate	110	0	0	0	0
Total	11,581	156	1.35%	41	26.28%

Table 2. Primers used for PCR detection of pathogens*: Haplosporidium nelsoni, Perkinsus spp.* and *Bonamia ostreae*

Parasites	Primers	Sequences (5'—— 3')	Length of PCR products (bp)	Reference
Haplosporidium nelsoni	MSX-A	CGACTTTGGCATTAGGTTTCAGACC	573	11
	MSX-B	ATGTGTTGGTGACGCTAACCG		
Perkinsus spp.	PerkITS-85	CCGCTTTGTTTGGATCCC	703	10
	PerkITS-750	ACATCAGGCCTTCTAATGATG		
Bonamia ostreae	CF	CGGGGGCATAATTCAGGAAC	760	7
	CR	CCATCTGCTGGAGACACAG		

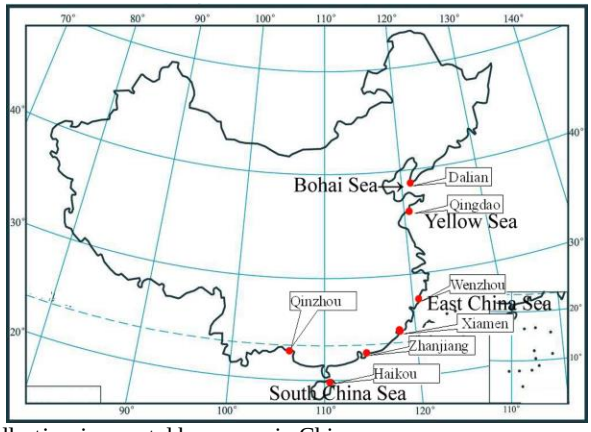

Figure 1. Sites for shellfish collection in coastal bay areas in China.

study, of which 156 (1.35%) were *Marteilia* spp. positive by PCR detection. The detection rate in the 13 shellfish species tested ranged from 0% to 6.67% (Table 1 and Figure 2). *R. philippinarum* (6.67%) and *S. constricta* (5.14%) showed the highest detection rate, whereas no *Marteilia* spp. positive was detected in *Haliotis discus hannai, P. undulate, T. dorsatus* or *C. antiquate* samples. The presence of *Marteilia* spp. in the other five shellfish species was lower than 2.17%.

Prevalence of Marteilia spp. in Three Different Oyster Species

A proportion of *C. rivularis*, *C. gigas* and *C. angulata* was *Marteilia* spp. positive**.** The detection rate of these three oyster species ranged from 0.15% to 1.97% (Table 1). *C. angulata* samples showed the highest detection rate (1.97%), whereas *C. gigas* was the least positive oyster species.

Prevalence of Marteilia spp. in the Oysters from Different Locations and Season

The detection rate of oysters from different locations ranged from 0% to 2.30% (Table 3). *Marteilia* spp. positive samples were not present in the seawaters of Dalian, Wenzhou, Zhanjiang and Haikou bay areas in China. The oysters (*C. angulata*) in Xiamen bay (2.30%) had the highest detection rate of *Marteilia* spp. among the seven tested bay areas. There is no significant difference in the detection rate of *Marteilia* spp. in different seasons (data not shown).

Phylogenetic Analysis

The *Marteilia* spp. sequence of the individual positive sample from *C. rivulari, C. nobilis, R. philippinarum, P. viridis, T. granosa* and *S. constricta* had a high sequence homology (97.4% to 99.6%) with the sequences of other *Marteilia* type "M" from GenBank (Figure 3).

Mixed Positive of Marteilia spp. with Other Protozoan Parasites

Among all 11,581 shellfish samples tested, 156

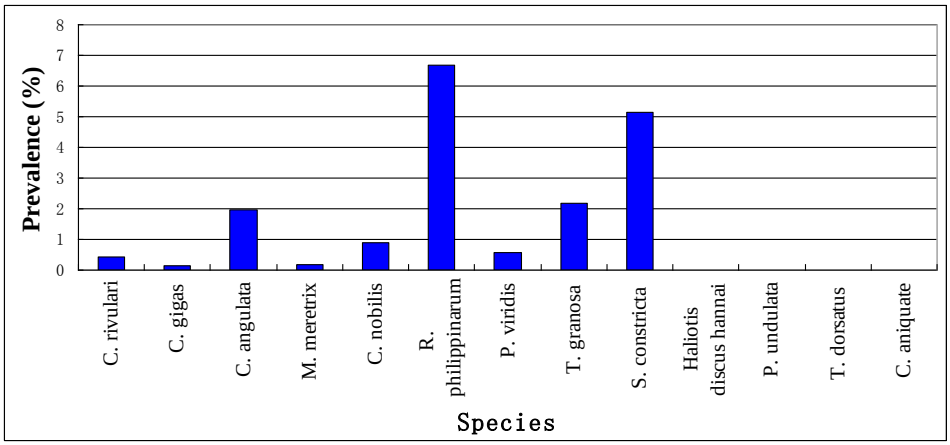

Figure 2. Prevalence of *Marteilia* spp. in 13 shellfish species.

