Molecular phylogenetic analysis of the coccidian cephalopod parasites *Aggregata octopiana* and *Aggregata eberthi* (Apicomplexa: Aggregatidae) from the NE Atlantic coast using 18S rRNA sequences

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Abstract

The coccidia genus *Aggregata* is responsible for intestinal coccidiosis in wild and cultivated cephalopods. Two coccidia species, *A. octopiana,* infecting the common octopus *Octopus vulgaris*, and *A. eberthi*, infecting the cuttlefish *Sepia officinalis*, are identified in European waters. Their morphology has been extensively studied and *A. octopiana* was redescribed in octopuses from the NE Atlantic Coast (NW Spain) to clarify confusing descriptions recorded in the past. The present study sequenced the 18S rRNA gene in *A. octopiana* and *A. eberthi* from NE Atlantic coast to assess their taxonomic and phylogenetic status. Phylogenetic analyses revealed conspecific genetic differences (2.5%) in 18S rRNA sequences between *A. eberthi* from the Ria of Vigo (NW Spain) and the Adriatic Sea. Larger congeneric differences (15.9%) were observed between *A. octopiana* samples from the same two areas, which suggest the existence of two species. Based on previous morphological evidence, host specificity data, and new molecular phylogenetic analyses, we suggest that *A. octopiana* from the Ria of Vigo is the valid type species.

Keywords: *Aggregata octopiana; Aggregata eberthi;* Coccidia; *Octopus vulgaris*; *Sepia officinalis*; 18S rRNA

Introduction

Coccidians are obligate intracellular parasites that cause severe injuries mainly in poultry and livestock (Levine, 1985), but are also able to infect marine fishes and molluscs causing a detrimental effect on their physiological condition (Kent and Hedrick, 1985; Lom and Dyková, 1992). Cephalopods are specifically infected by coccidians of the genus *Aggregata* (Hochberg, 1990), which are heteroxenous parasites transmitted through the food-web. Sexual stages (gamogony and sporogony) occur inside the digestive tract of the definitive cephalopod host, whereas asexual stages (merogony) can be found inside the digestive tract of the intermediate crustacean host (Hochberg, 1990).

The genus *Aggregata* has a complex taxonomic history. It was first described by Lieberkuhn (1854) as a gregarine infecting *Sepia officinalis*. Schneider (1875) described a similar parasite infecting *Octopus vulgaris*, and later the genus was correctly classified as a coccidium (Schneider 1883). Then, the genus *Aggregata* was assigned by Frenzel (1885), who described merogonic stages of the parasite in *Portunus arcuatus.* Finally, the cephalopod coccidia were classified into the family Aggregatidae by Labbé (1899). The taxonomy of the *Aggregata* species has been controversial (Hochberg, 1990), and confusing descriptions have been recorded in the past. The species *A. octopiana* was first described by Schneider (1875) in *O. vulgaris* from the English Channel and Western Mediterranean Sea (Banyuls-sur-Mer, France), and redescribed in samples from the NE Atlantic Ocean (Gestal et al., 1999b). Comparative ultrastructural studies revealed that the taxon described by others as *Aggregata spinosa* in the same host and locations using light microscopy (Moroff, 1908), was synonymous to *A. octopiana* (Gestal et al., 1999b). Consequently, ten *Aggregata* species have been described to date (see Table 1), and three of them are found in European waters: i) *A. eberthi*, which is the representative type-species of the genus *Aggregata* and infects the cuttlefish *Sepia officinalis* from the Mediterranean Sea, English Channel and NE Atlantic Ocean (Dobell, 1925); ii) *A octopiana*, which infects the common octopus *O. vulgaris* and has

been re-described in hosts from the NE Atlantic Ocean (Gestal et al., 1999b); and iii) *A. sagittata,* which infects the flying squid *Todarodes sagittatus* (Gestal et al., 2000).

