ORIGINAL PAPER

Morphological and Molecular Characterization of *Aggregata* spp. Frenzel 1885 (Apicomplexa: Aggregatidae) in *Octopus vulgaris* Cuvier 1797 (Mollusca: Cephalopoda) from Central Mediterranean

Running title: *Aggregata* spp. In *Octopus vulgaris*

Perla Tedesco^{a,b,1,2}, Camino Gestal^c, Kristina Begić^d, Ivona Mladineo^d, Sheila Castellanos-Martínez^{c,e}, Gaetano Catanese^{f,g}, Antonio Terlizzi^{a,h}, and Graziano Fiorito^a

^aDepartment of Biology and Evolution of Marine Organisms, Stazione Zoologica Anton Dohrn, Napoli, Italy

^bDipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento, CoNISMa, Lecce, Italy

^cInstituto de Investigaciones Marinas (CSIC), Vigo, Spain

^dInstitute of Oceanography and Fisheries, Split, Croatia

^eInstituto de Investigaciones Oceanológicas (IIO-UABC). Ensenada, Baja California, México

^fLaboratorio de Investigaciones Marinas y Acuicultura (Govern de les Illes Balears), Port d'Andratx, Spain

^gINAGEA (INIA-CAIB-UIB). Palma de Mallorca - Illes Balears, Spain

^hDipartimento di Scienze della Vita, Università degli Studi di Trieste, Trieste, Italy

Submitted November 16, 2016; Accepted August 22, 2017

Monitoring Editor: Frank Seeber

¹Current address: Dipartimento di Scienze Mediche Veterinarie, *Alma Mater Studiorum* Università di Bologna, Via Tolara di Sopra 50, 40064 Ozzano Emilia (BO), Italy

²Corresponding author; e mail perla.tedesco@unibo.it (P. Tedesco).

Coccidian parasites of the genus *Aggregata* are known to parasitize cephalopods as definitive hosts, however one of the genus members, *A. octopiana*, has shown an unresolved phylogeny within the same definitive host, the common octopus (*Octopus vulgaris*). Our study represents a large-scale investigation aimed at characterizing morphological traits and phylogeny of *A. octopiana* isolated from *O. vulgaris* inhabiting three distinct geographic areas of the central Mediterranean: The Adriatic, Ionian and Tyrrhenian Seas. The morphology of sporogonic stages of the parasite in octopus tissues was assessed by light and electron microscopy; molecular characterization has been carried out using the 18S rRNA locus. Our results support the hypothesis that two morphologically and genetically different *A. octopiana* infect *O. vulgaris* in the investigated areas of the Mediterranean Sea. Additional nuclear and mitochondrial markers for *Aggregata* should provide further information and better resolution of its phylogeny.

Keywords: *Octopus vulgaris*; *Aggregata octopiana*; Mediterranean Sea; Cephalopod; parasite.

Introduction

Coccidians of the genus *Aggregata* are among the most important infectious agents of wild and cultured cephalopods, infecting their digestive tract (Estévez et al. 1996). In the heteroxenous life cycle of this taxon, cephalopods act as definitive hosts where

sexual gamogonic and sporogonic stages of the parasite development occur, while the merogonic asexual stages are found in the intermediate crustacean host (Dobell 1925; Hochberg 1983).

Ten species are currently recognized worldwide (Castellanos-Martínez and Gestal 2013). Nevertheless, the taxonomic status of *Aggregata* in the Mediterranean is controversial and debated.

Moroff (1908) described 11 species of *Aggregata* infecting the common octopus *Octopus vulgaris* from Mediterranean Sea, and up to five species infecting the European cuttlefish *Sepia officinalis*. However, Moroff's systematic classification has been considered as too speciose (Naville 1925) and debated. As a consequence, most of the different species described by Moroff have been proposed as synonymous species (Dobell 1925).

Currently, *Aggregata octopiana* Schneider is considered as the sole valid species infecting *O. vulgaris* (Gestal et al. 1999b), while *A. eberthi* Labbé is the one known to parasitize *S. officinalis* (Dobell 1925). Molecular analyses have allowed the assessment of the taxonomic and phylogenetic status of these two *Aggregata* species occurring in European waters (Castellanos-Martínez et al. 2013).

Finally, Mladineo and Jozić (2005) suggested the possibility that morphologically and maybe phylogenetically different *Aggregata* spp. infecting *O. vulgaris*, may occur in the North Adriatic Sea.

Although *Aggregata* parasites are traditionally considered as species specific of their definitive host (Hochberg 1990), possibly displaying a lower species specificity in the crustacean intermediate host, knowledge on the biology and ecology of *Aggregata* is still too limited to support these assumptions.

From a taxonomic perspective, the fact that only ten *Aggregata* species are recognized to be parasitic in over six hundred living cephalopod species may support the view that this parasitic taxon is highly under-investigated. Thus, in most cases the species identification of the parasite infecting decapods and octopods worldwide remains unresolved (Hochberg 1990). Our knowledge is even more limited with respect to *Aggregata* spp. infecting the crustacean intermediate host.

The morphological characterization of coccidians is generally performed in sporogonic stages, while the characterization of merozoite morphology, even at an ultrastructural level, is difficult because taxonomical features in merozoites are still being validated, and successful experimental infections are required to establish their identity (Gestal et al. 2002). Therefore, despite the fact that merogonic stages of the genus *Aggregata* were reported from different species of brachyurans, anomurans and carideans from the Mediterranean Sea and the Atlantic Ocean (McDermott et al. 2010; Pixell-Goodrich 1950; Sardella and Martorelli 1997; Sprague and Couch 1971; Théodoridès 1965; Théodoridès and Desportes 1975), it is still difficult to associate any of these stages to its respective form infecting the cephalopod definitive host.

