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Identity and Systematic Position of *Paradinium poucheti* and Other *Paradinium*-Like Parasites of Marine Copepods Based on Morphology and Nuclear-Encoded SSU rDNA

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Paradinium **and** ***Paradinium*****-like parasites were detected in various copepod hosts collected in the NW Mediterranean Sea, the North Atlantic Ocean, and the Godthåbsfjord (Greenland). The identity and systematic position of the parasitic, plasmodial protist** ***Paradinium*** **was investigated on the basis of SSU rDNA and morphology. SSU rDNA sequences were obtained from 3 specimens of** ***Paradinium poucheti*** **isolated from their cyclopoid copepod host,** ***Oithona similis*****. In addition, a comparable sequence was obtained from a hitherto undescribed species of** ***Paradinium*** **from the harpactacoid copepod** ***Euterpina acutifrons*****. Finally, SSU rDNA sequences were acquired from 2 specimens of a red plasmodial parasite (RP parasite) isolated from** ***Clausocalanus*** **sp. Both morphological and SSU rDNA sequence data supported that** ***P. poucheti*** **and** ***Paradinium*** **sp. are closely related organisms. In phylogenetic analyses based on SSU rDNA sequences,** ***Paradinium*** **spp. clustered with sequences from an uncultured eukaryote clone from the Pacific Ocean and two sequences from haplosporidian-like parasites of shrimps,** ***Pandalus*** **spp. This** ***Paradinium*** **clade branched as a sister group to a clade comprising the Haplosporidia and the Foraminifera. The RP parasite had a superficial morphological resemblance to** ***Paradinium*** **and has previously been interpreted as a member of this genus. However, several morphological characters contradict this and SSU rDNA sequence data disagree with the RP parasite and** ***Paradinium*** **being related. The phylogenetic analyses suggested that the RP parasite is a fast-evolved alveolate and a member of the so-called marine alveolate Group I (MAGI) and emerging data now suggest that this enigmatic group may, like the syndinian dinoflagellates, consist of heterotrophic parasites.**

Key words: *Paradinium*; RP parasite; marine alveolates; MAGI; Galathea 3 expedition.

Introduction

Paradinium Chatton is a genus of parasitic protists that infect marine, planktonic copepods. Several copepod species have been reported as hosts for the 3 presently known species of *Paradinium* (Chatton 1920). *Paradinium poucheti* Chatton was first observed by Pouchet (1890) off the coast of Brittany, France. However, not until 20 years later

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were the genus and species names introduced in a preliminary note by Chatton (1910), who described the organism in brief as a parasite of *Acartia clausi* Giesbrecht from Mediterranean waters. It is not known with certainty which systematic group of organisms *Paradinium* is related to. Chatton (1910) argued that *Paradinium* had resemblance with the syndinian dinoflagellates, because they possessed common characters such as the plasmodium and the flagellated spores. Chatton (1920) described *Paradinium* in more detail, holding on to the hypothesis that the parasite was related to the syndinian dinoflagellates. In this monograph, he also reported *Paradinium*-like parasites infecting *Clausocalanus arcuicornis* Dana, *Oithona plumifera* Baird, and *Centropages typicus* Kröyer in French Atlantic and Mediterranean coastal waters. He also noted that the "parasite 21" observed by Apstein (1911) in *Oithona* sp. from the Kattegat, Denmark, was most likely a species of *Paradinium*. In subsequent studies, Chatton tentatively placed *Paradinium* among the cryptomonads (Chatton 1927, 1952) due to the shape of the spore. Finally, Cachon et al. (1968), based on characters of the nuclear division, concluded that *Paradinium* had affinities with the Mycetozoa. *Paradinium* is still included in the dinoflagellate literature by some authors (e.g. Shields 1994) because its taxonomic relationship has remained unresolved.