Understanding cephalopod pathogens is particularly relevant to the worldwide aquaculture of octopus species, which has to satisfy the global demand of cephalopods for human consumption (Iglesias et al., 2004; Domingues et al., 2007; Solorzano et al., 2009). The coccidian *A. octopiana* is known to cause heavy infections in the digestive tract of *O. vulgaris* (Pascual et al., 1996). Gamogonic and sporogonic stages cause the host digestive tissue to rupture (Gestal et al., 2002a). Malabsorption syndrome is a secondary effect of high infection rates, reducing the growth and condition of infected octopuses (Gestal et al., 2002b) and negatively impacting octopus culture (Gestal et al. 2007). Moreover, food sanitary regulations forbid commercialization of parasitized fishery and aquaculture products; hence, although *Aggregata* spp. are not zoonotic parasites, if oocysts are present in muscle, the octopus is rejected for human consumption (Peñalver et al., 2008).

Due to the increasing importance of coccidian diseases, particularly those caused by *Aggregata* species, the use of highly sensitive molecular methods for parasite diagnosis becomes crucial. Furthermore, molecular approaches are also useful to characterize parasites, complementing morphological descriptions, and phylogenetic classification (Jirků et al., 2009; Rueckert et al., 2011). The species *A. octopiana* and *A. eberthi* have been identified and characterized in the NE Atlantic coast according to morphological characters and host specificity (Gestal et al., 1999b; 2002c; Gestal and Pascual, 2002). In contrast, very little is known about their molecular classification and phylogenetic position, which could confirm their taxonomic affiliation within the genus and validate conservative and robust phenotypic characters used for species diagnosis. Kopečná et al. (2006) generated the first 18S rRNA sequences for *A. octopiana* and *A. eberthi* from Croatia (Adriatic Sea); however, the phylogenetic position of both coccidians remained unresolved.

In this study, we generated new 18S rRNA nucleotide sequences for *A. octopiana* and *A. eberthi* from the NE Atlantic coast (Galicia, NW Spain) to assess their phylogenetic position, complement existing morphologic descriptions and validate their phenotypic characters.

Material and Methods

Sampling and microscopic identification

Aggregata octopiana was isolated from a pool of ten infected octopuses of the species *Octopus vulgaris*, while *Aggregata eberthi* was isolated from a pool of ten infected cuttlefishes of the species *Sepia officinalis*. Both cephalopod species were collected by traps, an artisanal gear used by local fishermen from the Ria of Vigo, Spain (24º 14.09'N, 8º 47.18'W). The oocysts are easily observed as white spots on the digestive tract. Thus in the laboratory, the presence of *Aggregata* was assessed macroscopically in each of the cephalopod hosts, white oocysts were extracted from fresh caecum and intestine. Coccidians were identified using light microscopy and Scanning Electron Microscopy (SEM) to analyse morphology and dimensions of the fresh sporocyst and by histological analysis of the caecum, which is the target organ of the infection. The infected tissue was fixed in Davidson, embedded in paraffin wax and sectioned using a Microm HM-340 E microtome. Sections at 4 µm were stained with H-E according to standard procedures (Culling et al., 1985). For Scanning Electron microscopy (SEM), purified oocyst suspension was fixed 4 h in 2.5% glutaraldehyde in 0.2M cacodylate buffer (ph 7.4) at 4ºC and washed for 30 min in the same buffer. After dehydration in ethanol series, samples were critical point dried in CO_2 using a Polaron E3000 and sputter-coated in a Polaron SC500 using 60% gold-palladium. Analysis was performed with a Philips XC30 SEM operated at 10-20kV.

Isolation and purification of the parasite

The infected digestive tract of cephalopods was dissected and homogenized in 10 ml of filtered sea water (FSW) 1% Tween80 using an electric tissue grinder (IKA-Ultra Turrax T-25). Tissue homogenates were filtered twice with nylon meshes of 100 µm and 41 µm, respectively, to remove tissue fragments. The filtrate was then centrifuged at 1000 *x g* for 5 min in a centrifuge Beckman GS-15R. The sporocyst were purified by density gradient centrifugation method according to Gestal et al. (1999a), counted in a Neubauer chamber to standardize the sample at 2 x 10^6 spororocyst/ml and finally, sporocyst were preserved in 70% ethanol.