To improve our understanding of the biological diversification of this species, multiple approaches have been suggested, including the search for additional morphological characters to complement the classical diagnostic ones and for molecular data that may support the phylogenetic classification of *Aggregata*.

To date, reports of *Aggregata* spp. infection in *O. vulgaris* from the Mediterranean lack important information about the description of key-taxonomic characters of the parasite, such as the appearance of the sporocyst surface (which can be revealed solely by ultrastructural analyses), as well as molecular data; an exception is

represented by the work of Kopečná et al. (2006) that attempted a phylogenetic analysis of coccidian parasites by using nuclear SSU rRNA genes.

On the basis of the current knowledge, it is not possible to confirm the identification of *Aggregata* spp. infecting Mediterranean octopuses to the species level, and a detailed re-description of the parasite morphology as well as its molecular characterization through the use of genetic markers is still required.

Ultrastructural analysis of the sporocyst wall provides essential morphological information for the correct characterization of *Aggregata* spp. and may help in resolving taxonomic uncertainty. No ultrastructural characterization aimed at confirming the identity of *A. octopiana* in *O. vulgaris* from the Mediterranean Sea has, to the best of our knowledge, been carried out until now.

In this study we characterized parasitic stages of *Aggregata* spp. in specimens of *O. vulgaris* collected from three distinct geographic areas of the central Mediterranean: The Adriatic, Ionian and Tyrrhenian Seas. We applied morphological analysis of fresh mounts and histological sections (light microscopy), complemented by ultrastructural analyses sporogonic stages by transmission (TEM) and scanning electron microscopy (SEM). In addition, we carried out molecular analyses using the 18S rRNA locus and compared our data with those of *A. octopiana* described from Northeastern Atlantic.

A network based on the sequence data was constructed by NETWORK 4.6.1.1 (<http://www.fluxus-engineering.com>) using the median-joining network approach (Bandelt et al. 1999) with default settings.

Population structure was measured by AMOVA using F-statistics. AMOVA calculations were performed in ARLEQUIN 3.5 (Excoffier and Lischer 2010). We

maintained $\alpha = 0.05$ among all pairwise tests by controlling for the false discovery rate (Narum 2006).

Results

Gamogonic and sporogonic stages were observed in octopuses from all the investigated areas, with a prevalence of around 98% for Tyrrhenian Sea and as high as 100% for Adriatic and Ionian Sea. The non-cuticularized spiral caecum and intestine were the most infected organs, although infection was also extended to the cuticularized crop and oesophagus. In massive infections, the gills and laying mesentery, especially over digestive gland and gonads, were also infected. Similarly, in heavy infections sporogonic stages were additionally observed in the mantle musculature.

In Adriatic octopuses, cysts were observed in the whole cephalopod body including digestive tract (oesophagus, spiral caeca, intestine, rectum), gills, mantle and arms, with intensities varying from mild (few cysts barely observed by stereomicroscope) to heavy (milialy disseminated and noticeable by naked eye).

We documented for the first time an extra-intestinal infection in octopuses from the Tyrrhenian and Ionian Seas. In specimens from the Tyrrhenian Sea the infection was mostly restricted to caecum and intestine, but in some cases (approximately 10% of the examined octopuses) the infection also spread to the crop and the laying mesentery. A different infection pattern was observed in the Ionian Sea where, in addition to caecum and intestine, also the crop, the mesentery and the mantle musculature were always infected. Developmental stages of the parasite were visibly more abundant in these tissues (especially in the crop) rather than in caecum and intestine.

Morphological Description

The observation of fresh mounts of infected tissues (Fig. 1) allowed the measurement of sporogonic stages (sporocysts and oocysts) as well as the counting of sporozoites inside the sporocyst.

Minor morphometric differences between sporogonic stages in samples from the different geographic areas have been observed, with larger average sizes of oocysts and sporocysts in samples from the Tyrrhenian Sea compared to Adriatic and Ionian Seas (Table 1).

Histological examination of the digestive tract of *O. vulgaris* allowed the detection of gamogonic and sporogonic stages (Fig. 2A-D) of the parasite in the caecum, intestine, oesophagus, crop and mantle. In sporogonic stages, whenever a favorable transverse section was obtained, it was possible to confirm the number of sporozoites inside the sporocyst (Fig. 2D), as a discriminative feature in the identification of *Aggregata* species. Fresh and histological samples from the three investigated areas allowed the counting of eight sporozoites inside the sporocyst.

Histopathological Changes in the Host Tissues

Histological sections of Tyrrhenian and Ionian *Aggregata*-infected octopus samples revealed the presence of gamogonic and sporogonic stages of *Aggregata* in the lamina propria and submucosa, showing cell hypertrophy, inflammation and hemocyte infiltration (data not shown). In heavily parasitized organs (particularly crop), large portions of tissue were replaced with developmental stages of the parasite (Fig. 2C). Tissue deterioration was observed as result of heavy infection (data not shown).

Ultrastructural Characterization

Examination of sporogonic stages by TEM (Fig. 3) allowed the observation of sporocyst ultrastructure. In the sporocyst, two valves and a prominent dehiscence suture were distinguishable, the latter in mature sporocysts allowing the opening of the two valves and the release of the sporozoites. In all samples from the Tyrrhenian Sea and in some collected from the Ionian Sea, the sporocyst wall appeared to form a double layer; the smooth external and the spiny inner layer, showing electron dense projections, conical in shape. In tangential sections the wall appeared striated, composed of regular, electron-dense, transversal grooves intercalated by thinner grooves. In all samples from the Adriatic Sea and in another subset of samples collected from the Ionian Sea (71% of the sampled octopuses), the inner layer of the cyst wall appeared smooth. A range of recorded values for each area is shown in Table 1.