Table 3. Prevalence of *Marteilia* spp. in the oysters form different locations

Location (species)	No. of oysters tested	No. of positive samples	Detection rate
Dalian (*C. gigas*)	694	0	0
Qingdao (*C. gigas*)	657	2	0.30%
Wenzhou (122 *C. angulata*+ 475 *C. rivularis*)	597	0	0
Xiamen (*C. angulata*)	739	17	2.30%
Zhanjiang (*C. rivularis*)	540	0	0
Haikou (*C. rivularis*)	454	0	0
Qinzhou (*C. rivularis*)	1,928	14	0.73%
Total	5,609	33	0.59%

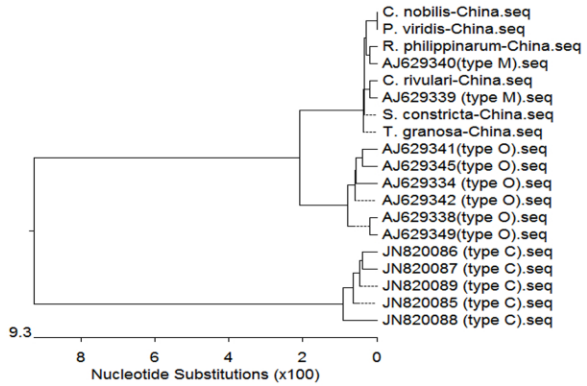

Figure 3. Phylogenetic sequences analysis of *Marteilia* spp. isolates.

shellfish were *Marteilia* spp. positive, and 41 samples (10 Oysters, 23 *R. philippinarums*, 6 *S. constrictas* and 2 *T. granosa*) were mixed positive, resulting in a mixed positive rate of 26.28% (41/156, Table 1). As shown in Table 4, 30 samples were mixed positive for *Marteilia* spp. with *Perkinsus spp.*(mixed positive rate: 19.23%, 30/156). Three samples were mixed positive for *Marteilia* spp. with *Haplosporidium nelsoni (*mixed positive rate: 1.92%, 3/156). Eight samples were mixed positive for *Marteilia* spp. with *Perkinsus spp.* and *Haplosporidium nelsoni* (mixed positive rate: 5.13%, 8/156). None of the samples were mixed positive for *Marteilia* spp. with *Bonamia ostreae*. Mixed positive rate for *Marteilia* spp. with *Perkinsus* spp. (19.23%) was the highest among all tested samples.

Discussion

R. philippinarum (6.67%) and *S. constricta*(5.14%) are the highly positive shellfish species among *Marteilia* spp. positive species (*C. rivularis*, *C. gigas* and *C. angulata*, *M. meretrix*, *C. nobilis*, *R. philippinarum*, *P. viridis*, T. *granosa* and *S. constricta*) in the tested seven bay areas in China. To our knowledge, this study is the first report on the prevalence of *Marteilia* spp. in oyster (*C. rivularis*, *C. gigas* and *C. angulata*), *M. meretrix*, *C. nobilis*, *R. philippinarum*, *T. granosa* and *S. constricta* in China. Previous studies have reported the detetion rate of *Marteilia* spp. in Mussel in China to be 2.8% (5/180) (Wang, Lu, & Liang, 2012). Little is known about mortalities or other pathological effects of *Marteilia* spp. infection on oysters, *M. meretrix*, *C. nobilis*, *R. philippinarum*, *P. viridis*, *T. granosa* and *S. constricta* in the seven bay areas in China, as have been documented for infection of *Ostrea edulis* and *Mytilus edulis* and *Mytilus galloprovincialis* in other parts of the world (Itoh, Momoyama, & Ogawa, 2005; Lopez-Flores *et al*., 2004; Fuentes *et al*., 2002; Villalba, 1993). Therefore, these need to be studied in the future.

