Parasitology Research
https://doi.org/10.1007/s00436-021-07389-0

PROTOZOOLOGY - ORIGINAL PAPER

Aggregata sinensis n. sp. (Apicomplexa: Aggregatidae), a new coccidian parasite from *Amphioctopus fangsiao* and *Octopus minor* (Mollusca: Octopodidae) in the Western Pacific Ocean

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Received: 24 August 2020 / Accepted: 17 November 2021

Abstract
A new species of parasitic *Aggregata*, *Aggregata sinensis* n. sp., is described from the octopuses *Amphioctopus fangsiao* (d'Orbigny, 1839–1841) and *Octopus minor* (Sasaki, 1920) (Mollusca: Octopodidae) in China based on morphological data and 18S ribosomal RNA (rRNA) gene sequences. The sexual gamogonic and sporogonic stages of the parasite were observed under light microscope. The sizes of oocysts were 207.35–618.74 μm in length and were 136.31–420.68 μm in width. Oocysts were fully sporulated and were shaped spherically or irregularly. Mature sporocysts within oocysts were spherical or slightly subovoid and were 19.42–22.32 μm in length and 19.12–21.55 μm in width. Sporozoites were typically spiral within sporocysts and were 17.81–24.90 μm long and 2.05–3.42 μm wide. Molecular phylogenetic analysis constructed using the 18S rRNA gene sequences supports the morphological classification that our species is placed within the genus *Aggregata*. Moreover, the sequence belonged to the genus *Aggregata*. However, the 18S rRNA gene sequence of *Ag. sinensis* was significantly different from that of other *Aggregata* available currently. *Ag. sinensis* is the first two-host parasitic species of *Aggregata* reported from definitive host *Am. fangsiao* and *O. minor* and is the only species of the genus *Aggregata* known in Western Pacific waters.

Keywords *Aggregata sinensis* · *Amphioctopus fangsiao* · *Octopus minor* · 18S rRNA · Parasite · Taxonomy

Introduction

A number of pathogens, fungi, labyrinthulomycetes, virus, bacteria, coccidia, ciliates, dicyemid, and metazoan, and diseases related to them which affect cephalopods have been described (Gestal et al. 2019). Coccidia are among the commonest parasites that infect the digestive tract of cephalopods. Nevertheless, the taxonomy of coccidia becomes a challenge because of their apparent simplicity (Castellanos-Martínez et al. 2019). To date, ten valid species are reported worldwide, and all of them belong to the genus *Aggregata* (Gestal et al. 2010; Roumbedakis et al. 2018).

Aggregata spp. are heteroxenous parasites transmitted through the food web (Dobell 1925). Sexual stages (gamogony and sporogony) occur inside definitive cephalopod host, and asexual stages (merogony) present the intermediate hosts of crustaceans (Dobell 1925; Hochberg 1990; Gestal et al. 2002c). Meronts (in asexual stages) ingested by cephalopods can lead to an infection of their entire digestive tract (Gestal et al. 2010; Castellanos-Martínez et al. 2019). The unequal fission of merozoites (asexual stages) inside a cephalopod digestive tract produces gametes (sexual stages), including macrogametes (♀) and microgametes (♂). The macrogamete fertilized by a microgamete gives rise to a zygote. The multiple fission of zygote produces oocysts, and sporocysts within oocyst contain sporozoites inside. The release of the sporocysts causes detachment of the epithelial cells and the connective tissue, the rupture of the epithelial cells, and the mucosal fold atrophy and ulcers (Castellanos-Martínez et al. 2019). The parasites are also reported to make the host more vulnerable to other biotic and abiotic stressors (Gestal et al. 2007).

Section Editor: Sven Klimpel

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For the parasites of *Aggregata*, traditional diagnostic characters among species rely on accurate morphological characterization. To date, molecular techniques are a complement to the assessment of the taxonomic and phylogenetic status. For example, 18S ribosomal RNA (rRNA) gene sequences for *Aggregata octopiana* Schneider, 1875 and *Aggregata eberthi* Labbé, 1895 are generated as molecular data to supplement traditionally morphological descriptions (Kopečná et al. 2006; Castellanos-Martínez et al. 2013; Tedesco et al. 2017).

The prevalence and distribution of the genus *Aggregata* has been reported in North-East Pacific Ocean, North-East Atlantic Ocean, North-West Indian Ocean, South-West Atlantic Ocean, and West Mediterranean Ocean (Gestal et al. 2010). Nevertheless, reports of pathology related to coccidian infections in cephalopods from Asia are scarce and lack detailed morphological information of the parasitic stages identified. A preliminary review shows that there are protozoan parasites of *Aggregata* infecting *Amphioctopus fangsiao* (d'Orbigny, 1839–1841) in large-scale concentrated deaths (Ren et al. 2019). However, in the mentioned review, the parasite is not identified to the species level. This study presented a new species of *Aggregata* found in *Am. fangsiao* and *Octopus minor* Sasaki, 1920 from the Western Pacific Ocean. The new species is to supplement the biological data of the genus *Aggregata*.