The appearance of purified sporocysts by SEM fully supported the TEM results, showing a spiny cyst surface in the Tyrrhenian Sea, and a smooth one in the Ionian and Adriatic Sea samples (Fig. 4).

Genetic Diversity and Population Divergence

A fragment of the partial 18S rRNA gene, with length of 662 bp, was sequenced from *A. octopiana* obtained from individuals of *O. vulgaris* sampled in the three investigated areas.

A total of 76 sequences of *A. octopiana* were aligned: 29 sequences from samples obtained from animals collected in the Tyrrhenian Sea (TYR), 28 sequences from octopuses collected in the Adriatic Sea (ADR), 15 sequences from animals collected in the Ionian Sea (ION), and finally 4 sequences from the Atlantic Ocean (ATL; Vigo - Spain).

Genetic diversity indices are presented in Table 2. The overall haplotype

diversity (hd) value was 0.819, higher for the Tyrrhenian and Ionian samples, but very low for the Adriatic that showed only 2 haplotypes. Only one haplotype was detected among the sequences of the 4 samples from the Atlantic. The values of nucleotide diversity were generally low, with the exception of the Ionian samples ($\pi = 0.056$).

The AMOVA analyses revealed significant values of variance (82.98%) among populations ($F_{ST} = 0.82978$; $P < 0.001$ and 17.02% of variance detected within populations (Table 3).

Phylogenetic Analysis

A total of 84 polymorphic sites, including 3 indels, were detected and 17 haplotypes were identified. Among all the haplotypes, 11 were unique and 6 were shared by more than one individual. All haplotype sequences of *A. octopiana* were deposited in GenBank under accession numbers: LC186909 - LC186925. When the *A. eberthi* sequence was included, we detected 126 polymorphic sites (Supplementary Material Table S1). Genetic distances among *A. octopiana* haplotypes ranged from 0.002 to 0.111 (Supplementary Material Table S2).

The tree topologies obtained using the different methods, gave similar results. Divergent *A. octopiana* 18S rRNA lineages split into three main clades (Fig. 5).

A consistent clustering of haplotypes from the Adriatic and part of the Ionian sequences (from H12 to H17) was evident, supported by high bootstrap values (Clade C). The remaining haplotypes clustered significantly in two supplementary separate groups, showing a monophyletic origin. One of these clusters resulted in only Atlantic and Tyrrhenian isolates (H1, H3, H4, H6) (Clade A), whereas the other group (Clade B) included Tyrrhenian and Ionian isolates. Haplotypes H1 and H17 were identical to those

of *A. octopiana* from Atlantic and Adriatic waters respectively, described in Castellanos-Martínez et al. (2013) and previously deposited in Genbank.

Likewise, the network analysis also revealed clusters with different distribution patterns, and the haplotypes in the clusters corresponded to those in the three clades presented in the phylogenetic tree (Fig. 6). Twenty-seven individuals from the Adriatic sampling locality shared the most common haplotype (H16). Five Ionian isolates (ION-05, ION-07, ION-10 ION-12, ION-13) showed a close relationship with Tyrrhenian isolates (H11), of which four presented a single haplotype (H7). The remaining ION isolates presented haplotypes (H12, H13, H14, H15) closely related to the Adriatic geographical area, whereas the only one Atlantic haplotype coincided with H1, also shown in Tyrrhenian samples (Fig. 6).

Genetic distances among *A. octopiana* clades were 0.022 (Clade A vs. B), 0.097 (Clade B vs. C) and 0.101 (Clade A vs. C). These last values were lower than those detected in the pairwise relations with others Apicomplexa genera, but not greatly distant to *A. eberthi* (ranging from 0.121 to 0.160; see Supplementary Material Table S2).

Discussion

The present study shows that infection by *Aggregata* coccidians is highly prevalent in *O. vulgaris* from all investigated areas. A high intensity of infection and histopathological effects were also observed, as previously reported for Mediterranean and non-Mediterranean areas (Gestal et al. 2002a; Licciardo et al. 2005; Mladineo and Jozić 2005; Mladineo and Bočina 2007).

Strong pathogenicity of the coccidia has been described in the Atlantic (Gestal et al. 2002a, c, 2007), Tyrrhenian (Licciardo et al. 2005) and Adriatic Seas (Mladineo and Bočina 2007; Mladineo and Jozić 2005). In the mucosa, ulceration and inflammation with occasional fibrosis and necrosis have been reported (Gestal et al. 2002a). A very similar infection pattern and pathogenicity have been documented for the Tyrrhenian Sea (Licciardo et al. 2005) and also observed in the present study.

An ultrastructural analysis of sporogonic stages of *Aggregata* found in samples of *O. vulgaris* across the Mediterranean was here performed for the first time and provided valuable taxonomic information.

Features of the outer sporocyst wall such as rough, spiny or smooth projection textures are key taxonomic features (Duszynski and Wilber 1997). In the present study, spiny protrusions were observed in sporocysts collected from the Tyrrhenian and Ionian Seas, coinciding with the ultrastructural characterization of *A. octopiana* in *O. vulgaris* from the NE Atlantic (Gestal et al. 1999b). In contrast, 71% of sporocysts from the Ionian and 100% of sporocysts from the Adriatic Sea appeared smooth. However, the wall thickness and the periodicity of transversal grooves partially overlaps with the ones reported for *A. octopiana* (Gestal et al. 1999), suggesting the existence of a different species/subspecies complex parasitizing *O. vulgaris* in the latter two areas.