DNA extraction

Genomic DNA was extracted from *A. octopiana* and *A. eberthi* sporocysts. Sporocysts were resuspended in 500 µl of extraction buffer (NaCl 100mM, EDTA 25mM pH 8, SDS 0.5%) and opened by sonication on ice (5 cycles, 40W, 50 s) to release sporozoites. After Proteinase K (Sigma) digestion (1 mg ml^{-1}) at 37ºC overnight, the DNA was purified following the phenol:chloroform:isoamil alcohol extraction method, as described by Sambrook et al. (1989). DNA was precipitated with ethanol and sodium acetate overnight at -20 ºC. The precipitated pellet was resuspended in 50 µl of Tris-EDTA (TE) buffer.

DNA amplification, cloning and sequencing

The small subunit 18S rRNA gene of both coccidia species was amplified by PCR using conserved primers designed for *Aggregata* spp. (Kopečná et al. 2006) and derived from GenBank sequences: (Aggregata 1-F: 5´-ATGATGAAACTGCGAAGAGC-3´; Aggregata 2-R: 5´-CGACGGTATCTGATCGTCTT-3´; Aggregata 3-F: 5´-GGGGGTATTTGTATTTAACAAGCA-3´; Aggregata 4-R: 5´-CCTACGGAAACCTTGTTACGA-3´). Aggregata primers 1-2 (positions 76-1008) amplify the initial 970 bp of the 18S rRNA gene, whereas Aggregata primers 3-4 (positions 871-1781)

amplify the next 915 bp. PCR reactions were performed in a total volume of 25 µl containing 1 µl 10mM dNTP mix, 0.25 µl *Taq* DNA polymerase (Roche), 2.5 µl *Taq* 10x buffer, 1 µl 2.5 mM $MgCl_2$, 1 µl of each primer (10 µM) and 1 µl of template DNA at 100 ng μl^{-1}. The temperature profile for primers 1-2 included an initial denaturation at 94°C for 10 min; 35 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. For primers 3-4, we used an annealing temperature of 55°C. PCR products were separated on 1% agarose in TAE 1x buffer gels (w/v), stained with ethidium bromide including a 100-bp ladder size standard (Invitrogen) and visualized using UV light. Fresh PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen) according to the protocol supplied by the manufacturers and transformed in TOP 10 F´competent bacteria *Escherichia coli* (Invitrogen). Screening of clones carrying 18S rRNA-coding region fragments was performed by PCR adding the positive colony directly to the PCR mixture reaction using the corresponding Aggregata primers. Positive clones were purified by digestion with the enzymes exonuclease I and shrimp phosphatase (SAP) (Amersham Pharmacia Biothech) for 1h at 37°C. The enzymes were then denatured for 15 min at 80°C. The purified PCR products were bi-directionally sequenced using the proper *Aggregata* pair of primers and using ABI 3130 Genetic Analyzer according to the manufacturer´s directions (Applied Biosystems). Sequenced fragments from multiple clones belonging to each of the two *Aggregrata* species were then assembled together into two consensus sequences (see below). Based on the obtained cloned sequences, the specific primers RV-F: 5´-GCTTATTAAATCAGTTATAGTT-3´; RV-R: 5´ATATTTACACACATTCTAATTC-3´(positions 20-1619) were designed and used to amplify almost complete 18S rRNA sequences for each species (annealing temperature of 54°C). Primers Aggregata 5-F: 5´-AAGCTCGTAGTTGCAGTTTTGA-3´; Aggregata 6-R: 5´ AACTAAGAACGGCCATGCAC-3´ (positions 544-1178) equivalent to 662 bp were designed to amplify the internal sequence at an annealing temperature of 54ºC. All the sites in

these new two sequences were also present in the two entire consensus sequences assembled from multiple *Aggregata* clones.