Material and methods

Sample collection and conservation

A total of 162 *Am. fangsiao* (OUC-LSGB-03162) were collected from two distinct geographic areas in China: Lianyungang in Jiangsu at 34° 49.00′ N, 119°20.06′ E in April 2018, March 2019, and January 2020 and Qingdao in Shandong at 36° 04.00′ N, 120°22.59′ E in October 2019 and January 2020. We obtained the specimens from fishermen at local fishing ports. The specimens obtained from Lianyungang in March 2019 were kept in six rectangular concrete tanks (7.5 m × 2 m × 0.8 m) indoors in the Ganyu Jiaxin Fishery Technical Development Co. Ltd. (Jiangsu Province) with open water circulation (temperature, 12.2–15.3 °C; salinity, 29–30). They were fed with fresh Manila clams (*Ruditapes philippinarum* A. Adams & Reeve, 1850, *Mactra veneriformis* Reeve, 1854) which were collected from the local sea. A total of 77 *O. minor* (OUC-LSGB-03077) were collected from five distinct geographic areas of China: Rongcheng in Shandong at 37° 09.43′ N, 122°29.10′ E in November 2018 and June 2020, Qingdao in Shandong at 36° 04.00′ N, 120°22.59′ E in October 2019, Lianyungang in Jiangsu at 34° 49.00′ N, 119°20.06′ E in March 2019 and June 2020, Yilan in Taiwan at 24° 41.36′ N, 121°44.45′ E in August 2013, and Nanjidao in Zhejiang at 27° 29.12′ N, 121°00.29′ E in July 2020. The specimens were examined in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for animal experiments. The digestive tract (including crop, esophagus, stomach, cecum, intestine) from all specimens that was dead no more than a few hours was dissected and examined for parasites. A Nikon D810 camera is used to observe infected specimens. Host species, number, the sampling site, and the other sampling dates were summarized in Table 1. All infected specimens are registered and deposited at the collection point of the Fisheries College, Ocean University of China.

Microscopic identification

Fresh oocysts from the intestine of host specimens were excised, squashed by cover glass pressure, and sporocysts were observed and measured directly under an Olympus BX53 light microscope. Some oocysts from intestine of host specimens were fixed in buffered 10% seawater formalin. Isolated sporocysts from fixed oocysts and sporozoites from fixed sporocysts were measured between microscope slide and the cover glass. For histological observations, fresh tissues from infected specimens were fixed in Bouin's fluid (Sbjbio) for 24 h, stored in 70% ethyl alcohol, dehydrated in increasing ethyl alcohol concentrations, embedded in paraffin wax, and sectioned using a Leica RM2016 microtome. Slices of 5 micron thickness were stained with hematoxylin and eosin based on standard procedures (Culling et al. 1985) and observed under an Olympus BX53 light microscope. Oocysts and sporocysts were measured based on histological sections under an Olympus BX53 light microscope. All measurements for oocysts, sporocysts, and sporozoites, in

Table 1 Sampling information of *Amphioctopus fangsiao* and *Octopus minor* examined for the presence of *Ag. sinensis* from the Western Pacific Ocean

Host	*Amphioctopus fangsiao*		*Octopus minor*			
Sampling site	Jiangsu	Shandong	Jiangsu	Shandong	Taiwan	Zhejiang
No. of host examined	130 (76♂ + 54♀)	32 (24♂ + 8♀)	13 (10♂ + 3♀)	55 (28♂ + 27♀)	5 (5 ♂)	4 (4♂)
No. of host infected	74 (44♂ + 30♀)	7 (4♂ + 3♀)	13 (10♂ + 3♀)	42 (24♂ + 18♀)	5 (5 ♂)	2 (2♂)
Prevalence (%)	56.90	21.88	100	76.36	100	50

micrometers (μm), were expressed as the mean followed by the standard deviation in parentheses. Histological sections of infected host tracts on slides are deposited at the Fisheries College, Ocean University of China with a serial number (OUC-LSGB-HE0036).

Statistical analysis

IBM SPSS 23.0 Statistics Software (https://www.ibm.com/products/spss-statistics) was used for statistical analyses. One-way analysis of variances was used to test for significant differences in sporocysts from different hosts with varying treatments. The differences between means were considered significant when $P < 0.05$.

DNA extraction and polymerase chain reaction (PCR) amplification

Genomic DNA was extracted from oocysts infecting the muscles and digestive tract of host specimens. Approximately 50 mg of oocysts was suspended in 500 μL of extraction buffer (Urea 6 M, NaCl 125 mM, Tris–HCl 10 mM, EDTA 10 mM, pH 7.5, SDS 1%) and opened by ultrasonic breaker on ice (5 cycles, 40 W, 50 s) to release sporozoites which were digested by proteinase K (Sigma). The genomic DNA extractions were carried out following the protocol of the phenol: chloroform: isoamyl alcohol extraction method (Sambrook 1989). The small subunit 18S rRNA gene was amplified by PCR with primers following previous research (Castellanos-Martínez et al. 2013). The specific primers were utilized (Aggregata1-F: 5′-ATGATGAAACTGCGAAGAGC-3′, Aggregata 1-R: 5′-CGACGGTATCTGATCGTCTT-3′, Aggregata 2-F: 5′-GGGGGTATTTGTATTTAACAAGCA-3′, Aggregata 2-R: 5′- CCTACGGAAACCTTGTTACGA-3′) to amplify a fragment of the small subunit 18S rRNA gene. Aggregata primers 1F/R amplified the initial 950 bp of the 18S rRNA gene, whereas Aggregata primers 2F/R amplified the next 950 bp. PCRs were carried out in the 25 μL total volume with 100 ng template DNA, 1.25 U of Taq DNA polymerase (TaKaRa), 0.25 μM each primer, 0.2 μM of each dNTP, 1 × PCR buffer, and 2 mM $MgCl_2$. PCR conditions were used as described by Castellanos-Martínez et al. (2013). For primers 1, we used an annealing temperature of 55 °C, while the annealing temperature for primers 2 was 53 °C. PCR products were checked by 1.5% agarose in TBE (1 × buffer) gels (w/v) and then sent directly to the BGI Genomics Company for sequencing via the primer-walking method. The partial 18S rRNA sequences have been deposited in GenBank under accession numbers MN275009, MN275010, MN275011, MN275012, and MN275013.