Another important taxonomic feature for the identification of coccidians is the number of sporozoites inside each sporocyst (Lom and Dyková 1992). Information provided by ultrastructural analysis in combination with fresh and histological examination allowed the counting of eight sporozoites inside the sporocysts of octopuses collected from the Tyrrhenian, Ionian and Adriatic Seas.

Measurements of sporogonic stages resulted in minor variability across the three geographical areas, particularly with respect to the size ranges of oocysts and

sporocysts. Nevertheless, both features are more similar in samples from the Ionian and Adriatic Sea even at the ultrastructural level (Table 1), whereas the sizes of oocysts and sporocysts from the Tyrrhenian Sea are more similar to *A. octopiana* described from the NE Atlantic (Castellanos-Martinez et al. 2013).

Information provided by morphological analysis is supported by molecular-based data of 18S rRNA sequences analysis. The molecular phylogeny indicated three lineages that might partly reflect a history of divergence within the species fragmented into separate areas. We found a significantly separate cluster of Adriatic sample haplotypes, clustered with those of the Ionian Sea in the same clade of haplotypes (clade C), which suggests an allopatric speciation probably derived from circulation of water masses.

The surface circulation of the Adriatic Sea consists of a northern flow along the eastern coast that returns along the western coast. Water exchange with the Ionian Sea occurs through a similar current pattern and even when more saline Mediterranean water flows in along the eastern coast, Adriatic water then flows out along the western coast (Orlić et al. 1992).

In contrast, the clustering of Ionian isolates in the same clade C could be the result of unlimited dispersion between close areas.

Therefore, a strong divergence of *A. octopiana* from Ionian/Adriatic areas with respect to other areas was supported by the several times greater values of genetic distances of clade C haplotypes detected in relation to other clades, and not very different from the *A. eberti* distance values (Supplementary Material Table S2). On the other hand, isolates with wider geographic distance (Atlantic and Tyrrhenian clade A) did not show significant differences.

We cannot exclude that such a diversity may be somehow linked to the dispersal of *Aggregata* through their intermediate hosts.

Finally, the differentiation pattern between the less distant geographic areas (Tyrrhenian vs Adriatic, and vs Ionian Sea) could be explained by gene flow of octopus populations adapted to local conditions or restricted movements. In the Mediterranean Sea, the octopuses are considered to exhibit inshore-offshore migrations mostly linked to reproductive events (Mangold 1983). In addition, octopuses are also showing high site fidelity, unless searching for partners to mate or a safe shelter (e.g. Mereu et al. 2015). Migrations related to reproductive events have been shown to be linked to environmental conditions (e.g. temperature, food availability).

Our molecular data support the view of a retention of eddies in the central part of the Ionian Sea (El-Geziry and Bryden 2010), similar to what occurs in *O. vulgaris* in a circulatory cell of the current system in the Ria of Vigo (Gonzalez et al. 2005). Samples from clade B (Ionian/Tyrrhenian Sea) showed significant differentiation from clade A (ancestral lineage) and C (haplotypes of Adriatic/ Ionian Sea). However, it might also respond to two distinct basins inside the Mediterranean that act as geographical and hydrological frontiers: the western basin, in which the Tyrrhenian Sea (clade A and B) is included; and the eastern basin, with the Ionian and Adriatic Seas (clade C; see also El-Geziry and Bryden 2010).

Discrepancies at molecular level suggested that a different *Aggregata* sp. infects different *O. vulgaris* populations in the Mediterranean Sea. Our findings support the genetic divergence between the coccidian analyzed (consider also the discussion about the evolutionary history of host changes and selective pressure in Barber et al. 2000), and supports the hypothesis of Castellanos-Martinez et al. (2013).

We suggest the presence of different forms of the *A. octopiana* ‘complex’ and that this may be the result of different populations of the definitive host species, *O. vulgaris*. This is supported by the existence of a significant genetic structure of *O. vulgaris* as recently described for Mediterranean *O. vulgaris* populations (De Luca et al. 2014, 2016). Significant differentiations in the pairwise genetic comparison of Adriatic specimens of octopus and the ones from the Balearic Sea and Strait of Messina (Italy) were observed, but these were not evident considering Adriatic (Croatia) and Ionian (Porto Cesareo, Italy) areas (De Luca et al. 2016).

Similarly, in this study no significant genetic differentiation was found between the two geographic isolates of coccidians from the Adriatic and Ionian Seas specimens, indicating the existence of gene flow between *Aggregata* inhabiting the two areas. On the other hand a clear border between Adriatic *Aggregata* genotypes and the genotypes sampled in western areas (Tyrrhenian and Atlantic) has been identified.

Although the data obtained so far do not allow an attempt to correlate genetic diversity of the parasite and the host, future studies may shed light on this hypothesis.

Based on the overall evidence, the description of sporogonic stages of *Aggregata* in octopuses from two of the three sampled areas is consistent with the description of *A. octopiana* found in *O. vulgaris* from NE Atlantic (Castellanos-Martínez et al. 2013; Gestal et al. 1999) and would therefore confirm the presence of this species in the Tyrrhenian and Ionian Seas.

This is in accordance with previous investigations (Licciardo et al. 2005) which identified *A. octopiana* in wild *O. vulgaris* specimens from the Tyrrhenian coasts off Sicily. However, the number of sporozoites reported by the latter authors (20-26 sporozoites) differs from that of other descriptions (Castellanos-Martínez et al. 2013;

Gestal et al. 1999b; Schneider 1875), as well as from observations in the present investigation.

