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Outbreak of mass mortality of yearling groupers of *Epinephelus* (Perciformes, Serranidae) associated with the infection of a suspected new enteric *Sphaerospora* (Myxozoa: Myxosporea) species in South China Sea

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Abstract

A suspected new enteric *Sphaerospora* species was believed to be directly associated with the mass mortality of yearling groupers of *Epinephelus* spp. in South China. The epizootic generally emerged from late September to late April of the following year. The infection prevalence and mortality rate were significantly negatively correlated with fish size. Clinical signs included anorexia, cachexia and extrusion of white pulp-like substance from anus after gentle pressure on the abdomen. Upon necropsy, severe intestinal oedema, thin and transparent intestinal wall, swollen spleen, kidney and gall bladder could be observed. Wet preparation of the infected samples showed large amount of typical disporous plasmodia of the genus *Sphaerospora*, but no mature spores were observed. Epidemiological investigation showed that this parasite exclusively infected *Epinephelus* groupers. Histopathologically, this species mainly infected the epithelium of intestine and kidney tubules and caused severe epithelia sloughing and the collapse of intestinal villus. Interestingly, this enteric myxosporidiosis did not cause severe emaciation of infected fish for mass mortality usually emerged within 2–3 days after appearance of clinical signs. The species was most genetically related to *Sphaerospora fugu* (89% sequence identity) and phylogenetically positioned within marine *Sphaerospora* lineage. This is the first report of enteric sphaerosporosis of groupers.

KEYWORDS
enteric sphaerosporosis, *Epinephelus*, groupers, myxosporea, *Sphaerospora*

1 | INTRODUCTION

Groupers are widely distributed in warm tropical and subtropical sea areas. Owing to their delicious meat, groupers has been become an important popular group of mariculture fish species in countries of East Asia and South-East Asia since the 1980s, especially after the success of artificial breeding (Lin, 2012). In China, sea areas off the coast of South China sea, including Taiwan, Fujian, Guangdong, Guangxi and Hainan provinces are the main production area of groupers and the annual production and value of artificially reared groupers here is above 100,000 tons and 10 billion RMB in 2015, respectively (Yuan & Zhao, 2016). Among more than 150 species of

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groupers, orange-spotted grouper *Epinephelus coioides* (Hamilton), Hong Kong grouper *Epinephelus akaara* (Temminck & Schlegel), brown-marbled grouper *Epinephelus fuscoguttatus* (Forsskål), giant grouper *Epinephelus lanceolatus* (Bloch), Malabar grouper *Epinephelus malabaricus* (Bloch & Schneider), Humpback grouper *Cromileptes altivelis* (Valenciennes) and leopard coralgrouper *Plectropomus leopardus* (Lacepède) are of the main production in China. In recent years, a hybrid species of *Epinephelus lanceolatus* (♂) × *Epinephelus fuscoguttatus* (♀) (hybrid grouper) has become the most popular cultured species due to its rapid growth. With the continual expansion of grouper aquaculture in South China, however, quite a few infectious diseases are emerging, which have been causing serious impediment for the sustainable development of this industry (Chi et al., 1997; Shen et al., 2017). Among them, infectious viral diseases represented the major threat and received many concerns, especially nervous necrosis virus that severely impacted the production of larval and juvenile groupers (Huang et al., 2015; Luo et al., 2013). Regarding epizootic parasitic diseases, *Cryptocaryon irritans*, *Pseudocohnilembus* spp., *Trichodina* spp., *Trypanosoma* spp., *Glugea* spp., *Petalosoma epinephelis*, *Neobenedenia* spp., *Pseudorhabdosynochus* spp., *Caligus* spp., *Ichthyoxenus* sp. and taxonomically unidentified leech were sporadically reported to be responsible for the mortality or morbidity of wild and cultured groupers (Li, Dan, Zhang, Luo, & Li, 2011; Luo et al., 2013; Su et al., 2014; Su et al., 2014; Zhang et al., 2005). Importantly, a new enteric intranuclear microsporidian was recently found to be the aetiological agent to result in the mass mortality of hatchery-bred juvenile groupers at the nursery stage; however, this microsporidiosis has not yet been found in groupers of grow-out stage in inshore and offshore net cages (Xu, Liu, Zhang, Liu, & Feng, 2017). Based on the provided information of disease surveillance by the local technicians, we conducted a parasitological investigation of fattening groupers of inshore net cages in two culture bays in Hainan provinces, South China. An enteric myxosporidiosis caused by a suspected new *Sphaerospora* species was found to be involved in an epizootic episode, causing mass mortality and severe economic losses. This *Sphaerospora* species is remarkably different from the previously reported *Sphaerospora epinepheli*, the pathogen responsible for renal sphaerosporosis of *E. coioides* and *E. malabaricus* in South-East Asia (U-taynapun, Chirapongsatonkul, Maneesaay, Itami, & Tantikitti, 2012; Xu, Zhang, Feng, & Wang, 2014). In the present work, the gross signs, prevalence and histopathological responses of this new sphaerosporosis and molecular characterization of the associated agent were documented. This is the second report of enteric myxosporidiosis in groupers (China et al., 2013).