Little is known about the life cycle of *Paradinium*, but the development and morphology of the *Paradinium* plasmodium have been investigated in some detail (Chatton 1910; Chatton and Soyer 1973; Cachon et al. 1968): Briefly, the parasite exists as 8—10 μm long amoeboid cells in the body cavity of its host. The individual cells are interconnected by thin pseudopodia thereby forming a reticulate plasmodium, a filoplasmodium. At more advanced stages of infection, a solid plasmodium is formed. Eventually, the plasmodium passes from the body cavity of the host and into the lumen of its intestine whereupon it is expelled through the anus. The plasmodial cell mass then forms a "cyst", a gonosphere (Chatton 1920), that attaches to the urosome of the host. The gonosphere is bounded by a mucoid wall with a reticulate surface. Within a few hours after the formation of the gonosphere, flagellated spores are formed. These spores measure approximately 12 μm in length and possess two flagella of unequal length. The spores are believed to represent the infectious stage (Chatton 1920), but this has never been documented.

Jepps (1937) found a *Paradinium*-like parasite in another host, *Calanus finmarchicus* Gunnerus, in the Clyde Sea area (United Kingdom). However, the morphology of the parasite observed by Jepps (1937) differed somewhat from the species described by Chatton (1910, 1920): the plasmodium was bright red-orange in color and the expelled cell mass did not have a well-defined size or shape and it lacked the mucoid, reticulate wall. The present study evaluates the morphological resemblance between *P. poucheti* and the *Paradinium*-like parasite observed be Jepps (1937). In addition, SSU rDNA sequences of both organisms are used to elucidate their taxonomic relationships.

Results

Paradinium poucheti

Paradinium poucheti was found in the cyclopoid copepod *Oithona similis* Claus in the NW Mediterranean Sea (Fig. 1A—C) and in the Godthåbsfjord, Greenland (Fig. 2A). Less than 20 *P. poucheti* gonospheres were detected during two extensive field campaigns (North Atlantic Ocean and Mediterranean Sea) even though the host was among the most abundant copepod species in many of the samples. Parasites were isolated from live animals at the stage of infection when gonospheres had been formed and could be observed attached to the urosome of the host. *Paradinium poucheti* gonospheres were ovoid, 200—250 μm long and 140 μm wide (Figs 1A—C, 2A). They were pale brownish with a reticulate surface. Each infected animal was always seen generating only one gonosphere. The gross morphology of live hosts appeared normal in respect of color, size, shape, and motility. Thus, the attached gonosphere was the only feature that revealed infection with *Paradinim* spp.

The SSU rRNA gene of *Paradinium poucheti* from *Oithona similis* was 2030 bp long including external PCR primers. Two complete sequences were obtained (isolates PaOi01 and PaOi21 collected in the NW Mediterranean Sea in spring 2004 and summer 2005, respectively) and these were 100% identical. For a third isolate, PaOi30 from the NW Mediterranean Sea, only a partial sequence of 1300 bp was obtained and this was 100% identical to the corresponding part of the two complete sequences. BLAST searches performed on the *P. poucheti* sequence yielded a sequence of an uncultured marine eukaryote