For the Ionian Sea, no epidemiological information on the presence of *Aggregata* spp. in wild cephalopods has so far been available. The current study would therefore represent a first screening with respect to cephalopod coccidia in the area.

In the Ionian and Adriatic Seas a new, previously undescribed *Aggregata* phenotype was also observed, which differs from *A. octopiana* species with respect to the appearance of the cyst wall.

In conclusion, phenotypic and genotypic traits of *A. octopiana* infecting common octopuses sampled across wide Mediterranean areas provide reasonable suspicion for the existence of a species complex composed of at least two different coccidian species/subspecies: one in Adriatic and Ionian Seas, and the other in the Tyrrhenian Sea and the Atlantic Ocean.

Phenotypic identification by light and electron microscopy coupled with molecular analysis may provide further insights on these differences and support the presumed existence of a new species/subspecies of *Aggregata*. Together with the 18S rRNA locus utilized herein, we believe that future studies will benefit from deeper analysis including additional, possibly faster-evolving, genetic markers or multilocus analysis that may further deepen our knowledge.

Methods

Areas selected for the collection of samples include one station for the Tyrrhenian Sea (Gulf of Napoli, Southern Italy: lat 40°41'N, long 14°08'E), three for the Eastern

Adriatic Sea (Kaštela Bay: lat 43°32'N, long 16°20'E; Island of Brač: lat 43°21'N, long 16°25'E; Island of Korčula: lat 42°57'N, long 16°38'E), and one for the Ionian Sea (Gulf of Taranto, Southern Italy: lat 40°14'N, long 17°49'E).

A total of 122 specimens of *Octopus vulgaris* were collected from the different Mediterranean localities (N = 10 Atlantic, N = 31 Tyrrhenian, N = 64 Adriatic, N = 17 Ionian). All specimens were dissected in order to macroscopically assess the presence of *Aggregata* in tissues. Fresh mounts of infected tissues were observed using light microscopy to examine the morphology of sporocysts and assess respective developmental stages.

Histological analyses: Infected tissue samples from different organs of the digestive tract (crop, intestine, caecum) were preserved in Davidson's fixative (Shaw and Battle 1957). Fixed tissues were embedded in paraffin wax, sectioned using a Microm HM-340-E microtome and stained with hematoxylin-eosin following Culling et al. (1985).

Ultrastructural analyses: For TEM analyses, small samples of infected tissue were fixed in 2.5% glutaraldehyde (0.1 M sodium cacodylate buffer, pH 7.4) for 8 h at 4 °C, washed for 12 h (4 °C) in the same buffer and post-fixed in buffered 2% OsO₄ for 6 h (4 °C). Samples were then dehydrated in graded ethanol series, washed in propylene oxide and included in EPON resin; polymerization was carried out at 60 °C for 48 h.

Blocks were cut using Leica Reichert's ULTRACUT ultramicrotome. Semi-thin sections obtained by diamond knife were stained with methylene blue to identify the area of interest. Ultrathin sections were double stained with uranyl acetate and lead citrate, and observed using a JEOL (JEM1010) TEM (80kV).

For SEM analyses, isolated and purified sporocysts were fixed for 4 h in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.3, 4 °C) and washed 30 min in the

same buffer. Samples were then dehydrated in a graded ethanol series, critical point-dried and sputter-coated with platinum. Observations and image acquisitions were made using a JEOL (JSM 6700F) SEM operating at 5.0 kV.

Isolation and purification of the parasite: For molecular analyses tissues infected with sporogonic stages of the parasite were dissected and homogenized in 10 ml of filtered sea water (FSW) using a tissue grinder (IKA-Ultra Turrax T-25). To facilitate removal of tissue fragments, homogenates were filtered twice with nylon meshes of 100 μ m and 40 μ m, respectively. After filtration, samples were centrifuged at $1000 \times g$ (5 min), sporocysts were cleaned according to Gestal et al. (1999a) and counted by a Neubauer chamber to adjust samples at 2×10^6 sporocysts/ml.

DNA extraction: Sporocysts were resuspended in 500 μ l of extraction buffer (NaCl 100 mM, EDTA 25 mM pH 8, SDS 0.5%) and disrupted by wet bead milling using a Retsch Mixer Mill MM 300 grinder. After Proteinase K (Sigma) digestion (1 mg/ml; 56 °C overnight), genomic DNA was purified following the phenol:chloroform:isoamil alcohol extraction method (Sambrook et al. 1989). DNA was precipitated with ethanol and sodium acetate overnight at -20 °C. The precipitated pellet was resuspended in 10-20 μ l of sterile water.

DNA amplification, cloning and sequencing: Specific primers designed for *Aggregata* spp. (Castellanos-Martínez et al. 2013) were utilized (Aggregata5-F: 5'-AAGCTCGTAGTTGCAGTTTTGA - 3'; Aggregata6-R: 5' - AACTAAGAACGGCCATGCAC - 3') to amplify a fragment of about 600 bp of the small subunit 18S rRNA gene.

PCR reactions were performed in a total volume of 25 μ l containing: 2.5 μ l of 10 mM dNTP mix, 0.25 μ l *Taq* DNA polymerase (Roche), 2.5 μ l PCR Reaction Buffer (+Mg) 10 $\times$, 1 μ l of each primer (25 pmol) and 2 μ l of DNA at 100 ng/ μ l. Followed an

initial denaturation at 94 °C for 10 min, we run 35 cycles (94 °C for 1 min), 56 °C for 1 min and 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were separated on 1% agarose in TAE 1× buffer gels (w/v), stained with SyBr Safe including a 100-bp ladder size standard (Invitrogen) and visualized on UV transilluminator.

PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen) according to the manufacturer protocol, and bi-directionally sequenced using ABI 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

Sequence analysis: Sequences were aligned and edited using the BioEdit v7.2.5 software (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) and MEGA 6.0 (Tamura et al. 2013). Two *A. octopiana* sequences previously identified and available in public database (from Atlantic and representing the valid recognized species; GenBank accession number: KC188342, from Croatia, Adriatic Sea, accession number: DQ096837) were added to the alignment. New clones of *A. octopiana* from NE Atlantic (ALT561, ALT562, ALT563, ALT564) were also included in the analyses.

Sequences of the 18S rRNA were employed to build Maximum likelihood (ML), neighbour-joining (NJ) and Bayesian inference (BI) trees, using: *A. octopiana*, *A. eberthi* (DQ096838), 10 sequences of Apicomplexa taxa available in GeneBank (with a Blast identity > 75% in relation to our sequences), and with a representative species of *Perkinsus* sp. (order: Perkinsida; AF488540) as an outgroup.

Because the TPM3uf+I model of sequence evolution is not implemented in the phylogenetic reconstruction tree software, we replaced it by the GTR model, conserving the proportion of invariable sites (I) defined in jModeltest.

Molecular diversity indices, such as the number of haplotypes (H), polymorphic sites (S), haplotype diversity (h; Nei 1987), nucleotide diversity (π ; Nei 1987) and the

average numbers of pairwise nucleotide differences (k ; Tajima 1983) were obtained by DNAsp v5.0 (Librado and Rozas 2009).

JModelTest v2.1.7 (Darriba et al. 2012) was used to select the appropriate model of evolution, as a guide to determine the best-fit maximum likelihood (ML) model. The Akaike Information Criterion (AIC; Posada and Buckley 2004) indicated the TPM3uf+I as the best fit model of DNA sequence evolution with proportion of invariable sites value ($I=0.539$). Overall distances among haplotypes were calculated in MEGA 6.0.

ML trees were built in RAxML v7.2.0 (Stamatakis et al. 2008) using 1,000 searches and 10 runs. In addition, a neighbour-joining (NJ) and Bayesian inference (BI) methods were adopted to reconstruct the phylogenetic relationships, using MEGA 6.0 with 1,000 bootstrap replicates and MrBayes v. 3.2 (Ronquist et al. 2012) with 2,000 replicates, respectively.

Acknowledgements

We thank F. Iamunno and R. Graziano (Electron Microscopy, Stazione Zoologica Anton Dohrn, Naples) and I. Pazos and J. Mendez (CACTI, University of Vigo) for their technical assistance.

This work has been initiated through the networking initiatives of the COST Action FA1301 (*CephsInAction*) and thanks to the support of a COST FA1301-Short Term Scientific Mission (P. Tedesco, from Italy to Vigo, Spain). The study has been supported through RITMARE Flagship project (MIUR-SZN) to PT and GF.

G. Catanese would like to thank INIA for the INIA/CCAA research contract program.

References

- Bandelt HJ, Forster P, Reohl A** (1999) Median-joining networks for inferring intraspecific phylogenies. *Mol Biol Evol* **16**:37–48
- Barber I, Hoare D, Krause J** (2000) Effects of parasites on fish behaviour: a review and evolutionary perspective. *Rev Fish Biol Fisher* **10**:131-165
- Castellanos-Martínez S, Gestal C** (2013) Pathogens and immune response of cephalopods. *J Exp Mar Biol Ecol* **447**: 14-22
- Castellanos-Martínez S, Perez-Losada M, Gestal C** (2013) Molecular phylogenetic analysis of the coccidian cephalopod parasites *Aggregata octopiana* and *Aggregata eberthi* (Apicomplexa: Aggregatidae) from the NE Atlantic coast using 18S rRNA sequences. *Europ J Protistol* **49**:373-380
- Darriba D, Taboada GL, Doallo R, Posada D** (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods* **9**: 772
- De Luca D, Catanese G, Procaccini G, Fiorito G** (2014) An integration of historical records and genetic data to the assessment of global distribution and population structure in *Octopus vulgaris*. *Front Ecol Evol* **2**:55 DOI:10.3389/fevo.2014.00055
- De Luca D, Catanese G, Procaccini G, Fiorito G** (2016) *Octopus vulgaris* (Cuvier, 1797) in the Mediterranean Sea: Genetic diversity and population structure. *PlosOne* **11**(2): e0149496
- Dobell CC** (1925) The life history and chromosome cycle of *Aggregata eberthi*. *Parasitology* **17**: 1-136
- Duszynski DW, Wilber PG** (1997) A guideline for the preparation of species descriptions in the Eimeriidae. *J Parasitol* **83**: 333-336
- Estevez J, Pascual S, Gestal C, Soto M, Rodriguez H, Arias C** (1996) *Aggregata octopiana* (Apicomplexa: Aggregatidae) from *Octopus vulgaris* off NW Spain.