Phylogenetic analysis

In addition to the new 18S rRNA sequences generated in this study for *A. octopiana* and *A. eberthi* from Ria of Vigo, sequences of 33 Apicomplexa taxa available in GenBank were used in the phylogenetic reconstruction. The GenBank accession numbers of the18S rRNA gene sequences used are as follows: *Theileria buffeli* (AF236097), *Theileria* sp. (U97055), *Babesia* sp. (AY048113), *Babesia conradae* (AF158702), *Eimeria alabamensis* (AF291427), *Eimeria bovis* (U77084) , *Eimeria falciformis* (AF080614), *Eimeria arnyi* (AY613853), *Cyclospora cayetanensis* (AF111183), *Cyclospora papionis* (AF111187), *Cyclospora colobi* (AF111186), *Isospora belli* (U94787), *Isospora felis* (L76471), *Goussia janae* (AY043206), *Goussia carpelli* (GU479640), *Goussia metchnikovi* (FJ009244), *Sarcocystis gracilis* (FJ196261), *Sarcocystis neurona* (U07812), *Toxoplasma gondii* (L37415), *Neospora caninum* (GQ899206), Neospora sp.(BPA1 U17345), *Hepatozoon canis* (EF622096), *Hepatozoon catesbianae* (AF130361), *Calyptospora spinosa* (FJ904637), *Calyptospora funduli* (FJ904645), *Adelina grylli* (DQ096836), *Adelina bambarooniae* (AF494059), *Adelina dimidiata* (DQ096835), *Tridacna* hemolymph apicomplexan (AB000912), *Klossia helicina* (HQ224955) clon 43, *Klossia helicina* (HQ224956) clone 26, *Aggregata octopiana* from the Adriatic Sea (DQ096837), *Aggregata eberthi* from the Adriatic Sea (DQ096838). Representative species of *Babesia* and *Theileria* were used as outgroups.

All sequences were aligned in MAFFT v6 (Katoh et al., 2005; Katoh, 2008) under the Q-INS-i algorithm, which takes into account RNA secondary structure. Ambiguous regions in the resulting alignment were identified and removed using GBlocks 0.91b (Castresana, 2000). *Aggregata* phylogenetic relationships were inferred using maximum likelihood (ML) and Bayesian inference coupled with Markov chain Monte Carlo (BMCMC). ML trees were built

in RAxML v7.2.0 (Stamatakis et al., 2008) using 1,000 searches and 10 runs. JModelTest v1.0.1 (Posada, 2009) was used to select the appropriate model of evolution under the Akaike Information Criterion (Posada and Buckley, 2004). The general time reversible (GTR) model (Tavaré, 1986), with invariable sites (I = 0.13) and gamma distribution (G = 0.63) to account for the among site rate heterogeneity was chosen. Clade support was assessed using the non-parametric bootstrap procedure (Felsenstein, 1985) with 5,000 bootstrap replicates run in the portal CIPRES Science Gateway portal (Miller et al., 2010). BMCMC trees were built in MrBayes v3.1.2 (Ronquist and Huelsenbeck, 2003). Three independent BMCMC analyses were run in CIPRES with each consisting of four chains. Each Markov chain was started from a random tree and run for $5x10^6$ cycles, sampling every 1,000th generation. Model parameters were unlinked and treated as unknown variables with uniform default priors and they were estimated as part of the analysis. Convergence and mixing were monitored using Tracer v1.5 (Rambaut and Drummond, 2009). All sample points prior to reaching stationary were discarded as burn-in. The posterior probabilities for individual clades obtained from separate analyses were compared for congruence and then combined and summarized on a 50% majority-rule consensus tree.