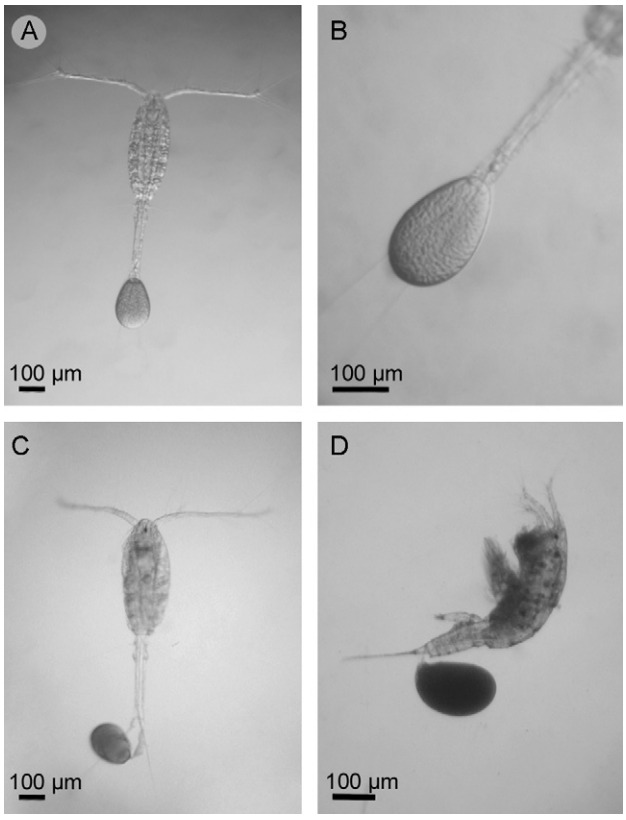

Figure 1. Living *Paradinium* spp.-infected copepods from the NW Mediterranean Sea. **A.** *P. poucheti* (PaOi21) gonosphere attached to the urosome of its host, the copepod *Oithona similis*. **B.** PaOi21 at higher magnification. **C.** *P. poucheti* (PaOi01) gonosphere attached to the urosome of the copepod *O. similis*. **D.** *Paradinium* sp. (PaEu41) gonosphere attached to the urosome of its host *Euterpina acutifrons*.

originating from a coastal sample from the Pacific Ocean (clone UEPAC05Hp2) as the closest match. This sequence was identical to PaOi01 and PaOi21 at 96% of the positions (including introduced gaps). Other matches revealed by the BLAST search were two sequences from a parasite of spot prawn, *Pandalus platyceros* Brandt (SPP, clones 3 and 16), which had 80% of the positions similar to sequences of *P. poucheti*. Finally, an uncultured clone (LC104_3EP_36) was 76% similar to *P. poucheti*.

Paradinium sp.

Two gonospheres of *Paradinium* sp. were found attached to specimens of the harpactacoid

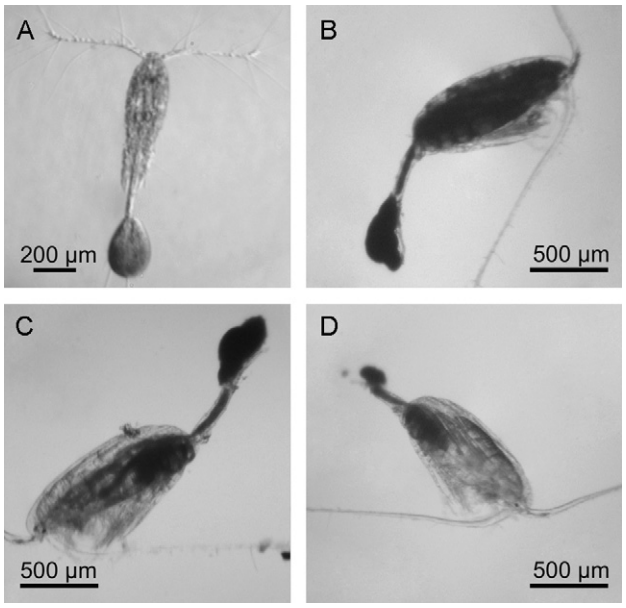

Figure 2. **A.** *Paradinium poucheti* gonosphere attached the gonosphere of its living host *Oithona similis* from the Godthåbsfjord, Greenland. **B—D.** RP parasite in living, unidentified calanoid copepod from the North Atlantic Ocean. **B.** Initial stage of expulsion of parasite mass, $t = 0$ h. **C.** $t = 2$ h. **D.** $t = 5$ h.