- Dis Aquat Org **27**: 227-231
- El-Geziry TM, Bryden GI** (2010) The circulation pattern in the Mediterranean Sea: issues for modeller consideration J Oper Oceanogr **3**:39-46
- Excoffier L, Lischer HEL** (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour **10**: 564–567
- Gestal C, Abollo E, Pascual S** (1999a) Evaluation of a method for isolation and purification of sporocysts of the cephalopod coccidian parasite *Aggregata Frenzel*, 1885 (Apicomplexa: Aggregatidae). Iberus 17:115-121
- Gestal C, Abollo E, Pascual S** (2002a) Observations on associated histopathology with *Aggregata octopiana* infection (Protista: Apicomplexa) in *Octopus vulgaris*. Dis Aquat Org **50**:45-49
- Gestal C, De La Cadena MP, Pascual S** (2002c) Malabsorption syndrome observed in the common octopus *Octopus vulgaris* infected with *Aggregata octopiana* (Protista: Apicomplexa). Dis Aquat Org **51**:61-65
- Gestal C, Guerra A, Pascual S, Azevedo C** (2002b) On the life cycle of *Aggregata eberthi* and observations on *Aggregata octopiana* (Apicomplexa, Aggregatidae) from Galicia (NE Atlantic). Europ J Protistol **37**: 427-435
- Gestal C, Pascual S, Corral L, Azevedo C** (1999b) Ultrastructural aspects of the sporogony of *Aggregata octopiana* (Apicomplexa, Aggregatidae), a coccidian parasite of *Octopus vulgaris* (Mollusca, Cephalopoda) from NE Atlantic coast. Europ J Protistol **35**:417-425
- González AF, Otero J, Guerra A, Prego R, Rocha FJ, Dale AW** (2005) Distribution of common *Octopus* and common squid paralarvae in a wind-driven upwelling area (Ria of Vigo, northwestern Spain). J Plankton Res **27**:271-277

- Hochberg FG** (1983) The parasites of cephalopods: a review. *Mem Nat Mus Victoria* **44**:109-145
- Hochberg FG** (1990) Diseases of Mollusca: Cephalopoda. In Kinne O (ed) *Diseases of Marine Animals Vol 3*. Biologische Anstalt Helgoland, Hamburg, pp 47-202
- Kopečná J, Jirků M, Oborník M, Tokarev YS, Lukeš J, Modrý D** (2006) Phylogenetic analysis of coccidian parasites from invertebrates: Search for missing links. *Protist* **157**: 173-183
- Labbé A** (1895) Sur le noyau et la division nucléaire chez les *Benedenia*. *C R Acad Sci Paris* **120**:381
- Librado J, Rozas P** (2009) DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**:1451-1452
- Licciardo G, Garziano A, Nocera G, Gaglio G, Marino F, De Vico G** (2005) Contribution to the knowledge of tissue damage due to *Aggregata octopiana* (Apicomplexa: Aggregatidae) in *Octopus vulgaris* in the southern Tyrrhenian Sea. *Ittiopatologia* **2**:193-198
- Lom J, Dyková I** (1992) Protozoan parasites of fishes. *Dev Aquacult Fish Sci* **26**:1–315
- Mangold K** (1983) *Octopus vulgaris*. In Boyle PR (ed) *Species Accounts. Cephalopod Life Cycles*, vol 1. Academic Press, London, New York, pp 335–364
- Mayo-Hernandez E, Barcala E, Berriatua E, Garcia-Ayala A, Munoz P** (2013) *Aggregata* (Protozoa: Apicomplexa) infection in the common octopus *Octopus vulgaris* from the West Mediterranean Sea: The infection rates and possible effect of faunistic, environmental and ecological factors. *J Sea Res* **83**:195-201
- McDermott JJ, Williams JD, Boyko CB** (2010) The unwanted guests of hermits: A worldwide review of the diversity and natural history of hermit crab parasites. *J*

Exp Mar Biol **394**:2–44

Mereu M, Agus B, Addis P, Cabiddu S, Cau A, Follesa MC, Cuccu D (2015)

Movement estimation of *Octopus vulgaris* Cuvier, 1797 from mark recapture experiment. J Exp Mar Biol Ecol **470**:64-69

Mladineo I, Bočina I (2007) Extraintestinal gamogony of *Aggregata octopiana* in the

reared common octopus (*Octopus vulgaris*) (Cephalopoda: Octopodidae). J Invert Pathol **96**: 261-264

Mladineo I, Jozić M (2005) *Aggregata* infection in the common octopus, *Octopus*

vulgaris (Linnaeus, 1758), Cephalopoda: Octopodidae, reared in a flow-through system. Acta Adriat **46**:193-199

Moroff T (1908) Die bei den Cephalopoden vorkommenden Aggregataarten als

Grundlage einer kritischen Studie über die Physiologie des Zellkernes. Arch Protistenkd **11**:1-224

Narum SR (2006) Beyond Bonferroni: less conservative analyses for conservation

genetics. Conserv Genet **7**:783-787

Naville A (1925) Recherches sur le cycle sporogonique des *Aggregata*. Revue suisse

Zool **32**:125-179

Nei M (1987) Molecular Evolutionary Genetics. Columbia. University Press, New York

512 p

Orlić M, Gačić M, La Violette PE (1992) The currents and circulation of the Adriatic

Sea. Oceanol Acta **15**:109-124

Pixell-Goodrich HLM (1950) *Aggregata leandri* n. sp. Q II Microsc Sci **91**: 465-467