Marteilia spp. prevalence is highly variable – up to 98% in *O. edulis*, and higher prevalence is expected depending on farming practices and in the areas that have had more than one year of exposure to infection (Feist, Hine, Bateman, Stentiford, & Longshaw, 2009; Berthe, Le Roux, Adlard, & Figueras, 2004). Stocking at low density has been shown to be effective on the control and prevention of *Marteilia* spp. Infection (Feist, Hine, Bateman, Stentiford, & Longshaw, 2009). Copepod *Paracartia grani* (Copepoda, Calanoida) could contribute to the transmission of *Marteilia refringens* (Arzul *et al*., 2013; Carrasco *et al*., 2008; Audemard *et al*., 2002). *Mecocyclops*, *Euchaeta concinna*, *Calanus sinicus* (Copepoda, Calanoida), *Paracalanus parvus* (Copepoda, Calanoida), *Calocalanus pavo* (Copepoda, Calanoida), *Phyllodiaptomustunguidus* (Copepoda, Calanoida), etc., have been reported in China. However, to our knowledge, no study has been reported on the *Paracartia grani* in China. The general husbandry practices are usually stocking at high density in China, but *Marteilia* spp. has the low prevalence in Chinese coastal bays, which may due to lack of intermediate host *Paracartia grani* (or *Paracartia grani* is not the dominant species) in these seven bay areas of China, so it needs to be further studied.

The detection rates of *Ostrea edulis*, *Mytilus edulis and Mytilus gulloprovincicillis* are up to 98%, 40% and 5% (Feist, Hine, Bateman, Stentiford, & Longshaw, 2009; Berthe, Le Roux, Adlard, & Figueras, 2004; Le Roux *et al*.,2001), respectively. But the detection rates among three oyster species in China are very low, ranging from 0.15% (*C. gigas*) to 1.97% (*C. angulata*), which suggests that the oyster species *Ostrea edulis* and *Mytilus edulis* may be more suitable for *Marteilia* spp. than other oyster species. The detection rates of oyster also different among the seven tested locations, ranging from 0% (Dalian, Wenzhou, Zhanjiang and Haikou) to 2.30% (Xiamen). *Marteilia* spp. positive was detected in Qingdao (0.30%), Xiamen (2.30%) and Qinzhou (0.73%), and Xiamen (2.30%) had the highest prevalence in the seven bay areas tested. These results suggest that the aquatic conditions in Dalian, Wenzhou, Zhanjiang and Haikou may not be optimal for the survival of *Marteilia* spp., and the aquatic conditions in Xiamen might provide a suitable environment for *Marteilia* spp. to infect oysters. The biological and environmental factors affecting the prevalence of

Table 4. Mixed positive of *Marteilia* spp. with other protozoan parasites

Shellfish species	Number of case			
	Marteilia spp. + *Perkinsus* spp.	*Marteilia* spp.+ *Haplosporidium nelsoni*	*Marteilia* spp.+ *Haplosporidium* nelsoni + *Perkinsus* spp.	*Marteilia* spp.+*Bonamia ostreae*
C. rivularis	4	1	0	0
C. angulata	5	0	0	0
R. philippinarums	14	1	8	0
S. constrictas	5	1	0	0
T. granosa	2	0	0	0
Total	30	3	8	0
Mixed positive rate	19.23%	1.92%	5.13%	0

Marteilia spp. in oysters should be further investigated.

The *Marteilia* spp. has three types "M", "O" and "C" (Carrasco *et al*., 2012). The *Marteilia* spp. sequence of the individual positive sample from *C. rivulari, C. nobilis, R. philippinarum, P. viridis, T. granosa* and *S. constricta* detected in this study, had a high sequence homology with the sequences of the *Marteilia* type "M". Because the number of the shellfish samples we sequenced was limited, there might be that also *Marteilia* type "O" and "C" in the shellfish of China, indicating further investigation is needed.

In this study, it is the first time to reveal that mixed positive of *Marteilia* spp. *with Perkinsus* spp. or *Haplosporidium nelsoni* was a common phenomenon, and that mixed positive of *Marteilia* spp. with *Perkinsus* spp. was the most common event (19.23%, 30/156). To our knowledge, this is the first report about the mixed positive of *Marteilia* spp. with other protozoan. It is still unknow whether the existence of *Perkinsus* spp. would bring a suitable living condition for *Marteilia* spp.. And more researches are requird to figure out whether these mixed positive observations affect the mortality or survival rate of shellfish.

The prevalence of *Marteilia* spp. in 13 species of shellfish in China is based on the PCR detection. In order to confirm the infection of *Marteilia* spp. inside the tissue of the shellfish in China, histological analyses or *in situ* hybridization will be performed in our further study.

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