copepod *Euterpina acutifrons* Brian in the NW Mediterranean Sea in March and October 2004 (Fig. 1D). These two gonospheres, which so far constitute the entire sample material of this organism, resembled those of *P. poucheti*, but appeared darker with no noticeable surface pattern. However, due to the limited material, the gonospheres were not studied at high magnification and it is possible that a surface pattern was obscured by the dark color of the gonosphere. The gonospheres on *E. acutifrons* were 190 µm long and 130 µm wide. Only one SSU rDNA sequence from *Paradinium* sp. in *E. acutifrons* was obtained (isolate PaEu41). This sequence was 2085 bp long including both external primers and it had 86% positions identical to the sequences from *P. poucheti* (including introduced gaps). Four indels of lengths between 5 and 19 bp constituted 4% of this dissimilarity. A BLAST search based on PaEu41 yielded the same closest matches as for PaOi01 and PaOi21: the uncultured marine eukaryote clone UEPAC05Hp2, parasites of spot prawn (SPP clones 3 and 16), and the uncultured clone LC104_3EP_36. The similarities to PaEu41 were 86%, 79%, and 75%, respectively.

RP Parasite

Another *Paradinium*-like parasite, a red plasmodial parasite (RP parasite), was found in one specimen of an unidentified calanoid copepod collected in the North Atlantic Ocean in September 2006 (Fig. 2B—D) and in two specimens of *Clausocalanus* sp. sampled in the Mediterranean Sea in September 2004 (Fig. 3). The RP parasite was considerably more conspicuous than *Paradinium* spp. This parasite formed a reddish cell mass that was visible inside the living host. The cell mass filled the intestinal tract with ramification into the body cavity, coloring almost the entire animal dark red (Figs 2B—D and 3A—D). After the infected copepod had been incubated overnight in filtered seawater, the RP parasite passed through the digestive tract of its host and was expelled through the anus. Here, a dark red, more or less amorphous cell mass remained attached to the urosome (Figs 2B—D and 3B, D, F). This cell mass was in one case shaped as one large sphere with a diameter of 395 μm (Fig. 3B). In another case, one large sphere with a diameter of 180 μm was followed by a string of 20—25 smaller spheres (Fig. 3D). This string of spheres had emerged from the host overnight, and the following day the string suddenly disintegrated into several rounded immobile bodies of variable sizes, 13—38 μm in diameter (Fig. 3E). However, a cell mass of irregular shape remained attached to the host (Fig. 3F). The expulsion process was initiated approximately 10 h after isolation of the host animal and continued for another 10—12 h, but the process never seemed to terminate completely in the sense that small amounts of reddish material were visible in the hosts until they died a few days afterwards. After a couple of days of incubation at 20 °C, the cell bodies produced by the two Mediterranean host specimens appeared degenerated, but this was not studied in detail. Cell mass from the host specimen collected in the North Atlantic was incubated at 10 °C, and the final stage observed 5 days after the expulsion from this host was a number of seemingly non-motile cells with a diameter of 5—6 μm. The limited sample material available was all preserved with glutaraldehyde for SEM and it was, therefore, not possible to follow a potential development in the morphology of these non-motile cells. As viewed in SEM these cells were spherical with a brain-like surface ornamentation (Fig. 4). Some of these cells seemed to be bearing appendices that had some resemblance to flagella, but the morphology of the appendices was variable. A total of more than 30 cells were observed in SEM, but it was not possible to rule out the possibility that the attached appendices could be structures produced by artifacts.

Complete SSU rDNA sequences were obtained from two individuals of the RP parasite (isolates RPP1 and RPP2 from the NW Mediterranean Sea). Both these sequences were 1781 bp long (including external PCR primers) and they were similar at 96% of the positions. A BLAST search performed on either of these sequences yielded only one close match: an uncultured eukaryote clone (SCM37C34) originating from an environmental sample from the Sargasso Sea. According to the BLAST search, this clone had 95% and 97% positions similar to RPP1 and RPP2, respectively. The second most similar sequence found through the BLAST search (uncultured eukaryote clone SCM27C35) had only 79% positions similar to RPP1. All other sequences among the 100 closest matches in the BLAST search were sequences from dinoflagellates or uncultured alveolates/eukaryotes.