Results

Phenotypic identification of both *A. octopiana* and *A. eberthi* was performed by light microscopy, histology and SEM (Fig. 1). The morphology of the analyzed oocysts, sporocyst and sporozoites was consistent with those previously described as the type species from the NE Atlantic (Dobell, 1925; Gestal et al., 1999b) (see Table 1). A total of 13 and nine 18S rRNA partial sequence clones of *A. octopiana* and *A. eberthi*, respectively, were sequenced and assembled to obtain two overlapping 50% majority-rule consensus partial 18S DNA sequences of 1624 bp for *A. octopiana* and 1686 bp for *A. eberthi*. Variation among *A.*

octopiana clones was <0.55%, while variation among *A. eberthi* clones was <0.25%. In addition, single sequences of similar length obtained in one single PCR amplification were obtained for each species in order to confirm the assembled fragments. We used the consensus sequences in all phylogenetic analyses to take into account intra-species genetic variation. The consensus sequences of *A. octopiana* and *A.eberthi* were deposited in GenBank under the accession numbers KC188342 and KC188343 respectively.

ML and BMCMC phylogenetic searches generated identical topologies, hence only the ML tree with corrected branch lengths is presented (Fig. 2). In our analysis, two main coccidian clades were recognized, and one of them, the adeleorinid clade, included *A. octopiana* and *A. eberthi* (Fig. 2). The aggregatids from the Ria of Vigo and the Adriatic Sea formed a highly supported monophyletic group [(bootstrap proportion (bp) = 100%, posterior probability (pP) = 1.0)].

In our 18S rRNA ML tree, the minimum genetic divergence (corrected branch lengths) observed between different recognized coccidian species pairs ranged from 0.1 to 15.1%, with most cases above 3%. A genetic divergence of 15.9% was observed between *A. octopiana* from the Ria of Vigo and *A. octopiana* from the Adriatic Sea, whereas a genetic divergence of only 2.4% was found between *A. eberthi* from the Ria of Vigo and *A. eberthi* from the Adriatic Sea (Fig. 2).

Discussion

According to their histological and ultrastructural features, *Aggregata octopiana* and *A. eberthi,* have been successfully characterized in samples from the NE Atlantic Ocean by Gestal et al. (1999b; 2002c) and Gestal and Pascual (2002). Now, the molecular characterization carried out in this work showed a high identity with the two *Aggregata*

sequences available in GeneBank. Our phylogenetic analyses were consistent with what is known from previous coccidian studies, where Eimeriidae and Sarcocystidae families formed well-supported monophyletic groups (bp = 100%; pP = 1.00). Both ML and Bayesian phylogenies strongly support (bp = 99%, pP = 0.99) a clade formed by Aggregatidae and Adeleidae species. Within this clade, *Aggregata* species are evolutionary close to the adeleorinid *Hepatozoon, Klossia* and *Adelina*, the latter being the most basal group, as also suggested by the ML tree in Kopečná et al. (2006). This makes adeleorinids the most primitive group of the Eucoccidiorida, as stated by Levin (1985), sharing with aggregatids the formation of the sporocyst and the excystation through a longitudinal suture (Gestal et al. 1999b; Kopečná et al., 2006). However, our 18S rRNA tree, as in Kopečná et al. (2006), cannot accurately discriminate the basal relationships and position of the genus *Aggregata*. As has been previously suggested for other Apicomplexa (Barta et al., 2012), additional taxa and new genetic markers will be required to resolve the relationships among these parasites.

Our tree shows that *A. octopiana* and *A. eberthi* from Ria of Vigo cluster with *A. octopiana* and *A. eberthi*, respectively, from the Adriatic Sea (Fig. 2); however, the high genetic divergence (15.9%) observed between the two *A. octopiana* samples suggests that they represent different species (congeneric divergence). On the contrary, the genetic divergence estimated between *A. eberthi* samples (2.5%) falls within the range observed among populations from the same species (conspecific divergence).