2 | MATERIALS AND METHODS

2.1 | Fish collection and microscopic examination

Groupers of different species were sampled from four different farming facilities located in XinChun bay, Lingshui city (18°24′47″N, 109°58′20″E), and HongSha bay (18°15′18″N, 109°33′54″E), Sanya city, two production areas of commercial groupers in Hainan provinces, South China, during the season of disease occurrence which were suggested by local technicians. Information on the fish examined and sampling location was given in Table 1. All collected fish were morphologically identified to species, following by measuring the body length and weight and recording the clinical signs. Wet squash mount of gut intestinal contents and Giemsa-stained imprints were examined on spot to determine the prevalence of this enteric myxosporean under light microscopy (Motic BA210, China). The data on sources of fry and the gross cumulative mortality were provided by fish farmers, and the information of disease history was obtained from the local technicians. For infected fish, gills, ulcer of body surface and internal organs, including gall bladder, liver, spleen, heart, kidney and blood, were also examined to find possible parasites by observing the fresh smear preparation and Giemsa-stained imprints under light microscopy as above. The photographs of thawed and Giemsa-stained myxosporean-containing tissue imprints were taken by Leica DC 350F (Germany). Three infected samples per farm were also routinely fixed in 10% neutral formalin and 100% ethanol and transferred to the laboratory of the Institute of Hydrobiology, Chinese Academy of Sciences, for histopathological and molecular characterization of the aetiological agent.

2.2 | Bacteria isolation

Bacterial isolation and identification were based on the protocol of Shen et al. (2017). Briefly, bacteria were isolated from the white nodules on liver, kidney and spleen and ascetic fluid of the moribund fish and streaked onto thiosulphate-citrate-bile salts-sucrose (TCBS) and 2216E agars under aseptic conditions. Following incubation at 28°C for 24–48 hr, morphologically uniform colonies were picked up and streaked onto new agars to obtain pure culture. Then, a single colony was inoculated into fresh 2216E broth and incubated at 28°C with shaking for 12 hr. Five millilitres of bacterial suspension was collected to extract genomic DNA. The 16S rDNA gene was amplified with primer pair 16S-F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 16S-R (5′-GGT TAC CTT GTT ACG ACT T-3′), and the obtained sequences were searched by BLAST to molecularly identify the bacteria.

2.3 | Histopathology

Tissue blocks of intestines and kidney of infected fish fixed in 10% neutral-buffered formalin were dehydrated through a serial of graded concentration of ethanol and embedded in paraffin wax. Tissue sections, 4–6 μm, were stained with haematoxylin and eosin (H&E) and examined under a light microscope (Leica DC 350F, Germany).

2.4 | Molecular characterization of the myxosporean

Disporous plasmodia including kidney, bile and intestinal contents fixed in 100% ethanol were used to extract the genomic DNA (gDNA) with Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germany),

TABLE 1 Information of sampled groupers and epidemiology of enteric sphaerosporosis caused by this novel myxosporidian in two production bay of ongrowing groupers in Hainan province

Farm	Bay	Sampling date	Fish species	No. fish examined	Mean body length (cm)	Prevalence[a] (%)	Mortality[b] (%)
A	XC	October 2014	*Epinephelus lanceolatus* × *Epinephelus fuscoguttatus*	50	8.9	82	90
A	XC	October 2014	*E. lanceolatus* × *E. fuscoguttatus*	50	21.6	24	20
A	XC	November 2015	*Epinephelus coioides*	50	10.8	94	90
A	XC	November 2015	*E. coioides*	50	23.7	12	5
A	XC	November 2015	*E. lanceolatus*	60	25.7	18	10
A	XC	November 2015	*Plectropomus leopardus*	50	9.7	0	10
A	XC	November 2015	*Cromileptes altivelis*	50	10.6	0	0
B	XC	March 2016	*E. lanceolatus* × *E. fuscoguttatus*	50	9.2	100	100
B	XC	April 2017	*E. coioides*	50	11.9	98	90
B	XC	April 2017	*Cromileptes altivelis*	50	10.6	0	15
C	HS	October 2014	*E. lanceolatus* × *E. fuscoguttatus*	50	8.3	86	100
C	HS	November 2015	*Epinephelus fuscoguttatus*	50	19.8	20	10
C	HS	November 2015	*Plectropomus leopardus*	50	8.8	0	25
C	HS	November 2015	*E. coioides*	50	17.8	22	10
C	HS	November 2015	*Plectropomus leopardus*	50	8.5	0	15
D	HS	April 2017	*E. lanceolatus* × *E. fuscoguttatus*	50	9.9	80	80
D	HS	April 2017	*Plectropomus leopardus*	50	17.6	0	10

LS and HS represent the XinChun bay and Huangsha bay, respectively.

[a]prevalence was determined by the occurrence of disporous plasmodia of this novel *Sphaerospora* species in the wet preparation of gut contents examined by light microscopy on spot.

[b]Mortality was grossly calculated based on the provided data by local farmers and technicians.

following the manufacturer's recommended protocol for animal tissue, after removing the ethanol remnants by centrifugation and wash. The gDNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) at 260 nm. The almost complete sequence of SSU ribosomal DNA (rDNA) gene of the myxosporean of interest here was obtained by assembling two overlapped parts. The first part was amplified by ERIB1/ACT1R primer pair (Barta et al., 1997; Hallett & Diamant, 2001), and the PCR cycling parameters included 95°C for 5 min, following by 35 cycles of 95°C for 1 min, 52°C for 50s, 72°C for 80s and a final extension of 7 min at 72°C. The second part was amplified by MyxospecF/18R primer pair (Fiala, 2006), and the PCR cycle consisted of an initial denaturation step at 95°C for 4 min, followed by 35 cycles of 95°C for 1 min, 46°C for 50 s, 72°C for 90 s and a final extension of 10 min at 72°C. All obtained PCR products were separated by agarose gel electrophoresis, purified with a PCR purification kit (CWBiotech, China) and then cloned into PMD–18T vector system (Takara, Japan). Then, positive clones were selected and sequenced with the ABI BigDye Terminator v3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyzer.