Data analysis

The 18S rRNA sequences obtained in this study were manually edited by SeqMan v.7.2. The following sequences of 36 Apicomplexa taxa retrieved from GenBank were used in the phylogenetic construction. These sequences were *Ag. octopiana* RV1 (KC188342), *Ag. octopiana* (DQ096837), *Ag. eberthi* RV2 (KC188343), *Ag. eberthi* (DQ096838), *Cryptosporidium meleagridis* (AF112574), *C. parvum* (AF115377), *C. wrairi* (U11440), *C. felis* (AF112575), *C. canis* (AF112576), *Tridacna hemolymph apicomplexan* (AB000912), *Adelina bambarooniae* (AF494059), *Ad. dimidiate* (DQ096835), *Ad. grylli* (DQ096836), *Hepatozoon canis* (EF622096), *H. catesbianae* (AF130361), *Cyclospora cayetanensis* (AF111183), *C. colobi* (AF111186), *C. papionis* (AF111187), *Eimeria alabamensis* (AF291427), *E. arnyi* (AY613853), *E. bovis* (U77084), *E. falciformis* (AF080614), *Calyptospora funduli* (FJ904645), *C. spinosa* (FJ904637), *Neospora caninum* (GQ899206), *Neospora* sp. (U17345), *Toxoplasma gondii* (L37415), *Isospora belli* (U94787), *I. felis* (L76471), *Sarcocystis gracilis* (FJ196261), *S. neurona* (U07812), *Goussia Janae* (AY043206), *Theileria buffeli* (AF236097), *Theileria* sp. (U97055), *Babesia conradae* (AF158702), and *Babesia* sp. (AY048113). As representative species, *Theileria* and *Babesia* were used as outgroups. All sequences were imported into MEGA v.6.0 and were aligned using default settings (Tamura et al. 2013). The best fitted nucleotide substitution model was used to select by jModelTest (Posada 2008) under the Akaike information criterion (Posada & Buckley 2004). GTR + G was the fittest for sequences used in the phylogenetic construction. Sequences of the 18S rRNA were employed to build maximum likelihood (ML) trees by MEGA v.6.0 and Bayesian inference (BI) trees using MrBayes 3.2 (Ronquist et al. 2012). BI trees were constructed with 500,000 generations under the Markov chain Monte Carlo (MCMC) command, and the trees were sampled every 1000 generations, with the first 25% removed as burn-in. Using Tracer v1.5 (Rambaut & Drummond 2009), convergence of the parameters was inferred. The results were exported to software FigTree v1.4.2 (Rambaut 2009)

Results

Family Aggregatidae Labbé, 1899.
Genus Aggregata Frenzel, 1885.
Aggregata sinensis n. sp.

Material examined

This was based on the examination of 162 *Am. fangsiao* specimens and 77 *O. minor* specimens (Table 1).

Phenotypic identification

At the macroscopic level, the distribution of the coccidian parasites in *Am. fangsiao* and *O. minor* was observed (Fig. 1). White cysts were present in the viscera (Fig. 1b–d) and the body surface (Fig. 1a, e). The oocysts were easily observed as white spots throughout the mantle of the *Am. fangsiao* and *O. minor* when the hosts had a severe infection (Fig. 1a).

Fresh squash preparation and histological sections were observed by light microscope examination (Fig. 2). Sporocysts were naturally sporulated in oocysts. Fresh sporocysts were spherical, and free sporozoite could be seen by crushed sporocysts between two microslides (Fig. 2a, b). The oocysts were typically spherical (Fig. 2c). Histological sections of heavily infected cecum in *O. minor* revealed large numbers of oocysts resulting in the destruction of the tissue architecture (Fig. 2d). Oocyst contained numerous sporocysts and mature sporocysts contained sporozoites inside (Fig. 2e, f). Young macrogamete from gamogony was found in arm of *Am. fangsiao* and cecum of *O. minor*, respectively (Fig. 2h, i). A zygote (Fig. 2g) was produced by the union of a microgamete and macrogamete.

Sporocysts of different treatments (fresh, fixed in buffered 10% seawater formalin, histological sections stained with hematoxylin and eosin) in *Am. fangsiao* and *O. minor* were