Coupled with molecular data, phenotypic characters are also required to classify coccidians (Tenter et al., 2002). Among them, one of the most conspicuous characters is the number of sporozoites per sporocyst (Lom and Dyková 1992). From the Adriatic Sea, scarce and confusing records about the sporozoite number of coccidians infecting cephalopods exist. Mladineo and Jozić (2005), for example, reported *O. vulgaris* infected by coccidian of the genus *Aggregata* with four to five sporozoites. The Adriatic coccidia fit with the usual size range of *A. octopiana* from the NE Atlantic (Gestal et al., 1999b), but it does not agree with

the number of sporozoites (eight sporozoites per sporocyst for *A. octopiana*), and spiny sporocyst wall, which are the most noticeable specific features. Based on the number of sporozoites, the *Aggregata* sp. from Adriatic Sea resembles *A. sagittata*, which infects only the squid *Todarodes sagitattus* (Gestal et al., 2000), or *A. valdesensis*, which infects *Octopus tehuelchus* in SW Atlantic (Sardella et al., 2000). Interestingly, a second record by Mladineo and Bočina (2007) also mentions coccidia with eight sporozoites infecting the Adriatic *O. vulgaris*, which suggest the presence of *A. octopiana*. Thus, following morphologic characters, these records suggest two different *Aggregata* species infecting *O. vulgaris* in the Adriatic Sea. In addition, the absence of consistent and reliable morphological information about the coccidia sequenced by Kopečná et al. (2006) makes it difficult to identify the Adriatic *Aggregata* sp. correctly. Therefore, a detailed morphological characterization and accurate identification of the *Aggregata* species occurring in the Adriatic Sea is needed.

Octopus vulgaris has a worldwide distribution including the Southern Indian Ocean (Roper et al., 1984; Guerra et al., 2010). This octopod is now considered to form different populations with differences in reproductive structures and parasite specificity (Mangold, 1998; Guerra pers. comm.). Because coccidia are host-specific parasites, the distinct number of sporozoites in coccidia recorded from the Adriatic Sea (Mladineo and Jozić; 2005; Mladineo and Bočina, 2007) suggests the possibility of different octopus populations harboring different *Aggregata* parasites.

Therefore, based on previous morphological evidence (Gestal et al. 1999b; Gestal and Pascual, 2002; Gestal et al. 2002c), host-specificity data and the new molecular phylogenetic analyses herein presented, we conclude that the *Aggregata* species parasitizing the common octopus *O. vulgaris* from the Ria of Vigo (NW Spain, NE Atlantic) is *A. octopiana*, the valid type species. We also confirm the identification of *A. eberthi* infecting the cuttlefish *S. officinalis* from the same locality, validating the known phenotypic characters as useful diagnostic tools.

Further effort is needed to sample cephalopod hosts harboring *Aggregata* species at different geographic locations in the NE Atlantic and worldwide. Moreover, new genetic markers need to be combined with the 18S rRNA gene to improve phylogenetic analysis and complement the morphological taxonomy and classification of this poorly understood coccidian group.

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Figure legends

Fig. 1. Morphology of *Aggregata octopiana* and *Aggregata eberthi*. (A) Histological section of the digestive tract of O*ctopus vulgaris* showing sporocyst of *A. octopiana* containing 8 sporozoites. (B) Histological section of the digestive tract of *Sepia officinalis* showing sporocyst of *A. eberthi* containing 3 sporozoites. (C) SEM photograph of *A. octopiana* sporocyst showing the spiny wall. (D)SEM photograph of *A. eberthi* showing the smooth sporocyst wall.

Fig. 2. Maximum likelihood cladogram of Apicomplexa evolutionary relationships. Corrected branch lengths estimated under the GTR+G+I evolutionary model are shown above branches and bootstrap proportions (if ≥70%)/posterior probability (if ≥0.95) are shown in bold below branches.