Phylogenetic Analyses

A phylogenetic tree (Fig. 5), based on SSU rDNA sequences of 101 taxa, was constructed by use of Bayesian interference (BI) with additional bootstrap values estimated by neighbor joining (NJ) and maximum parsimony (MP) methods. The two sequences from *Paradinium* spp. formed a well-supported clade with the uncultured marine eukaryote clone UEPAC05Hp2. The *Paradinium* clade formed a sister group to a clade comprising two sequences from the spot prawn parasite (SPP, clones 3 and 16) and these relations were well supported: Bayesian posterior probabilities (PP) of 1.0, NJ and MP bootstrap values of 78—100%. This *Paradinium*/SPP clade branched as a sister group to the Haplosporidia and Foraminifera in the BI and NJ analyses, but the MP analysis failed to resolve the position of the Foraminifera and placed these at the base of the phylogenetic tree (not shown). The uncultured clone LC104_3EP_36 and two isolates of *Gromia oviformis* branched basal to the *Paradinium*/SPP clade, the Haplosporidia, and the Foraminifera, although the NJ analysis placed them, with low support, as basal branches to the *Paradinium*/SPP clade only. All of the above-mentioned groups formed a monophyletic assemblage, corresponding to the phylum Rhizaria, together with the Cercozoa and the Phytomyxea (PP = 1.0, NJ bootstrap value = 58%). Rhizaria, excluding the Foraminifera, were monophyletic in the MP analysis with a bootstrap value of 78% (not shown).