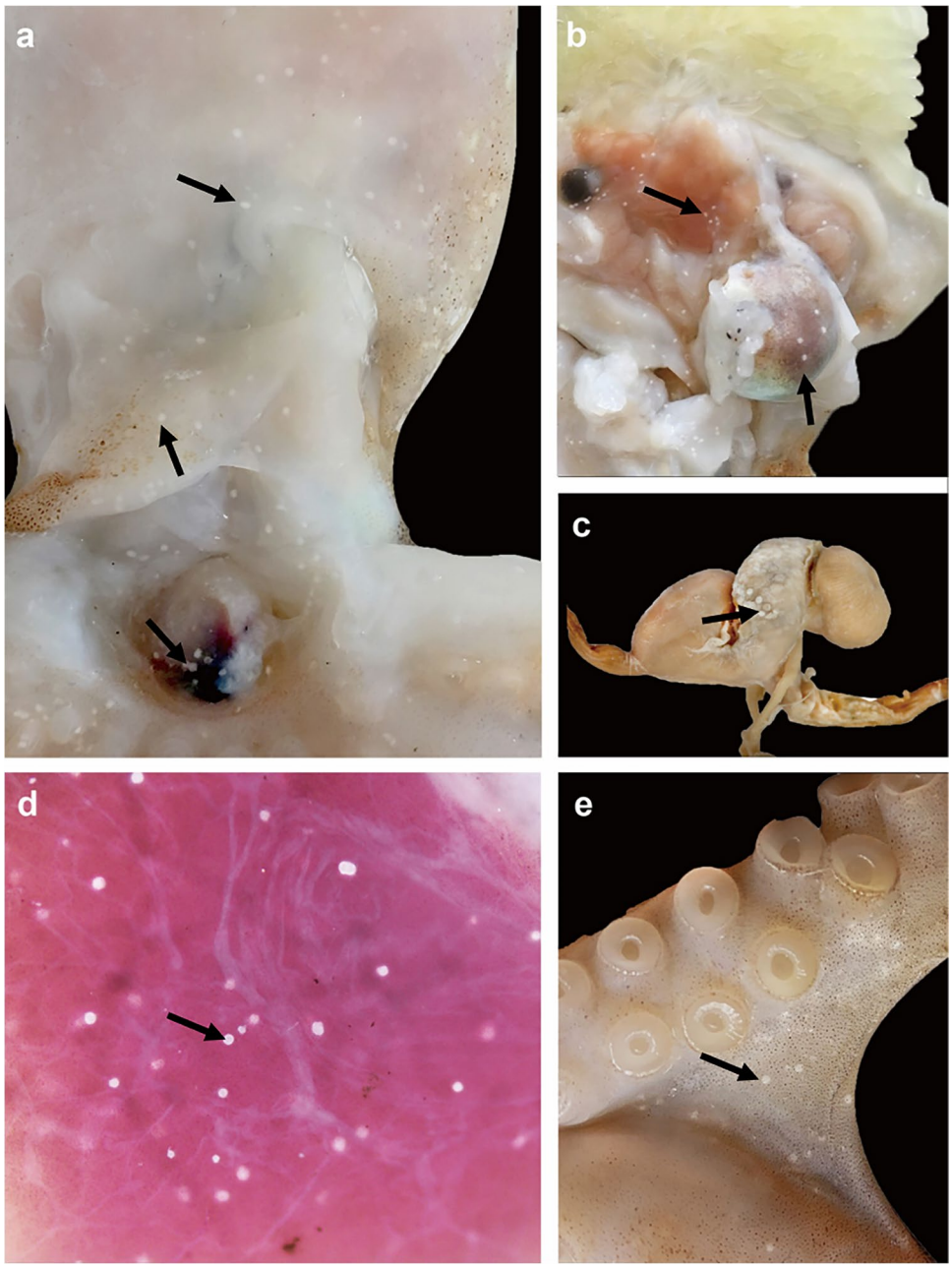

Fig. 1 Distribution of parasites in *Am. fangsiao* (**a**, **b**) and *O. minor* (**c**–**e**). **a** Severe infection of *Am. fangsiao*, arrows show large number of white cysts developing in the mantle, funnel, and buccal mass, respectively. **b** Anatomy of *Am. fangsiao*, arrows show white cysts in the digestive gland duct appendages and the digestive gland. **c** Part of the digestive system of *O. minor*, arrow shows white cysts distributed in the caecum. **d** Branchial heart of *O. minor*, arrow shows large number of white cysts in the branchial heart. **e** Arm of *O. minor*, arrow shows white cysts in the epidermis of interbrachial membrane