Figure

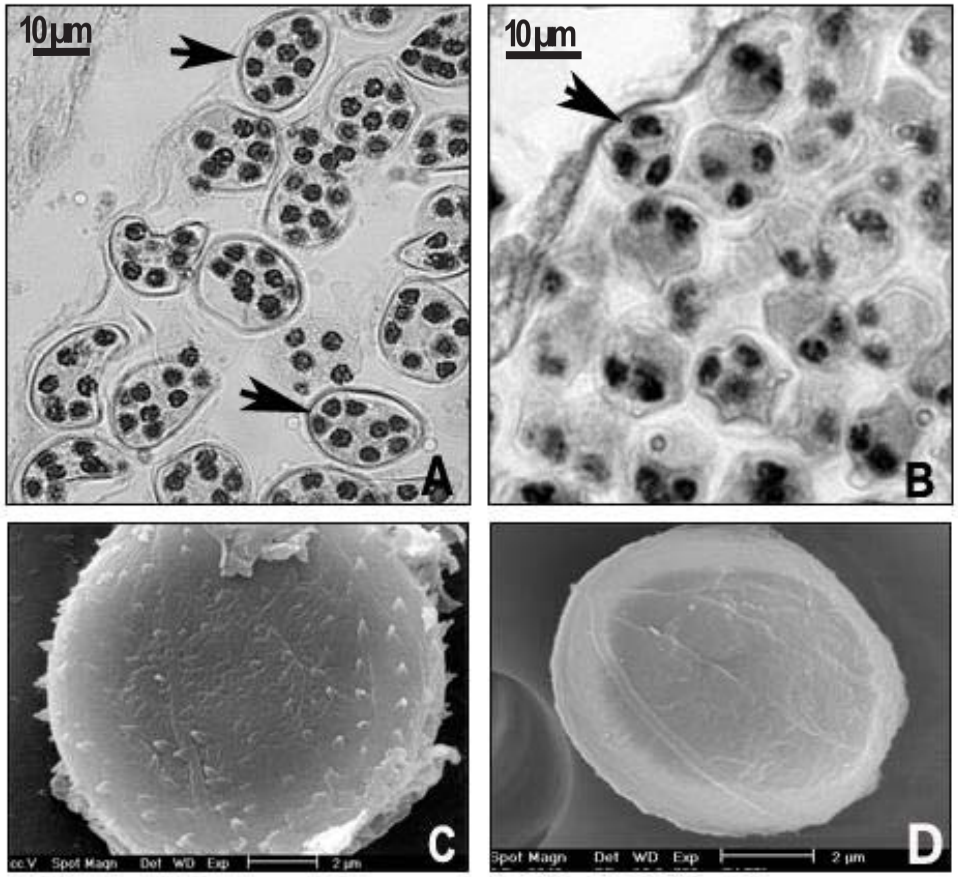
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Figure