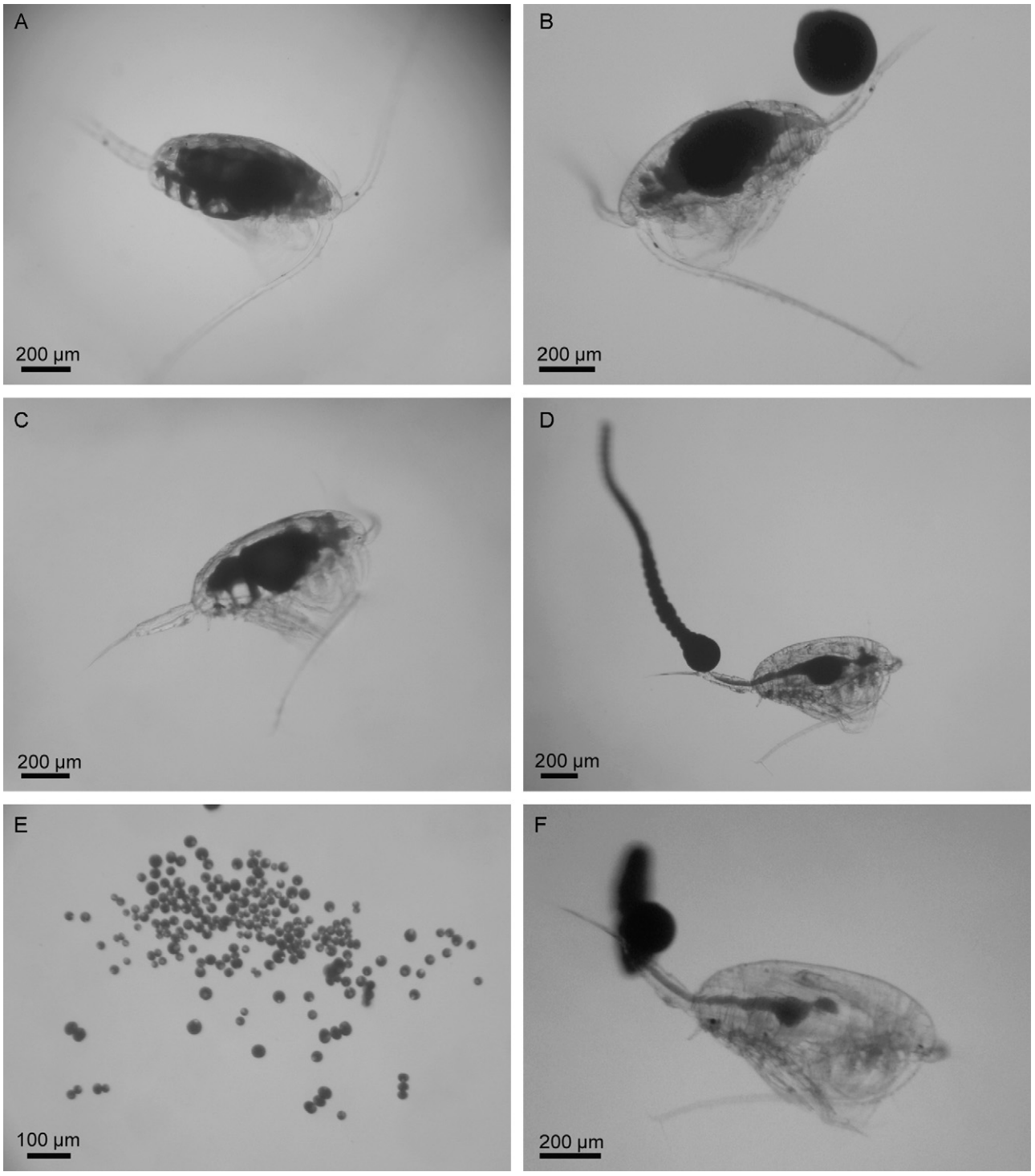

Figure 3. RP parasite in *Clausocalanus* sp. from the NW Mediterranean Sea. **A, B**. RPP2. **A.** Appearance of infected host at the time of discovery ($t = 0$ h). The host's body cavity is almost completely filled with parasite cell mass. **B.** $t = 22$ h, the parasite cell mass adheres as a large sphere to the host's urosome. **C—F.** RPP1. **C.** $t = 0$ h, infected host with parasite cell mass. **D.** $t = 19$ h, parasite cell mass adheres as a string of spheres connected to the host's urosome. **E.** $t = 21$ h, parasite cell mass uncoupled from the host. **F.** $t = 23$ h, additional parasite cell mass attached to the urosome of the host that now contains only a small amount of parasite cell mass.