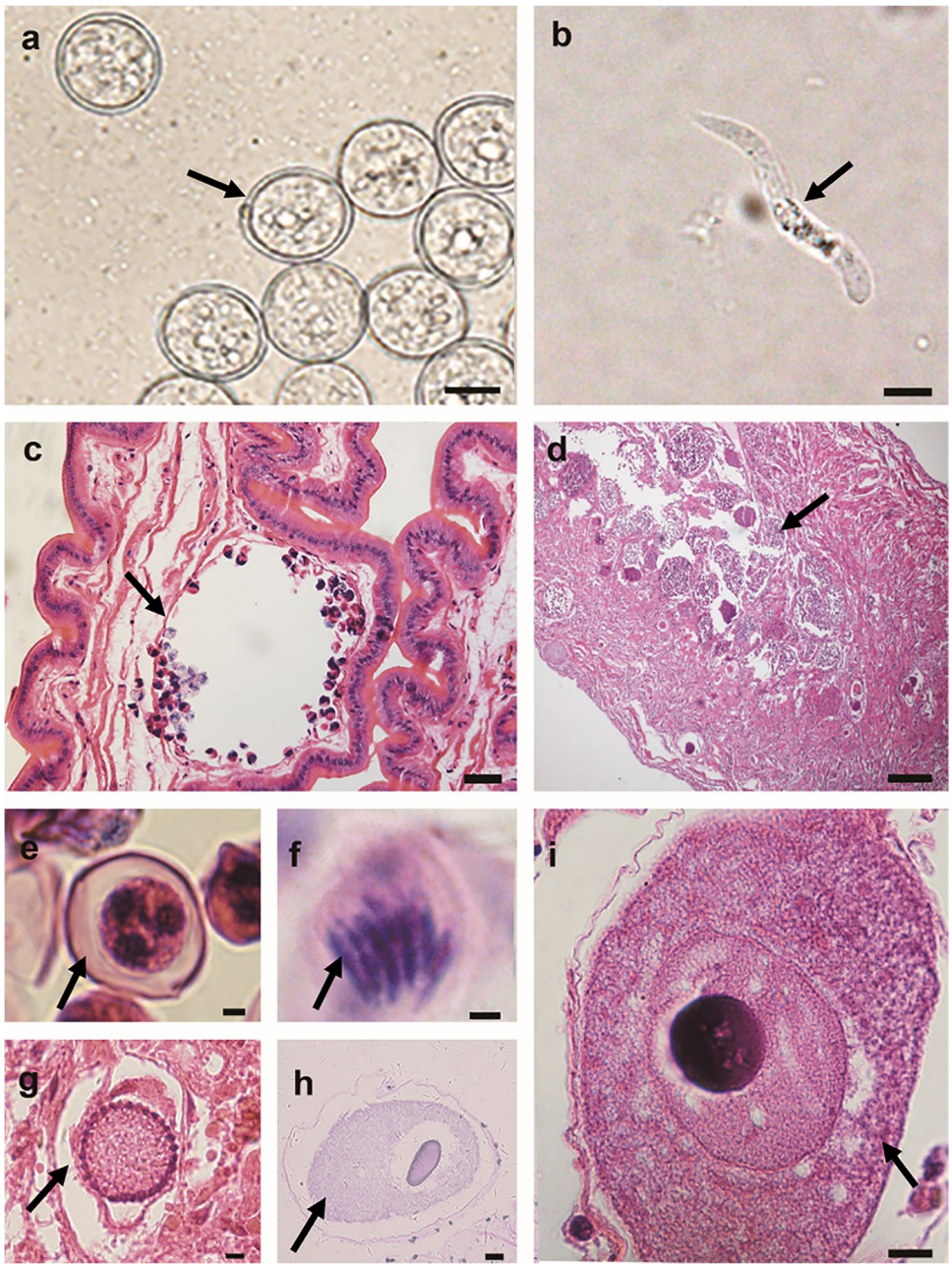

Fig. 2 *Aggregata sinensis*. Observation under an optical light microscope of fresh sporocysts and histological sections of heavily infected tissues in *Am. fangsiao* (**a**, **b**, **c**, **h**) and *O. minor* (**d**, **e**, **f**, **g**, **i**). **a** Fresh sporocysts, arrow shows a sporocyst, scale bars: 10 μm. **b** Free sporozoite from fresh sporocyst, scale bars: 5 μm. **c** Histological sections of *Am. fangsiao* crop, arrow shows oocysts, scale bars: 50 μm. **d** Histological sections of *O. minor* cecum infected by numbers oocysts (arrow), scale bars: 200 μm. **e** Mature sporocysts containing three sporozoites in transverse section (arrow), scale bars: 2 μm. **f** Mature sporocysts containing sporozoites, longitudinal section (arrow), scale bars: 2 μm. **g** Zygote after starting multiple divisions in cecum epithelium of *O. minor*, scale bars: 5 μm. **h** Young macrogamete in the arm of *Am. fangsiao* (arrow), scale bars: 20 μm. **i** Young macrogamete of *O. minor* cecum (arrow), scale bars: 10 μm

measured. Lengths and widths of sporocysts (Fig. 3) in *Am. fangsiao* and *O. minor* were estimated. There was no significant variation between *Am. fangsiao* and *O. minor* seen from the average values, but lengths of sporocysts had significant variation among different treatments (Fig. 3a), and widths of sporocysts had significant variation between hematoxylin and eosin-stained group and the other two groups (Fig. 3b).

Oocysts Oocysts were fully sporulated, shaped typically spherical or irregular; each oocyst contained numerous sporocysts. Oocysts were measured from histological sections stained with hematoxylin and eosin. Oocyst dimensions were measured from the ten longest oocysts in sections.

Oocysts in *Am. fangsiao* ($n = 10$, 7 hosts), lengths 246.61–492.44 (373.99 ± 73.57), widths 238.25–420.68 (308.29 ± 58.61)

Oocysts in *O. minor* ($n = 10$, 3 hosts), lengths 207.35–618.74 (403.57 ± 162.14), widths 136.31–350.52 (250.53 ± 87.45)

Sporocysts Mature sporocysts were typically spherical to slightly sub ovoid, and the surface of sporocyst capsule was spiny. Isolated sporocysts from formalin-fixed oocysts in *Am. fangsiao* and *O. minor* were measured as follows:

