

Short communication

Mass Mortality of Cage-cultured Orange-spotted Grouper *Epinephelus coioides* Associated with Renal Sphaerosporosis Caused by *Sphaerospora epinepheli* in South China Sea

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ABSTRACT—In November 2011, the renal sphaerosporosis was found in cage-cultured orange-spotted grouper *Epinephelus coioides* in a fish farm in Guangdong, China. The infected fish exhibited emaciation, anaemia, anorexia, renomegaly, slight splenomegaly, and occasionally skin ulcer. The cumulative mortality reached at 50% to 80% within 2 weeks, when water temperature ranged from 21°C to 27°C. The renal tubules were almost completely occluded by sporogonic pseudoplasmodia of a myxosporean, and mature spores were found in the lumen. Combined with morphological, histopathological, and molecular analyses, *Sphaerospora epinepheli* was suggested as the etiological agent. This is the first report of mortality case of cultured *E. coioides* associated with *S. epinepheli*.

Key words: renal sphaerosporosis, *Sphaerospora epinepheli*, *Epinephelus coioides*, grouper

The orange-spotted grouper *Epinephelus coioides* widely distributing in subtropical and tropical areas, represents an important mariculture species in South China Sea off the coasts of the Guangdong, Hainan, Guangxi, Fujian and Taiwan (Lin, 2012). However, serious diseases are increasingly reported to cause mass mortality of cage-cultured groupers with the rapid development of grouper aquaculture industry in China (Luo *et al*., 2013). Myxozoans represent an important group of pathological agents during the full culture cycle of groupers from fingerlings breeding to marketable fish fattening in China, and species belonging to genera *Henneguya*, *Sinuolinea*, *Ceratomyxa*, *Thelohanellus*, *Sphaerospora* and *Myxidium* have been found from cultured groupers (Chen, 1996; authors' unpublished data).

As a part of ongoing project on the fish myxosporean diversity and myxosporidiosis in South China Sea, the present work describes a *Sphaerospora* species based on morphological, histopathologial and molecular data.

Materials and Methods

From November 5 to 18 in 2011, a total of 116 cultured orange-spotted grouper (body length: 17–22 cm, body weight: 250–350 g) from 3 cage-culture sites in a fish farm, along the coasts of Shuidong town, Guangdong province, China, were randomly collected and transported to the laboratory of South China Sea Fisheries Research Institute (Guangzhou, Guangdong). Thereafter they are maintained in an aerated aquarium for a maximum of 48 h before dissection for parasitological and microbiological investigation. Fish specimens were necropsied by overdose of the anaesthetic MS-222 (Sigma), following recording external signs of the disease. Squash preparations of fresh tissues from kidney, liver, heart, intestine, gallbladder and other internal organs and gill filaments were examined with a compound microscope and photographed by a digital camera (DP20-5, Olympus). Kidney smears were also stained with Giemsa's solution. Fresh myxospores were measured on digital images using ImageJ v.1.44p (Wayne Rasband, http://imagej.nih.gov/ij) calibrated against a digital image of a graticule. Descriptions and measurements of spores (n = 50, four individual fish specimen) were performed following the guidelines of Lom and Arthur (1989). All measurements for myxospores are given as means ± SD (range) in μm unless otherwise stated.

For bacterial investigation, swabs taken from the external ulcerative skin, kidney, spleen and liver were streaked onto tryptic soy agar (TSA), TSA with 5% goat blood, 1.5% NaCl, brain heart infusion agar, Sabouraud's dextrose agar and Lowenstein-Jensen medium. Plates were then incubated at 25°C for 20 days.

For histological characterization, infected renal tissues were fixed in 10% neutral buffered formalin, dehydrated through a graded ethanol series, embedded in paraffin wax and sectioned at 5 μm. Sections were stained with hematoxylin and eosin (H & E).

Infected renal tissues of four individual fish were preserved in 95% ethanol for molecular characterization.