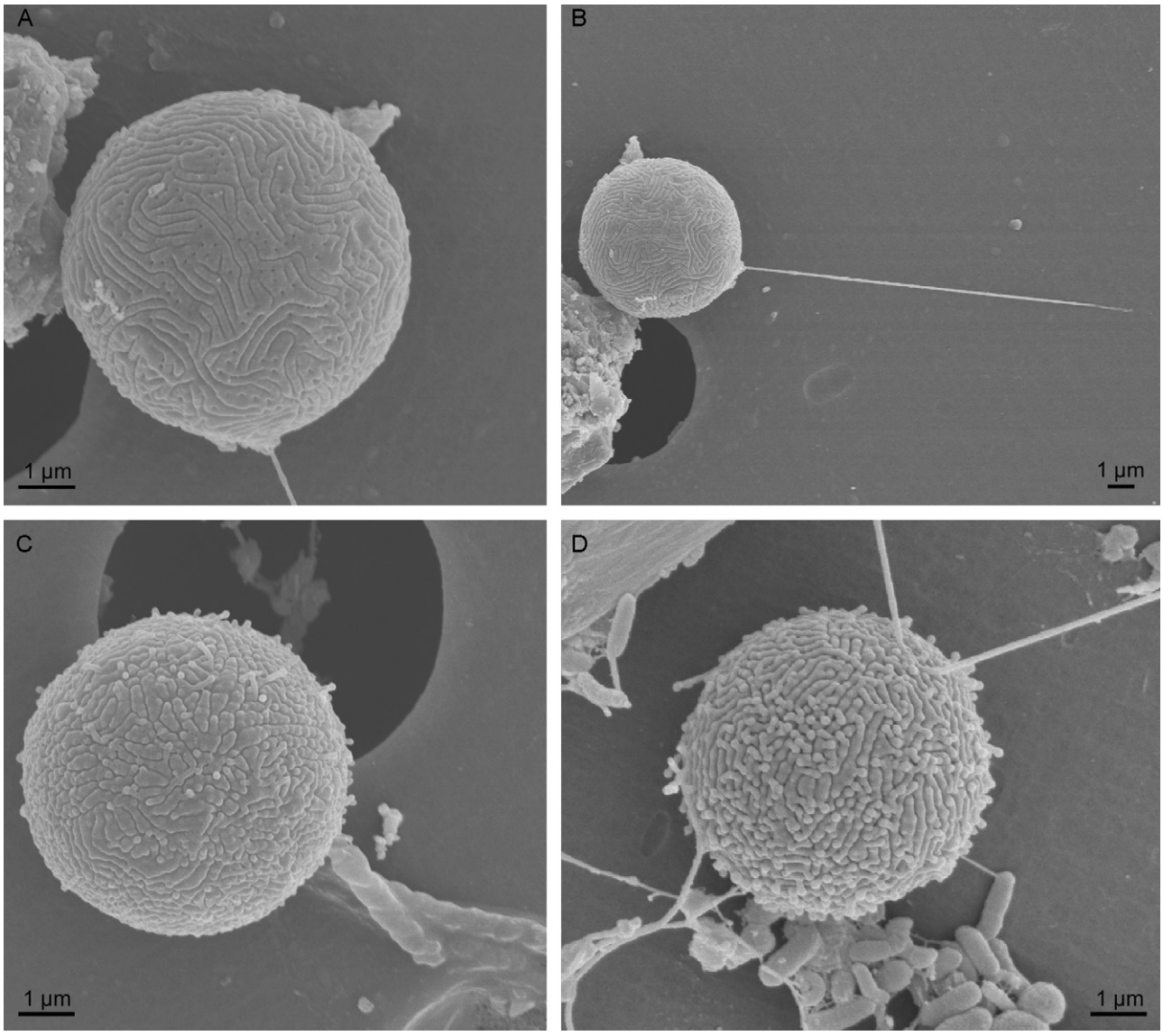

Figure 4. **A—D** SEM micrographs of unicellular stages of RP parasite from the calanoid host in Figure 2B—D. Cells were fixed at $t = 4$ d. **A.** Cells with brain-like surface ornamentation and minute pores. **B.** Cell from A at lower magnification showing full length of appendix. **C.** Cell with slightly different surface ornamentation than the cell depicted in A—B, with papillae and no visible pores. **D.** Cell that is seemingly bearing two appendices.

The two sequences of the RP parasite (RPP1 and RPP2) branched distantly from the *Paradinium* sequences (Fig. 5). RPP1 and RPP2 comprised a clade together with a sequence from the uncultured eukaryote clone SCM37C34, and this clade had maximum support in all three phylogenetic analyses. This RPP clade branched with members of Marine alveolate Group I (in the following abbreviated "MAGI") with PP of 1.0 and NJ bootstrap values of 66%. Several alveolate groups were unsupported in both the NJ and the MP analysis, but the Alveolata, excluding the Ciliata (i.e. including RPP1 and RPP2), constituted a clade in both these analyses.

Discussion

Paradinium spp.

The parasite of *Oithona similis* examined in the present study was identical to *Paradinium*