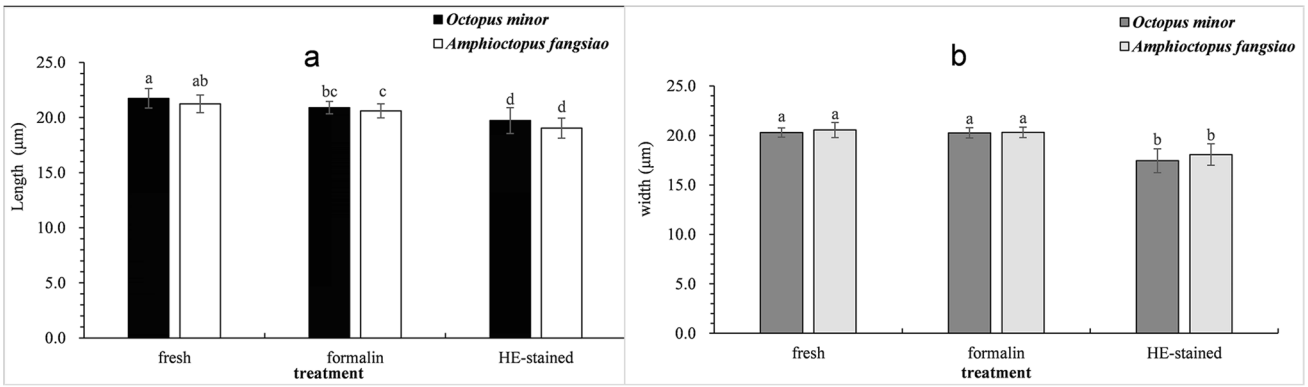

Fig. 3 The average (±SD) of sporocysts' size on different treatments (fresh, fixed in buffered 10% seawater formalin, histological sections stained with hematoxylin and eosin). Values with different letters indicate significant differences ($P < 0.05$)

Sporocysts in *Am. fangsiao* ($n = 30$, 1 host), lengths 19.42–21.98 (20.61 ± 0.63), widths 19.12–21.55 (20.31 ± 0.53)
Sporocysts in *O. minor* ($n = 30$, 3 hosts), lengths 19.89–22.32 (20.90 ± 0.57), widths 19.25–21.30 (20.26 ± 0.52)

Sporozoites They were typically curled in sporocysts. Isolated sporozoites from formalin-fixed sporocysts in *Am. fangsiao* and *O. minor* were measured. The number of sporozoites in sporocysts were observed and measured based on histological sections.

Sporozoites in *Am. fangsiao* ($n = 10$, 1 host), lengths 17.81–24.90 (22.77 ± 2.02), widths 2.22–3.42 (2.74 ± 0.41); the number of sporozoites in sporocysts (n = 18, 3 hosts) of *Am. fangsiao* included 7–18

Sporozoites in *O. minor* ($n = 10$,1 host), lengths 19.93–23.18 (21.72 ± 1.06), widths 2.05–3.32 (2.68 ± 0.35); the number of sporozoites in sporocysts (n = 10, 3 hosts) of *O. minor* included 5–16

Phylogenetic analysis

A total of five 18S rRNA gene partial sequence were assembled to obtain partial 18S DNA sequences. The BI and ML phylogenetic trees constructed based on 18S rRNA gene sequence of 1864 bp showed similar topologies (Fig. 4) and illustrated the evolutionary relationships of Apicomplexa. *Ag. sinensis* (the sequences JS.1 and JS.2 amplified from *Am. fangsiao*, the sequences JS.3, SD.1 and TW.1 amplified from *O. minor*) formed a consistent cluster, and *Ag. sinensis* was a part of the branches of Aggregatidae. The Aggregatidae clade included *Ag. octopiana*, *Ag. eberthi*, and *Ag. sinensis*. The family Aggregatidae clustered as a sister group with the

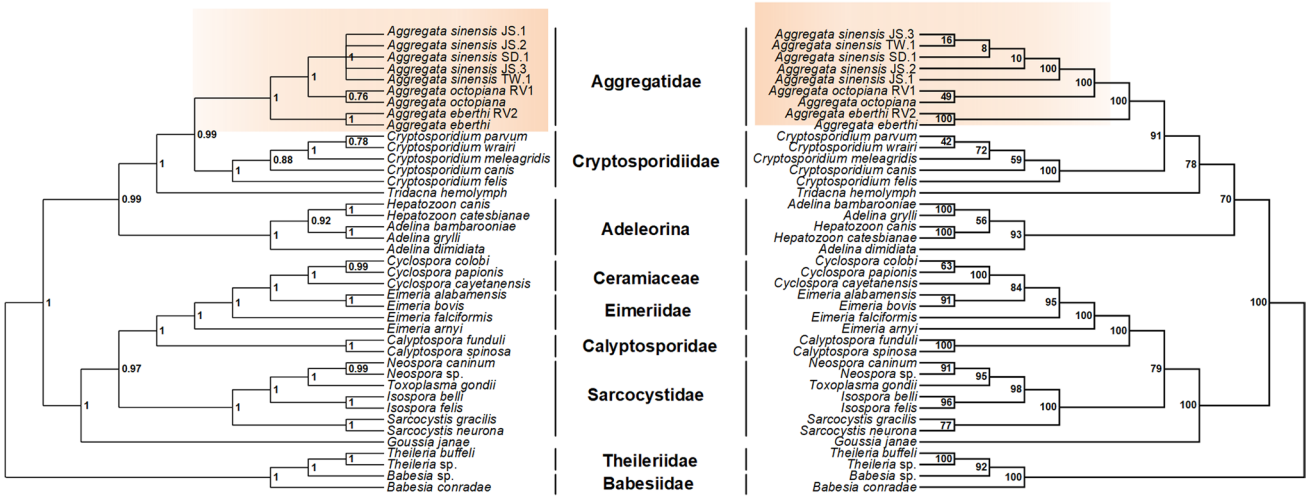

Fig. 4 Phylogenetic relationships of Conoidasida. Bayesian (left, numbers in nodes are posterior probabilities) and maximum likelihood (right, numbers in nodes are bootstraps) of Conoidasida evolutionary relationships under the GTR + G evolutionary model are shown above branches

family Cryptosporidiidae, successively. Adeleidae clustered as a sister group with the Aggregatidae-Cryptosporidiidae cluster.