Posada D, Buckley TR (2004) Model selection and model averaging in phylogenetics:

advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst Biol **53**:793-808

- Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP** (2012) MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. *Syst Biol* **61**:539–542
- Sardella NH, Martonelli SR** (1997) Occurrence of merogony of *Aggregata* Frenzel 1885 (Apicomplexa) in *Pleoticus muelleri* and *Artemesia longinaris* (Crustacea: Natantia) from patagonian waters (Argentina). *J Invertebr Pathol* **70**:198–202
- Semmens JM, Pecl GT, Gillanders BM, Waluda CM, Shea EK, Jouffre D, Ichii T, Zumholz K, Katugin ON, Leporati SC, Shaw PW** (2007) Approaches to resolving cephalopod movement and migration patterns. *Rev Fish Biol Fisheries* **17**:401–423
- Shaw BL, Battle HI** (1957) The gross and microscopic anatomy of the digestive tract of the oyster *Crassostrea virginica* (Gmelin). *Can J Zool*, **35**:325–347
- Sprague V, Couch J** (1971) An annotated list of protozoan parasites, hyperparasites, and commensals of decapod crustacea. *J Protozool* **18**:526–537
- Stamatakis A, Hoover P, Rougemont J** (2008) A rapid bootstrap algorithm for the raxml web servers. *Syst Biol* **57**:758–771
- Tajima F** (1983) Evolutionary relationship of DNA sequences in finite populations. *Genetics* **105**:437–460
- Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S** (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* **30**:2725–2729
- Theodorides J** (1965) Parasitisme de Decapodes Natantia de Banyuls par *Aggregata leandri* Pixell Goodrich, 1950 (Coccidia: Aggregatidae). *Vie Milieu* **16**:229-230
- Theodorides J, Desportes I** (1975) Sporozoaires d'invertebres pelagiques de Villefranche-sur-Mer (etude descriptive et faunistique). *Protistologica* **11** 205-

220

Villanueva R, Nozais C, Boletzky SV (1995) The planktonic life of octopuses. *Nature*
377:107

Figure Legends

Figure 1. Fresh preparation of cleaned *Aggregata* sporocysts with visible sporozoites obtained from tissues of *O. vulgaris* sampled in Tyrrhenian Sea. **sc**: sporocyst

Figure 2. Histological preparations of *Aggregata* infected tissues. **A:** gamogonic stage (macrogamont); **B:** sporogonic stage (sporont); **C:** heavy infection with sporogonic stages (oocyst containing sporocysts); **D:** detail of sporocyst showing eight sporozoites.

mg = macrogamont; **sr** = sporont; **oc** = oocyst; **sc** = sporocyst.

Figure 3. TEM micrographs of sporocysts from the three sampled areas **A:** Ionian Sea, sporocyst with smooth wall surface; **B:** Ionian Sea, dehiscence suture; **C:** Ionian Sea, sporocyst with spiny surface; **D:** Tyrrhenian Sea, detail of the spine over the sporocyst wall; **E:** Tyrrhenian Sea, detail of the dehiscence suture; **F:** Adriatic Sea, detail of the sporocyst wall showing a smooth surface and the inner structure with regular transversal grooves. **sz** = sporozoite; **ds** = dehiscence suture; **cw** = cyst wall; **sp** = spine.

Figure 4. SEM micrographs of purified sporocysts from **A:** Tyrrhenian Sea; **B:** Adriatic Sea; **C:** Ionian Sea. **ds** = dehiscence suture; **sp** = spine.

Figure 5. Phylogenetic relationships among *Aggregata octopiana* haplotypes and Apicomplexa species. Maximum likelihood, neighbor-joining and Bayesian bootstrap values higher than 50% are indicated, respectively (below nodes).

Figure 6. Median-joining network of haplotypes. The sizes of the circles are proportional to frequencies of haplotypes. The black points on the lines correspond to missing haplotypes. The numbers on the lines represent the number of mutations between two close haplotypes.

Table 1. Morphological and morphometric differences between sporogonic stages in samples from sampling areas. All measurements are given in micrometers unless otherwise stated. **OL** = oocyst length; **OW** = oocyst width; **SL** = sporocyst length; **SW** = sporocyst width; **WS** = wall surface; **WT** = wall thickness; **GD** = electron-dense grooves distance; **nSp** = number of sporozoites; **SpT** = sporozoite thickness; **SP** = spiny; **SM** = smooth

Sampling Area	OL	OW	SL	SW	WS	WT	GD (nm)	nSp
Tyrrhenian Sea	300 - 1300	300 - 1300	15 -18	15 -18	SP	0.4 - 0.5	15 - 17	8
Adriatic Sea	250 - 300	250 - 300	11 – 13	11 - 13	SM	0.3 - 0.4	11 - 16	8
Ionian Sea	250 - 500	250 – 500	10 – 12	10 - 12	SP / SM	0.3 - 0.4	11 - 16	8
Atlantic	100 - 1000	100 - 1000	11 – 15	11 - 15	SP	0.4 - 0.5	15 - 17	8

Table 2. Sampling localities and descriptive statistics of genetic diversity of *Aggregata octopiana* based on 18S rRNA sequence data. **n** = sample size; **H** = number of haplotypes; **S** = number of segregating sites; **hd** = haplotype diversity; π = nucleotide diversity; **k** = mean pairwise difference.

Region and site	abb.	n	H	S	hd	π	k
Tyrrhenian Sea	TYR	29	10	22	0.813	0.01157	7.012
Ionian Sea	ION	15	6	67	0.810	0.05346	32.343
Adriatic Sea	ADR	28	2	3	0.071	0.00035	0.214
TOTAL		73	17	81+3 indels	0.819	0.05160	31.215

Table 3. Analysis of molecular variance (AMOVA) results showing genetic variance for *A. octopiana* populations based on 18S rRNA sequence data. **d.f.** = degrees of freedom.

Source of variation	d.f.	Sum of	Variance	Percentage
		squares	components	of variation
Among populations	2	831.860	19.72402	82.98
Within populations	65	262.993	4.04604	17.02
Total	67	1094.853	2377.007	
Fixation Index		FST=0.82978		P=0.0000