The obtained DNA sequences were firstly assembled and edited in BioEdit (Hall, 1999), and the homologous positions with differences among contigs were checked by inspecting the chromatograms. Sequences of different tissue resources of an individual infected grouper were compared by Clustal X 1.8 (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997) to validate a single or mixed myxosporean infection. Then, the consensus sequence was submitted to GenBank with accession number MF431725 and used as a query to search GenBank database by BLASTN to identify the most genetically closely related organisms. The delimitation of variable regions of the present species was followed by Holzer, Wootten, and Sommerville (2007) and compared with those of other myxozoans based on phylogenetically related species (Bartošová et al., 2013). To uncover the phylogenetic position of the present species among the genus *Sphaerospora*, all available *Sphaerospora* SSU rDNA sequences were retrieved from GenBank and aligned with Clustal X 1.8 (Thompson et al., 1997) by default setting. The alignment was corrected manually by MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Phylogenetic analysis of aligned sequences, excluding the gaps, was performed by Bayesian analysis in Mr. Bayes (Ronquist & Huelsenbeck, 2003). Optimal evolutionary model was determined using jModeltest (Posada, 2008) which identified the best evolutionary model as the general time-reversible model (GTR + I + G), judging by the Akaike information criteria (AIC). Two independent runs were performed with four chains for a million generations. Phylogenetic trees were sampled every 100 generations. The first 25% of the samples were discarded from the cold chain (burninfrac = 0.25). *Buddenbrockia plumatellae* (AY074915) and *Tetracapsuloides bryosalmonae* (U70623) were used as outgroup. Trees were initially examined in TreeView X (Page, 1996), edited and annotated in Adobe Illustrator (Adobe Systems USA).

3 | RESULTS

3.1 | Clinical signs, light microscopy and epidemiology

This epizootic myxosporidiosis was of seasonal pattern and generally emerged in the autumn and winter (from late September to late April of the following year) when water temperature ranged from 22 to 28°C and salinity from 32 to 34 ppt. Disease generally occurred for the yearling fish below 20 cm in body length and 50 g in body weight. No infection was found by visual detection of disporous plasmodia in the intestinal contents from early May to early September based on the surveillance data of local technicians. Diseased fish exhibited anorexia, cachexia, dispersedly floating at the water surface, no responses to external stimulators and extrusion of white pulp-like contents after gentle pressure on the abdomen of infected fish. Outbreak of mass mortality emerged generally within 2–3 days after appearance of the above clinical signs, and the final cumulative mortality in investigated cages ranged from 10% to 100%, depending upon the size and species of infected fish. After necropsy of moribund fish, remarkably swollen intestine, thin and transparent intestinal wall, extended spleen, kidney and gall bladder could be observed. At the early stage of disease, gut of diseased fish presented as a sausage for cheese-like substance was fully filled within the intestinal cavity (Figure 1). Squash preparation of the shedding intestinal contents showed that the cheese-like substance consisted of large number of *Sphaerospora*-typical disporous plasmodia, sloughed enterocytes and cell debris. And, these disporous plasmodia could be found in the renal tubules and gall bladder of severely infected fish (Figure 2), but no infection was found in the liver, heart, spleen and blood under light microscopy. The size of disporous plasmodia was highly variable, with length ranged from 25.8 to 41.4 μm and width ranged from 18.6 to 25.4 μm, respectively (n = 100), although the size of spherical polar capsules was usually consistent, with 0.8–1.5 μm in diameter. No mature *Sphaerospora* spore was found from all examined samples (Figure 2a). Giemsa-stained imprint of intestinal

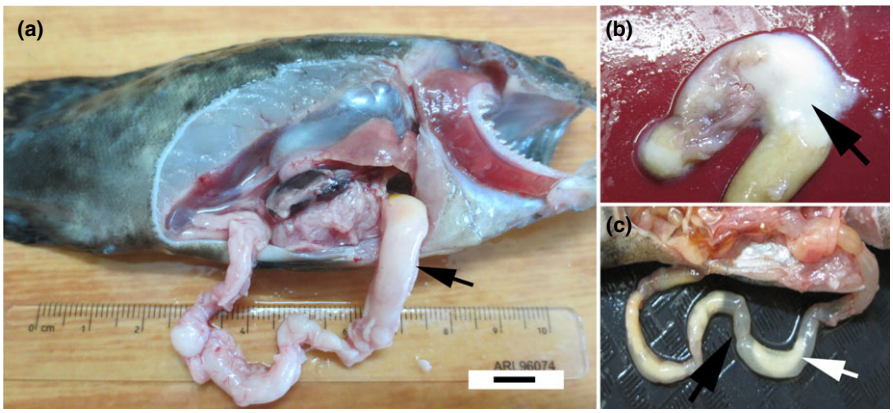

FIGURE 1 Clinical signs of infected hybrid groupers (*Epinephelus lanceolatus* ♂ × *Epinephelus fuscoguttatus* ♀) by the present enteric *Sphaerospora* sp., showing (a) advanced infection stage with severely enlarged and swollen intestine, especially foregut, which seemed like a sausage (arrow) fully filled with cheese-like substance; (b) extruded white pulpy contents after opening the intestinal cavity (arrow) with thin intestinal wall; (c) late infection stage with severely thin and transparent intestine wall (black arrow) after excreting large parts of intestinal contents and remained white pulpy contents (white arrow); scale bar = 1 cm [Colour figure can be viewed at wileyonlinelibrary.com]