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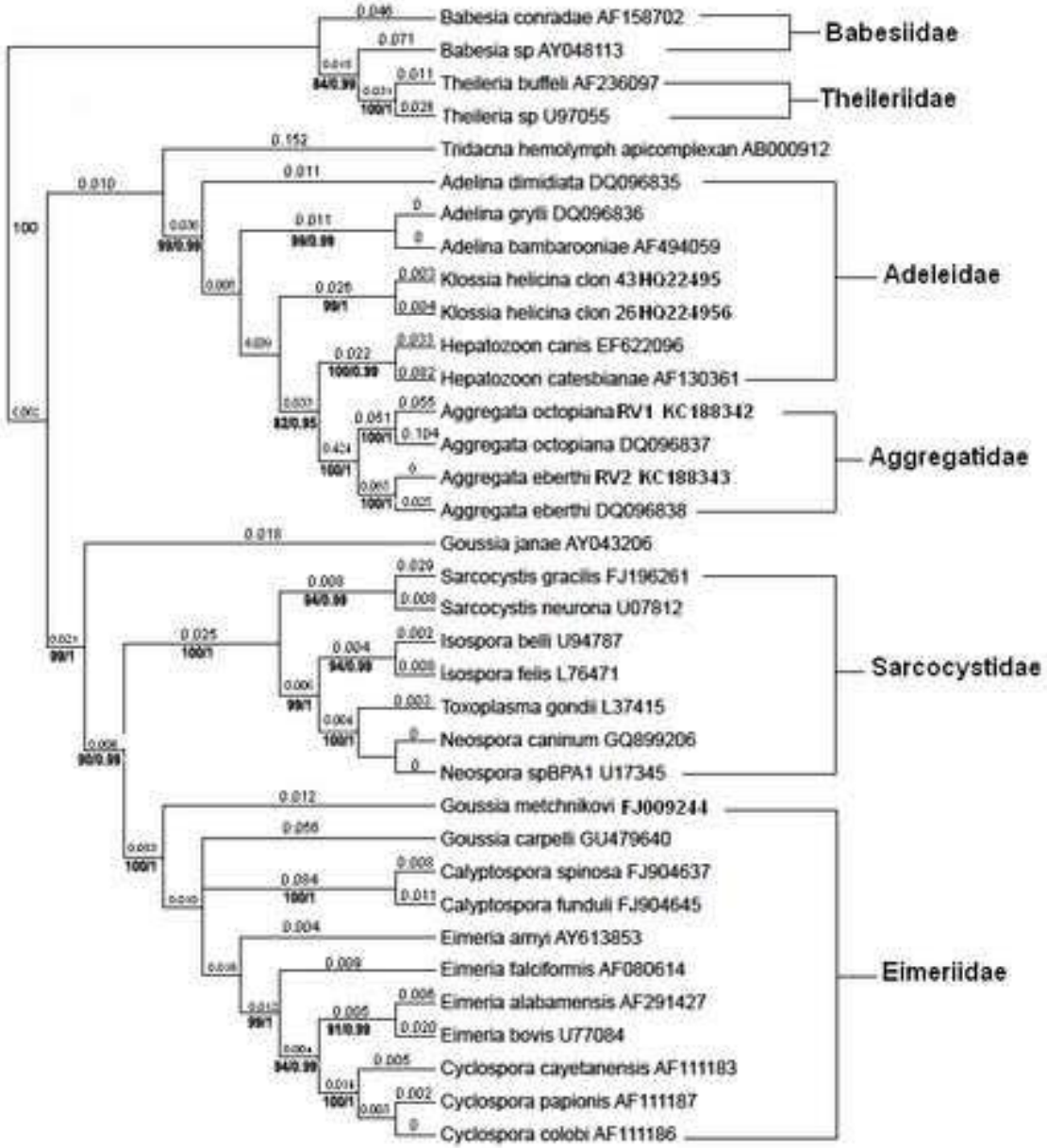

Table

Table 1. *Aggregata* species recorded from cephalopod hosts. Length and width measurements are given as ranges (- denotes no data available).

Aggregata species	Host	Locality (Ocean/Sea)	Sporocysts			Sporozoites		References
			length	width	cyst wall	nº	length	
octopiana	*Octopus vulgaris*	NE Atlantic, W Mediterranean	11-15	11-15	spiny	8	16-24	Gestal et al. (1999b) Schneider (1875)
Aggregata sp.	*O. vulgaris*	E Mediterranean (Adriatic Sea)	-	-	-	4-5 8	-	Mladineo and Jozic (2005) Mladineo and Bocina (2007)
dobelli	*Enteroctopus dofleini*	NE Pacific	18-31	15-27	smooth	9-22	18-23	Poynton et al. (1992)
millerorum	*O. bimaculoides*	NE Pacific	12-20	11-17	smooth	8-10	18-31	Poynton et al. (1992)
patagonica	*E. megalocyatus*	SW Atlantic	13	12	smooth	8	18	Sardella et al. (2000)
valdesensis	*O. tehuelchus*	SW Atlantic	10	10	-	4-8	17	Sardella et al. (2000)
bathytherma	*Vulcanoctopus hydrothermalis*	NE Pacific	27-32	24-32	smooth; thick	14-17	49	Gestal et al. (2010)
sagittata	*Todarodes sagittatus*	NE Atlantic	17	15	smooth; thick	4-8	12	Gestal et al. (2000)
andresi	*Martialia hyadesi*	SW Atlantic	9.7	8.2	smooth; thick	3	16-20	Gestal et al. (2005)
eberthi	*Sepia officinalis*	NE Atlantic, W Mediterranean	8-9	-	smooth	3	15-17	Labbé (1895)
kudoi	*S. elliptica*	NW Indian	9-14	-	smooth	6-12	16-18	Narasimhamurti (1979)