Taxonomic summary

Type-host: *Amphioctopus fangsiao* (d'Orbigny [in Férussac & d'Orbigny], 1839–1841) (Octopoda: Octopodidae).

Other host: *Octopus minor* (Sasaki, 1920) (Octopoda: Octopodidae).

Type locality: Lianyungang in Jiangsu at 34° 49.00′ N, 119°20.06′ E, Qingdao in Shandong at 36° 04.00′ N, 120°22.59′ E, Rongcheng in Shandong at 37° 09.43′ N, 122°29.10′ E, Yilan in Taiwan at 24° 41.36′ N, 121°44.45′ E, Nanjidao in Zhejiang at 27° 29.12′ N, 121°00.29′ E.

Type specimens (Syntypes): histological section in cecum of *O. minor* containing mature sporocysts was deposited in at the Fisheries College, Ocean University of China, (OUC-LSGB-HE0002).

Prevalence: see Table 1.

Site of infection: Gamogonic and sporogonic developmental stages located in the mucosa of the host digestive tract and in the muscle layer of arms.

Etymology: The specific name "sinensis" is derived from the geographic area of the type-locality (China) where the parasites were found.

Discussion

At present, 10 named *Aggregata* species have been reported to occur in 10 species cephalopod hosts worldwide including octopus, squid, and cuttlefish (Schneider 1875; Poynton et al. 1992; Gestal et al. 1999, 2000, 2005, 2010; Sardella et al. 2000). Traditionally, species of *Aggregata* are distinguished on the phenotypic characteristics, such as aspects of sporocyst structure (shape, size, and thickness of the outer surface wall) and number and size of the sporozoites contained within each sporocyst and data on host specificity (Sardella et al. 2000; Gestal et al. 2005, 2010). Comparing the sporocysts of *Aggregata* in different hosts (*Am. fangsiao* and *O. minor*), lengths and widths of different treatments (fresh, fixed in buffered 10% seawater formalin, histological sections stained with hematoxylin and eosin) in different hosts showed no significant variation (Fig. 3). The results of phenotypic characteristics (Table 2) in this study showed little variation between the parasites in *Am. fangsiao* and *O. minor.* Furthermore, based on the molecular phylogenetic analyses presented herein, *Ag. sinensis* clustering (the sequences JS.1 and JS.2 amplified from *Am. fangsiao*, the sequences JS.3, SD.1 and TW.1 amplified from *O. minor*) in a single branch indicated that *Ag. sinensis* was a new species (Fig. 4). Therefore, this study extended the species number of *Aggregata* to 11, adds two species of octopus (*Am. fangsiao* and *O. minor*) to the host list making it 12 host species,

Table 2 Comparative data on the morphology of the sporogonic stages for species of *Aggregata* described from cephalopod hosts. Length and width measurements are given in µm as means. –: no data

Species	Cephalopod host	General locality	Oocysts		Sporocysts		Sporozoites		Reference
			Length	Width	Length	Width	No	Length	
Aggregata eberthi	*Sepia officinalis*	NE Atlantic, W Mediterranean	469	433	8.5	–	3	16	Labbé (1895)
A. kudoi	*S. elliptica*	NW Indian	260	180	12	–	6–12	17	Narasimhamurti (1979)
A. andresi	*Martialia hyadesi*	SW Atlantic	431	391	9.7	8.2	3	16–20	Gestal et al. (2005)
A. sagittata	*Todarodes sagittatus*	NE Atlantic	287	280	17	15	4–8	12	Gestal et al. (2000)
A. bathytherma	*Vulcanoctopus hydrothermalis*	NE Pacific	245.6	255.0	28.7	27.9	14–17	49	Gestal et al. (2010)
A. dobelli	*Enteroctopus dofleini*	NE Pacific	736	312	23	21	9–22	21	Poynton et al. (1992)
A. patagonica	*E. megalocyathus*	SW Atlantic	519	346	13	12	8	18	Sardella et al. (2000)
A. octopiana	*Octopus vulgaris*	NE Atlantic, W Mediterranean	612	402	11–15	–	8	20	Schneider (1875) Gestal et al. (1999)
A. millerorum	*O. bimaculoides*	NE Pacific	441	226	16	14	8–10	24	Poynton et al. (1992)
A. valdessensis	*O. tehuelchus*	SW Atlantic	250	169	10	10	4–8	17	Sardella et al. (2000)
A. sinensis n. sp.	*Amphioctopus fangsiao*	W Pacific	373.99	308.29	20.61*	20.31**	7–18	22.77***	Present study
	O. minor	W Pacific	403.57	250.53	20.90 *	20.26 **	5–16	21.72 ***	Present study

*, **, ***Values in *A. fangsiao* and *O. minor* indicate significant differences ($P < 0.05$)

and extends the distribution for *Aggregata* to Western Pacific region (Table 2).

Comparative data on morphology and morphometry of *Aggregata* species based on sporogonial stages are shown in Table 2, including size of oocysts, size of sporocysts, and number and size of sporozoites contained within sporocysts. *Aggregata sinensis* is principally distinguished from the other 10 species by the large size of its sporozoites. Another distinct difference between these species is the size of oocysts and sporocysts. *Aggregata sinensis* has smaller oocysts and larger sporocysts as compared to *Ag. octopiana* and *Ag. millerorum* (Poynton et al. 1992) (Table 2).