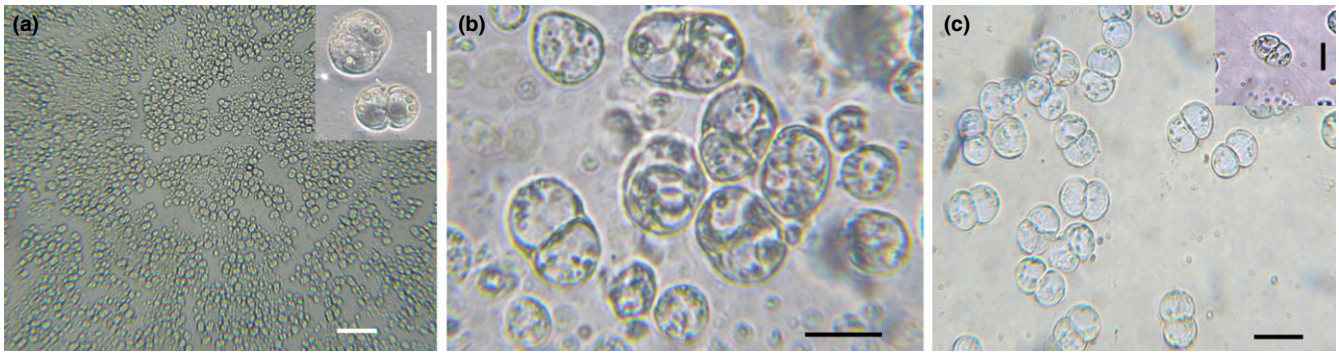

FIGURE 2 Photographs of fresh smear of intestinal contents, bile and kidney of infected samples by the present enteric *Sphaerospora* sp., (a) showing great number *Sphaerospora*-typical disporous plasmodia in the intestinal contents (scale bar = 100 μm), with an insert of two plasmodia of variable size (scale bar = 20 μm); (b) showing plasmodia in the wet preparation of kidney (scale bar = 20 μm); (c) showing plasmodia in the bile smear, with an insert of a plasmodium of ejecting four polar filaments (scale bar = 20 μm) [Colour figure can be viewed at wileyonlinelibrary.com]

contents and kidney showed that proliferative and sporogonic stages of the parasite and infiltrated inflammatory cells (mainly monocytes) also presented besides disporous plasmodia (Figure 3). The emaciation of infected and moribund fish was not significant, although the body weight of diseased fish, especially at advanced stage of disease, was slightly lower than that of uninfected fish (data not shown). Occasionally, white round nodules with 0.5–4.0 mm in diameter could be observed in the spleen, kidney and liver of suffered fish. No enteric microsporidian causing severe emaciation disease of juvenile groupers was observed among all examined fish. Molecular comparison by BLAST search identified the isolated bacteria from the white nodules and ascites of severely infected samples to be *Vibrio harveyi*.

Preliminary epidemiological investigation on the four facilities in the two production area of Hainan province obviously indicated that the infection prevalence and intensity of this enteric myxosporean in fish with above 20 cm in body length were significantly lower than that of fish with about 10 cm in body length. As such, the mortality of infected fish with above 20 cm in body length was remarkably reduced, compared with fish of below 10 cm in body length. Generally, yearlings of 8–20 cm in body length and 25–50 g in body weight were more susceptible and suffered, compared with big fish. Actually, fish of above 30 cm in body length was almost found to be uninfected according to the surveillance data of the local technicians. More interestingly, this myxosporean was found to exclusively infect *Epinephelus* groupers; however, no infection was found in *Plectropomus leopardus* and *Cromileptes altivelis*, which were cultured in the same facility of two investigated production areas, no matter what size of these two no-*Epinephelus* groupers was (Table 1).

3.2 | Histopathology

The histological observations demonstrated the presence of severe enteritis in the affected fish. At the early stage of disease, intestinal mucosa and submucosa layer could still keep integrity, but it was remarkable that large number of *Sphaerospora*-typical disporous plasmodia and early sporogonic stages severely invaded the epithelium, causing hypertrophy and haemorrhages of the subepithelial connective tissue. Few sloughed epithelial cells, together with various sporogonic stages of the parasites, were present in the intestinal cavity. Many cytoplasmic vacuolation of epithelial cells and infiltrated inflammatory cells could be observed in the affected mucosa and submucosa layer, although the brush border kept almost complete (Figure 4a–c). Meanwhile, early sporogonic stages and disporous plasmodia could also be observed in the epithelium of renal tubules and renal lumen, but the integrity of the epithelia layer of renal tubules maintained (Figure 5a). With the disease progress, the epithelium of intestinal mucosa and submucosa were completely lost and the intestinal lumen was fully filled with the sloughed enterocytes and cell debris, as well as the various sporogonic stages of parasite. And, the muscular layer of affected intestine significantly became thin, together with severely atrophied intestinal villus, although no infection of this myxosporean was found to invade the muscular layer (Figure 4d). Furthermore, the epithelium of glomerulus was completely destroyed and the renal tubules were fully congested with early sporogonic stages and disporous plasmodia of the myxosporean (Figure 5b). Cytoplasmic vacuolation of epithelial cells of renal tubules was also evident, especially to which the disporous plasmodia attached. Occasionally, a few melanomacrophages could be observed present in the interstitium. Compared with gut, however, the impairment extent of kidney was significantly milder. Occasional appearance of white nodules in kidney was histologically approved to a bacterial granuloma, with surrounding infiltrated inflammatory cells (Figure 5c).