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Genomic DNA was extracted from ethanol-preserved kidney specimen after removing completely remnant ethanol using QIAamp DNA mini kit (Qiagen Inc.). Obtained DNA was dissolved in DNAse-free water and stored in −20°C for the following use. The partial sequence of small subunit ribosomal DNA (SSU rDNA) of the present myxozoan was amplified by the previously reported primer pairs, including 18E/18G, MyxospecF/18R, MyxGP2F-ACT1R (Fiala, 2006; Bartošová *et al*., 2013). PCR was carried out in a 50 μL reaction volume using 20 pmoL of each primer, 1 μL of Takara Ex Taq^{TM} HS (Takara Inc.), 5 μL 10 × PCR buffer (Ex Taq Buffer), 250 μM of each deoxyribonucleotide triphosphate. The reactions were run on an iCycler (Biorad). Amplified target products were gel-purified by Quick Gel Extraction Kit (CoWin Bioscience Inc.). Both strands of the purified PCR product were sequenced using PCR primers and dye termination PCR sequencing in a commercial company (SinoGeno). The obtained partial SSU fragments were assembled and aligned in SeqMan (Lasergen Inc.) and the consensus sequence was submitted to GenBank with accession number KJ939364 and check for the closest relatives by BLAST tool. Variable SSU rRNA regions of all 3 available *S. epinepheli* isolates were delimited by comparison with those of *Sphaerospora truttae*, for which the variable regions have been defined (Holzer *et al*., 2007; Bartošová *et al*., 2013) to identify dissimilar positions. Also, the similarity of all available SSU rRNA sequences of marine *Sphaerospora* species were analyzed by Clustal X (Larkin *et al*., 2007) and BioEdit software (Hall, 1999).

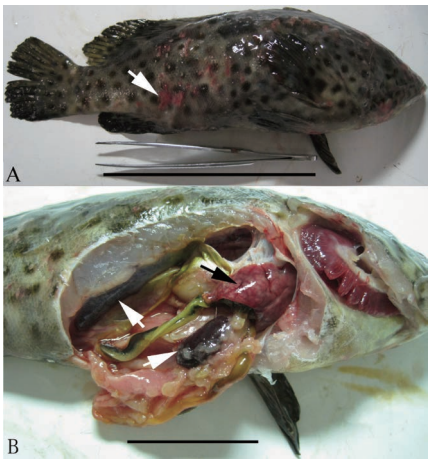

Fig. 1. Gross observation of orange-spotted grouper exhibiting renal sphaerosporosis. **A**: External signs. Note the skin ulcer (arrow), Bar = 15 cm. **B**: Internal signs. Note the renomegaly (white arrow), splenomegaly (arrowhead) and some whitish patchy lesions on the liver (black arrow), Bar = 5 cm.

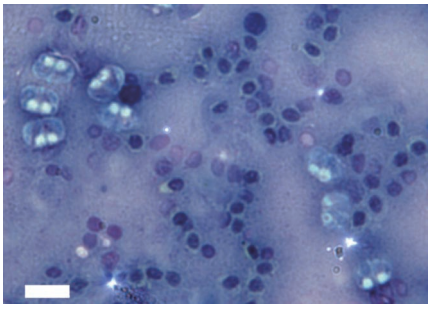

Fig. 2. Mature spores of *Sphaerospora epinepheli* in a kidney smear. Giemsa stain. Scale bar = 20 μm.

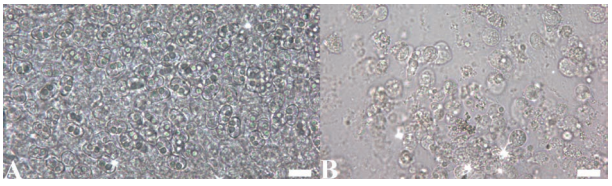

Fig. 3. Fresh spores (A) and developing stages (B) of *Sphaerospora epinepheli* in the kidney. Scale bars = 20 μm.

Results and Discussion

Affected fish exhibited emaciation, anaemia and anorexia, slight splenomegaly and renomegaly, occasionally with skin ulcer. Remarkable whitish patchy lesions were also found on the liver of infected fish. Haemorrhages on the mouth, caudal fins and body surface occurred on some diseased fish. (Fig. 1A & B). The disease occurred when water temperature ranged from 21°C to 27°C. The cumulative mortality in three different cages within the two weeks ranged from 50% to 80%.