Traditionally *Aggregata* parasites were considered to host specificity of their definitive host, cephalopods (Hochberg 1990). Poynton et al. (1992) have reported that *Ag. dobelli* and *Ag. millerorum* only infected *Enteroctopus dofleini* Wülker, 1910 and *Octopus bimaculoides* Pickford and McConnaughey, 1949, respectively. *Aggregata bathytherma* (Gestal et al. 2010) has been found in the digestive tract of *Vulcanoctopus hydrothermalis* González & Guerra, 1998. However, *Aggregata sinensis* had two hosts comprising two genera. So identification of new *Aggregata* species based on host specificity was not corroborated by our data.

The study found the presence of large number of visible oocysts in *Am. fangsiao* and *O. minor* infection. When in the hosts of mild infection (number of oocysts /$cm^2 < 20$ in crop or cecum), oocysts were only found in the digestive tract. Based on macroscopic level observation and analysis of histopathological characteristics, we consider that infected tissue area is similar to that previously described for *Aggregata* species (Mladineo & Bočina 2007; Gestal et al. 2010). In hosts with severe infection (number of oocysts / $cm^2 \geqq 20$ in crop or cecum), the parasites were irregularly distributed in digestive tract and the unusual organs such as the connective tissue of buccal mass, digestive gland duct appendages, and the digestive gland, mesentery, mantle, and arms of the infected octopus (Fig. 1a–e). The parasites that migrate outside the digestive tract lead to an extra-intestinal coccidiosis that is easily identified because of white oocysts on the tissue evident on gross exam (Mladineo & Bočina 2007; Castellanos-Martínez et al. 2019). Our study shows that the infection first appears in the digestive tract, and it may further support for the mode of transmission through the food web (Hochberg 1990). Similar results have been reported (Gestal et al. 2010). When a cephalopod ingests an infected intermediate host, the whole digestive tract of the cephalopod may be infected, leading to impaired absorption of nutrients and deterioration of fitness due to "malabsorption syndrome" (Gestal et al. 2002b).

Based on the histopathological examination of this study, the seriously infected host organ architecture was replaced by the oocysts of *Aggregata* (Fig. 2d). This result was coincident with the previously described findings for other *Aggregata* species (Gestal et al. 2002a; Baldascino et al. 2017). Poynton et al. (1992) have showed that the octopods such as *O. bimaculoides* and *O. dofleini martini* acquired *Ag. millerorum* and *Ag. dobelli*, respectively, in the wild. As an important infectious agent, *Aggregata* infects *Am. fangsiao* that may have affected the process of artificial temporarily culture (Ren et al. 2019). However, *Aggregata* infection may not be a lethal factor for octopus (Hochberg 1990).

Topologies resulting from molecular-based BI and ML analyses showed a highly supported monophyletic clade containing Aggregatidae, Cryptosporidiidae, and Adeleorina (posterior probability (PP) = 1, bootstrap (BS) = 100, Fig. 4). Within this clade, *Ag. sinensis* (the sequences JS.1 and JS.2 amplified from *Am. fangsiao*, the sequences JS.3, SD.1 and TW.1 amplified from *O. minor*) could be considered a new species (PP = 1, BS = 100, Fig. 4), and it constituted a clade along with the members of *Ag. octopiana* and *Ag. eberthi*. Our phylogenetic analyses suggested that the family Aggregatidae and Cryptosporidiidae should constitute a clade as a sister group with high nodal support (PP = 0.99, BS = 91, Fig. 4). Adeleidae were ancestral to the family Aggregatidae and Cryptosporidiidae. However, our results were not consistent with the previous studies (Kopečná et al. 2006). The ML phylogenetic analyses (Kopečná et al. 2006) showed that *Aggregata* species were evolutionarily close to *Adelina* rather than *Cryptosporidium*. In addition, the close relationship of *Aggregata* and *Tridacna hemolymph apicomplexan* (PP = 1, Fig. 4) was strongly supported rather than *Adelina* genus, which was different from the previous coccidian studies, where the *Aggregata* genus was evolutionarily closer to *Adelina* genus (Castellanos-Martínez et al. 2013).

The description of *Ag. sinensis* in *Am. fangsiao* and *O. minor* represents the first two-host parasitic species of *Aggregata* in Western Pacific waters. Further investigations are warranted of the life cycle and pathologic features of *Aggregata* in cephalopods of the Western Pacific.

Acknowledgements We thank Prof. Chungcheng Lu (Museum Victoria, Australia) and Mr. Qingqi Zhang for providing experimental samples, respectively. In addition, we would like to thank Dr. Jinhai Wang and Dr. Yan Tang for the advice of the experiments design, as well as Prof. CC Lu for reviewing the manuscript and his valuable comments.

Author contribution Jing Ren and Xiaodong Zheng conceived and designed the experiments together. Jing Ren contributed significantly to analysis and manuscript preparation. Xiaodong Zheng helped perform the analysis with constructive discussions and revise the manuscript.

Funding National Natural Science Foundation of China, Grant/Award Number: 31672257; Fundamental Research Funds for the Central Universities, Grant/Award Number: 201762017.

Declarations

Ethics approval Approval was obtained from the ethics committee of Ocean University of China. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent for publication Manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

Conflict of interest The authors declare no competing interests.

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