3.3 | Molecular characterization

Almost the entire length of the SSU rRNA genes (2091 bp vs. 2169 bp of expected length) of enteric, renal and gall bladder resources of this myxosporean were amplified, cloned and sequenced which was approved to be 100% identical. Homology analysis by BLAST showed that the present species was most genetically related to *Sphaerospora fugu*, with 89% sequence similarity over 1641 bp. However, the present parasite was just 85% similarity with that of another grouper-infecting congener, *S. epinepheli*. The variable regions (V) of SSU rDNA of the present *Sphaerospora* species was delimited based on Holzer et al. (2007), including V1 of 33 bp, V2 of 243 bp, V3 of 60 bp, V4 of 389 bp, V5 of 46 bp, V7 of 200 bp and V8 of 60 bp which was located in the length range of the corresponding variable regions of lineage A (marine lineage) of

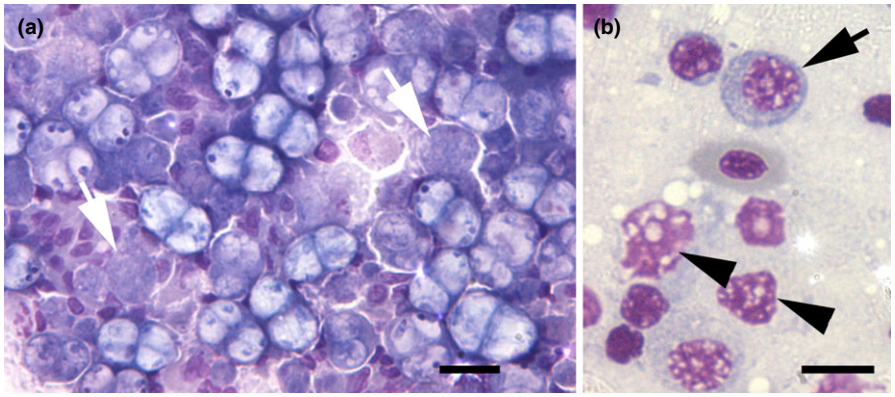

FIGURE 3 Giemsa-stained freshly smear preparation, showing (a) imprint of intestinal contents containing proliferative stages (arrow) and disporous plasmodia; (b) imprint of kidney containing proliferative stages (arrowhead) and infiltrated monocytes (arrow); scale bar = 20 μm [Colour figure can be viewed at wileyonlinelibrary.com]

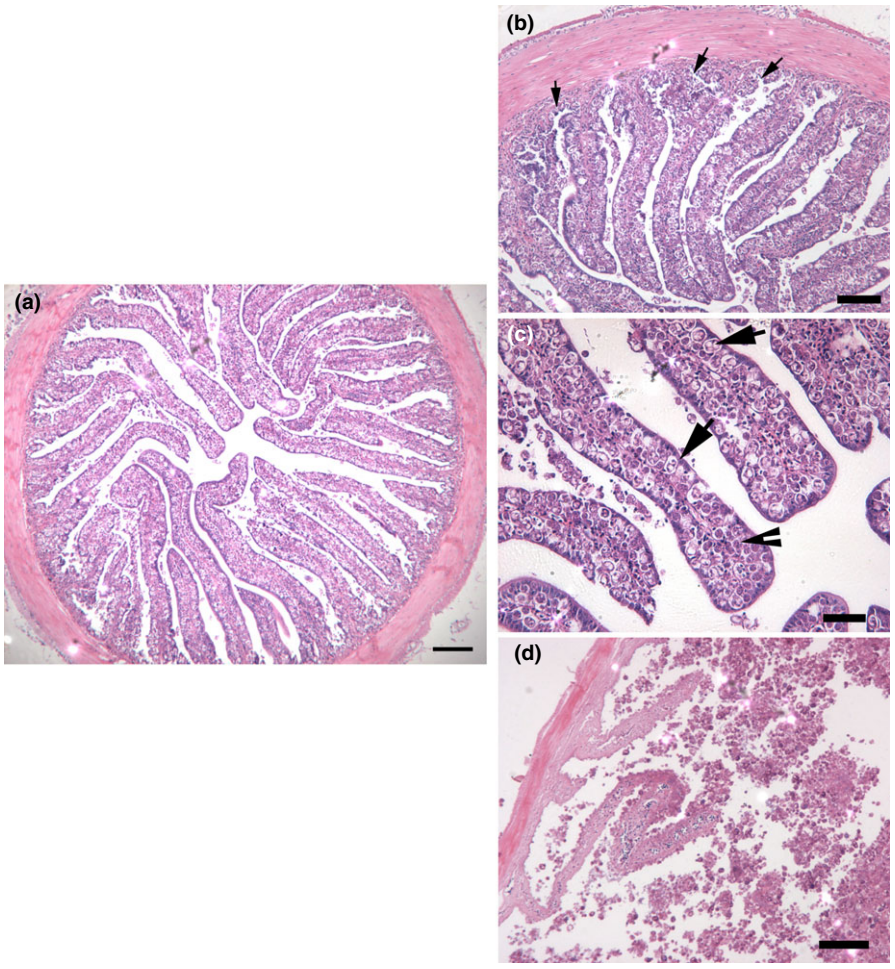

FIGURE 4 Histopathological changes of intestine of an infected hybrid grouper, (a) the panoramic view of affected intestine showing severely invaded epithelium of the entire mucosa layer by large number of disporous plasmodia, scale bar = 200 μm; (b) showing the severely destroyed epithelial cells in submucous layer by the infection of the present *Sphaerospora* sp. (arrow), scale bar = 200 μm; (c) showing the details of the distribution of disporous plasmodia (arrow) and early sporogonic stages (arrowhead) of the present myxosporean in the mucous layer, with severe cytoplasmic vacuolation of epithelial cells and some parasites of different stages and epithelia cell debris shedding in the intestinal lumen, scale bar = 100 μm; (d) showing the almost completely detached gut epithelium, thin muscular layer, large number of shedding parasites, sloughed host epithelial cell debris and inflammatory cells in the intestinal lumen at the advanced stage of the disease and thin muscular layer, scale bar = 200 μm [Colour figure can be viewed at wileyonline library.com]