General parasitological investigation revealed that low numbers of a monogenean *Pseudorhabdosynochus epinepheli* was generally found in the gills of affected fish, but also in unaffected fish (data not shown). Of the 116 examined fish, renal tubules of 105 specimens (90.5% prevalence) were infected with a myxosporean. Squash preparations of fresh tissues from trunk and posterior kidney showed that renal tubules lumens were heavily filled with mature spores and developing stages of a myxosporean. Both monosporous and disporous pseudoplasmodia were found in the renal tubule lumens (Figs. 2 & 3). The morphological features (Figs. 2 & 3) and measurements (Table 1) of the mature myxospores were almost identical to those of *Sphaerospora epinepheli* in the original description by Supamattaya *et al.* (1991). Some round and irregular vegetative stages, containing some refractile granules were found in wet mount preparation of the kidney (Fig. 3). The myxospores contained two uninucleate sporoplasms (data not shown). Anterior and posterior notches occurred in some mature spores. No remarkable blood stages could be found from blood smears in the infected specimens. This myxosporean was not found in the other external and internal organs. Also, no other myxosporeans such as *Enteromyxum leei* were found

Table 1. Morphological comparison of *S. epinepheli* from the present and previous reports and *S. korean*a from *Sebastes schlegeli*. Measurements were presented as means ± SD (range) if available

Myxosporean	Host	Infection sites	SL (μm)	ST (μm)	SW (μm)	Spore shape	Developmental mode	PC size	Turns of PF	References
S. epinepheli	*Epinephelus malabaricus*	Kidney tubules, Glomerulus	8.7 ± 0.4 (7.8–10.0)	13.4 ± 0.5 (12.3–14.5)	8.2 ± 0.5 (7.0–9.5)	Subspherical, spherical	Monosporous, disporous	3.7 ± 0.3 (2.9–4.4)	6–7	Supamattaya *et al*. (1991)
S. epinepheli	*E. malabaricus*	Kidney tubules, Glomerulus	8.7 ± 0.5	13.5 ± 0.4	8.3 ± 0.6	Subspherical, spherical	Monosporous, disporous	3.6 ± 0.4	6–7	U-taynapun *et al*. (2012)
S. epinepheli	*E. coioides*	Kidney tubules	8.8 ± 0.4	13.4 ± 0.5	8.3 ± 0.5	Subspherical, spherical	Monosporous, disporous	3.6 ± 0.4	6–7	U-taynapun *et al*. (2012)
S. epinepheli	*E. coioides*	Renal tubules	8.6 ± 1.4 (7.9–10.3)	13.4 ± 1.2 (12.1–14.9)	8.1 ± 0.6 (6.9–9.5)	Subspherical	Monosporous, disporous	3.8 ± 0.4 (2.9–4.5)	6–7	The present work
***Sphaerospora koreana* n. comb (Syn. *Leptotheca koreana*)**	*Sebastes schlegeli*	Renal tubules	8.59 ± 1.25	13.42 ± 1.0	8.13 ± 0.52	Broadly oval	Disporous	3.86 ± 0.45	6–7	Cho & Kim (2001)

SL; spore length, ST; spore thickness, SW; spore width, PC; polar capsule, PF; polar filament

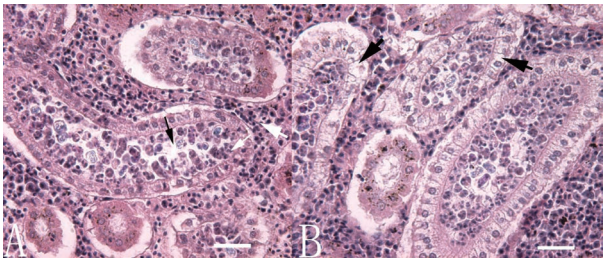

Fig. 4. Histological sections of the kidney of affected orange-spotted grouper. **A:** A large number of spores (black arrow) and developing stages (white arrow) are filled in the renal tubule. Arrowhead shows the occurrence of some inflammatory cells in the kidney interstitium. **B:** Vacuolation (arrowheads) of tubular epithelial cells are conspicuous. Scale bars = 50 μm.

from other examined tissues. Additionally, an unidentified digenetic trematode with eggs was found in the kidney of diseased and clinically healthy specimens, but the infection intensity is low. No bacteria were isolated from the diseased fish.