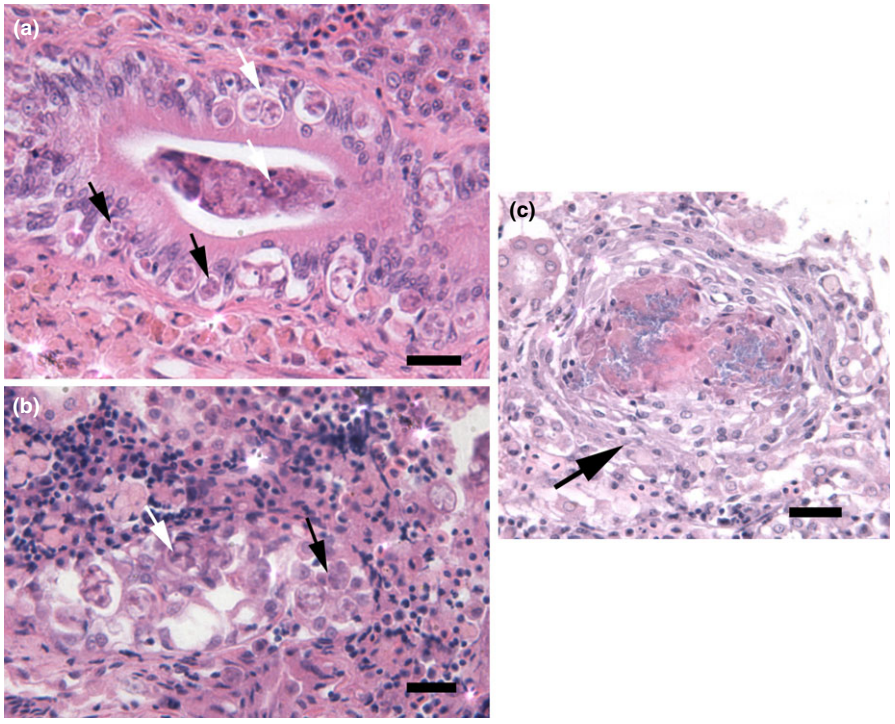

FIGURE 5 Histopathological changes of kidney of an infected hybrid grouper; (a) showing many disporous plasmodia (white arrow) and early sporogonic stages (black arrow) dwelling in the epithelium of renal tubules and few suspected early sporogonic stages in the renal lumen at the early stage of disease, scale bar = 50 μm; (b) showing that many disporous plasmodia (white arrow) and early sporogonic stages (black arrow) occluded in glomerulus at the late stage of disease, scale bar = 50 μm; (c) showing the occasional occurrence of white nodule granuloma (arrow), possibly caused by Vibrio harveyi in the kidney of infected fish, scale bar = 100 μm [Colour figure can be viewed at wileyonline library.com]

Sphaerospora sensu stricto clade (Bartošová et al., 2013). The length of V4 region of the species is similar with that of *S. fugu*; however, the length of V7 of the former is significantly longer than that of the latter (200 bp vs. 142 bp). Interestingly, V4 regions of the present species and *S. fugu* are shortest among the genus *Sphaerospora* (Table 2). SSU rDNA-based phylogenetic analysis also showed that the present *Sphaerospora* species firstly clustered with *S. fugu* to form an independent group within the lineage A of *Sphaerospora sensu stricto* clade, with high nodal support value. Again, the genus *Sphaerospora* was approved to cover several distinct lineages in the present phylogenetic analysis, as previous reports (Figure 6).