Histological observation of the kidney of infected *E. coioides* further revealed that large number of mature spores and developmental stages of *S. epinepheli* occluded the renal tubule (Fig. 4), with mature spores generally locating in the centre of lumen and presporogonic stages in the peripheral regions attaching to the brush border of the epithelium of renal tubules (Fig. 4A). Vacuolation of tubular epithelial cells were generally associated with affected tubules and some tubular epithelial cells became necrotic (Fig. 4B). No spores or pseudoplasmodia could be found in renal corpuscles and Bowman's spaces. Numerous inflammatory cells occurred in the interstitial regions of infected kidney. The above histopathological observations suggest that the renal function is inevitably impaired. However, the infection of this myxosporean is not likely responsible for other clinical signs of the infected fish, including skin ulcer and whitish patchy liver. The cause of these lesions was still unknown, because histopathological studies were done only on the kidney. Although *Nocardia* spp. were intensively reported to cause nodules or patchy lesions of the liver of cultured fish in South China Sea, such bacteria were not isolated from the liver of the infected fish. Further studies are necessary to elucidate the pathological mechanisms in the present disease.

The almost full length SSU rDNA sequences (about 2,280 bp) of all clones isolated from four individual fish were almost identical, only with 1–3 bp differences (data not shown). Similarity search by Blast tool showed that the present sequence is the most similar to that of *S. epinepheli* isolates from *E. coioides* (HQ871153) and *E. malabaricus* (HQ871152) with 99.7% homologous over 2,223 bp and 99.4% homologous over 2,218 bp, respectively. Sequence analysis showed that an insertion of "GGTGG" in the V4 E23_15 region of SSU rRNA gene sequence of *S. epinepheli* isolates from *E. malabaricus*, which may differentiate it from *E. coioides* isolates in China and Thailand. This insertion may be used as a potential marker to differentiate these two hosts or locality isolates. The obtained SSU rRNA gene sequence will benefit to the further molecular epidemiological investigation of *S. epinepheli* infection in groupers in South China Sea. Comparisons with freshwater lineages and polysporoplasmid lineages of *Sphaerospora* sensu strict, marine lineages have less expansion segments of SSU rRNA sequences, especially in V4 and V7 regions. The percent identities of the SSU rDNA sequences among all

available marine disporoplasmid *Sphaerospora* sensu stricto were presented in Table 2.

Morphological, molecular and histopathological analyses in the present study indicate that *S. epinepheli* is a probable aetiological agent which caused this mass mortality of cage-cultured *E. coioides* in South China Sea. *S. epinepheli* was firstly recorded in *E. malabaricus* and then *E. coioides* along the coastlines of Thailand and Malaysia (Supamattaya *et al.*, 1990, 1991; U-taynapun *et al.*, 2012). Our study extends the locality record of *S. epinepheli* and is the first report of *Sphaerospora* species in South China Sea. However, it remains unknown whether other grouper species in South China Sea are likewise susceptible to *S. epinepheli*.

Interestingly, morphological comparisons showed that *S. epinepheli* was indistinguishable from *S. koreana* described from *Sebastes schlegeli* in South Korea (Table 1) which was originally named as *Leptotheca koreana* (Cho and Kim, 2001; Gunter and Adlard, 2010). Sphaerosporid species, however, were generally host specific and previous phylogenetic analyses indicated *Sphaerospora* sensu stricto usually clustered according to the host family (Lom and Dyková, 2006; Bartošová *et al*., 2013). Additionally, only disporous (no monosporous) pseudoplasmodia were observed for *S. koreana*. Unfortunately, the molecular data of *S. koreana* is unavailable for genetic and phylogenetic comparison. So, *S. koreana* was here not suppressed to be conspecific to *S. epinepheli* although its validity was highly suspected.

Considering that the grouper seedlings in South China Sea including *E. drummondhayi*, *E. fuscoguttatus*, *E. coioides*, *E malabaricus* and *E. awoara* are becoming an important resources for grouper culture in China, South-East Asia, Taiwan and Japan (Lin, 2012), myxosporean parasites are increasingly representing important pathogens for cage-cultured grouper (China *et al*., 2013). Thus thorough investigation of myxosporean fauna of groupers in South China Sea is of great importance for monitoring the transmission route of grouper myxosporidiosis.

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