4 | DISCUSSION

Myxozoans are microscopic parasites belonging to the phylum Cnidaria which are well known as an important group of pathogenic parasites to continually threaten the development of aquaculture and wild fish stocks (Okamura, Gruhl, & Bartholomew, 2015). More than 2000 species have been found, belonging to 60 morphologically taxonomic genera (Lom & Dyková, 2006), among which the genus *Sphaerospora* contains around 80 reported species, although the species diversity is probably underestimated. For no mature spores were found, the present species could not be taxonomically identified to species. However, the typical disporous plasmodia, extensive nucleotide insertions in the V4 region of the SSU rDNA (Jirků, Fiala, & Modrý, 2007) and genetically relatedness can definitely demonstrate the present myxosporean to be a *Sphaerospora* species within *Sphaerospora sensu stricto* clade. Genetically, the present species is most closely related to *S. fugu*, which is a notorious enteric myxosporean to cause emaciation disease of tiger puffer *Takifugu rubripes* (Temminck & Schlegel) by invading the intestine of fish and causing severe catarrhal enteritis (Kodama et al., 2014). Host habitat and tissue tropism of myxosporeans have been widely reported and accepted to be important taxonomic criteria to infer the phylogenetic relationships of myxosporeans and differentiate species with similar morphological features (Eszterbauer, 2004; Fiala, 2006; Okamura et al., 2015; Zhang, Wang, Li, & Gong, 2010). So, the clustering of these two enteric marine *Sphaerospora* species further supports the previous contention at some extents, although sequence data of only two enteric *Sphaerospora* species are available in the database. The present species did not form an independent group with *S. epinepheli*, another *Epinephelus*-infecting *Sphaerospora* species. Although host affinity tends to give the strongest phylogenetic signal for myxosporean (Carriero, Adriano, Silva, Ceccarelli, & Maia, 2013), they are many exceptions and this is just another one. Furthermore, similar length of V4 of the present species and *S. epinepheli* can be suggested to be a possible biomarker of enteric marine *Sphaerospora* lineage, for it is shortest within *Sphaerospora sensu stricto* clade and longer than that of all other myxozoans. Given the basal phylogenetic position of *Sphaerospora sensu stricto* clade to all myxosporean species, it can be supposed that the enteric *Sphaerospora* species are possibly a transition group between lineage B of *Sphaerospora sensu stricto* clade (Bartošová et al., 2013) and other myxosporeans. The phylogenetic position of the present histozoic enteric marine species among the genus *Sphaerospora* warrants to be further discussed after obtaining its sufficient morphologically taxonomic data which are out of the aim of the present work.

Although *V. harveyi*, recently identified to be the causative agent of skin ulcer disease in juvenile hybrid grouper in China (Shen et al., 2017), was isolated from the white nodule on liver, spleen and kidney of some severely diseased fish, histopathological analysis explicitly demonstrated that the enteric *Sphaerospora* species was directly associated with the epizootic outbreak of groupers. *V. harveyi* was a

TABLE 2 SSU rRNA sequence data of this novel *Sphaerospora* species (Bold values), compared with other myxozoans: lengths of variable regions (in base pairs); length amplified in this study; estimated total length of gene based on related taxa amplified with primer pair ERIB1-ERIB10. GC content (%) in parentheses

Clades and taxa	V1	V2	V3	V4	V5	V7	V8	Amplified Length (bp)	Estimated total length (bp)
Sphaerospora s.s. clade	33 (46)	254 (46)	60 (44)	824 (49)	52 (46)	307 (49)	58 (47)	—	2726 (49)
Lineage A	33 (48)	243 (36)	58 (35)	492 (38)	42 (31)	168 (37)	59 (48)	—	2254 (43)
***Sphaerospora* sp. (MF431725)**	**33 (48)**	**243 (30)**	**60 (21)**	**389 (33)**	**46 (28)**	**200 (29)**	**60 (38)**	**2091**	**2169 (40)**
Sphaerospora fugu (AB195805)	33	246	59	388	46	142	60	2087	2131
S. olsoni (KJ526213)	—	—	59	565	41	155	59	1934	2462
Swainsona formosa (GQ374533)	33	222	58	543	40	160	59	2050	2276
Sphaerospora epinepheli (HQ871153)	33	266	58	544	40	201	59	2226	2369
S. sparidarum (JX286620)	33	239	58	491	43	168	59	2240	2240
Lineage B	33 (45)	258 (49)	61 (47)	926 (51)	56 (50)	362 (52)	57 (46)	—	2871 (50)
Freshwater myxosporeans	33 (42)	186 (44)	59 (41)	372 (45)	41 (50)	174 (47)	57 (42)	—	≥2000 (47)
Marine myxosporeans	33 (35)	158 (40)	57 (37)	254 (42)	35 (53)	47 (38)	57 (47)	—	~1800 (46)
Malacosporeans	33 (42)	171 (40)	58 (44)	266 (41)	38 (53)	47 (34)	57 (46)	—	~1800 (46)

The delimitation of variable regions was followed by Holzer et al. (2007); and the data of other species were cited from Bartošová et al. (2013).

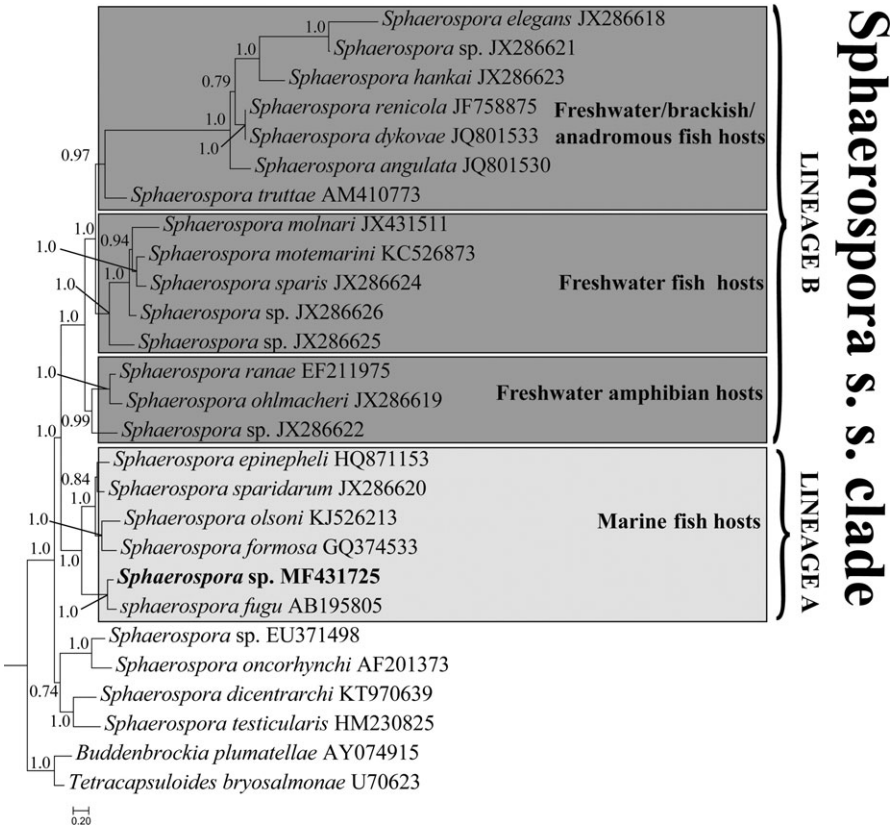

FIGURE 6 Phylogenetic tree by Bayesian analysis of the aligned small subunit rDNA sequences of all available *Sphaerospora* species. GenBank accession number of species was listed adjacent to the corresponding species name, respectively. Posterior probability was listed on the branch nodes. Lineage groups were followed by Bartošová et al. (2013)

possible secondary infected agent. Among the reported pathogenic *Sphaerospora* species, most were coelozoic to infect the urinary system of fish and amphibian, for example, *S. ictaluri*, *S. dykovae*, *S. testicularis*, *S. epinepheli* and *S. ranae* (Lom & Dyková, 2006). Among histozoic species, *S. dicentrarchi*, one of most common parasites of European seabass, *Dicentrarchus labrax* (L.), is a systemic parasite and predispose the connective tissue rather than epithelium, although intestine, gall bladder and kidney are the target organs (Sitjà-Bobadilla & Alvarez-Pellitero, 1993), as same as the species of interest here. Moreover, the disease caused by *S. dicentrarchi* is generally chronic, but the present enteric sphaerosporosis of groupers is acute and outbreak of mass mortality usually occurs 2–3 days after appearance of clinical signs. The histopathological changes of the present myxosporean infection were similar with infection of *S. fugu* in tiger puffer (Tun, Ogawa, & Wakabayashi, 2002) and *Enteromyxum scophthalmi* in turbot, *Psetta maxima* (L.) (Bermúdez et al., 2010), all of which provoked catarrhal enteritis, including severe cytoplasmic vacuolation of epithelial cells, infiltration of inflammatory cells, detachment of epithelium and congestion of intestinal lumen by various developmental stages of parasites and sloughed cell and cell debris. No clinical appearances of emaciation diseases of tiger puffer (Tun et al., 2002), turbot (Bermúdez et al., 2010) and juvenile groupers (Xu et al., 2017), like sunken eyes and bony ridges, however, were observed for this new epizootic. Osmoregulatory failure and impaired intestinal nutrient absorption were considered to the pathogenesis of emaciation disease in the tiger puffer for severe damage of intestinal epithelium (Ishimatsu, Hayashi, Nakane, & Sameshima, 2007). Thus, one of the possible reasons is the occurrence of acute death, although we cannot exclude the important roles of secondary infection and environmental stressors during the disease course. For no formation of mature spores and provoked strong pathological responses of host, it can be suspected that *Epinelphelus* groupers are the accidental or opportunistic vertebrate host of the present *Sphaerospora* species, like *Tetracapsuloides bryosalmonae* in salmonid species (Okamura et al., 2015). Further work should be conducted to find the natural host of the present enteric myxosporean by widening the examined fish host range in the epizootic area, including cultured and wild fish by applying more sensitive molecular detection methods which can be developed by the amplified SSU rDNA gene here. Also, possible overlooked spores in the examined uninfected fish in the present work should be seriously re-examined to validate the host specificity.

A relatively narrow host spectrum of *Sphaerospora* species has been previously intensively reported, irrespective of histozoic and coelozoic group. *S. elegans* and *S. truttae* parasitize only related fish species of the family Gasterosteidae and Salmonidae, respectively (Lom & Dyková, 2006). No infection of *S. epinepheli* was detected in *E. stictus*, *E. fuscoguttatus* and *E. bleekeri* in the same grouper production area in Thailand where prevalence of the myxosporean infection in *E. coioides* and *E. malabaricus* was high (U-taynapun et al., 2012). *S. ranae* only infected two species of the genus *Rana* of five sympatric amphibian species (Jirků et al., 2007). In the present work, this enteric *Sphaerospora* species was also found to exclusively infect *Epinephelus* groupers, but not two other groupers, belonging

to the same family Serranidae (Perciformes), although it awaited more data to finally demonstrate it for variable biological and environmental factors that could drive the myxosporean infection (Okamura et al., 2015). Additionally, host fish with small size (generally below 20 cm) presented greater susceptibility to the infection of this myxosporean which could be partially explained by the more developed immunity and acquired immunity of fish with big size after exposure to the infection. However, *S. dicentrarchi* was previously reported to predispose sea bass greater than 10 g in weight and the infection intensity also progressively increased with the size of host fish (Sitjà-Bobadilla & Alvarez-Pellitero, 1993), which possibly represented a different myxosporean fish interaction pattern from that of the present *Sphaerospora* grouper.

In conclusion, this study reported an episode of acute enteric sphaerosporosis of fattening *Epinephelus* groupers in inshore net cages in South China which has caused significant losses and will be an important limited factor to threaten the sustainable development of grouper production in China. Future studies should be conducted to sufficiently identify it to species, uncover the infection dynamics and elucidate the presumptive two alternate host life cycle of the causative myxosporean to develop practical control strategies against this enteric myxosporidiosis of economic importance in China.

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