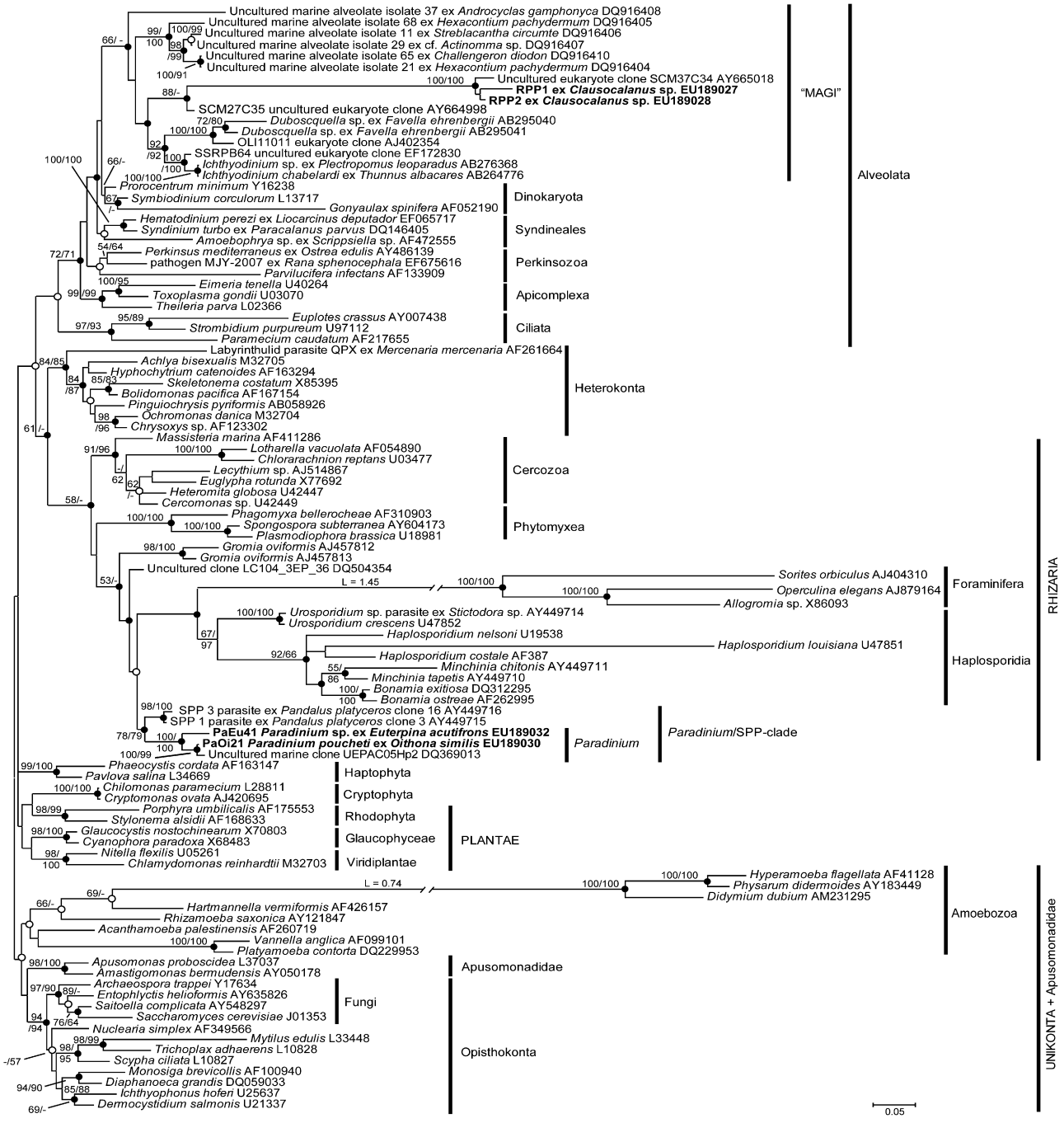

Figure 5. Phylogenetic tree constructed using Bayesian analysis of a 1283 base-pair long alignment comprising 101 SSU rDNA sequences. The tree is rooted with the Unikonta/Apusomonadidae. Last numbers in taxon names are GenBank accession numbers. Numbers above internal branches are Neighbour-Joining and Maximum Parsimony bootstrap values (BS), respectively. BS $<$ 50% are symbolized with a dash. Filled circles at nodes denote that the clade had Bayesian posterior probabilities (PP) of 1.00; open circles denote PP of 0.95—0.99. PP $<$95 are not shown and neither are BS when both values were $<$50%.

poucheti studied by Cachon et al. (1968) in all respects, i.e. gonospheres were of similar size, color, and shape, and the host species was the same. In addition, the geographical sampling areas were overlapping. *Paradinium poucheti* was originally described as a parasite of the calanoid copepod *Acartia clausi*, but Cachon et al. (1968) considered that the *Paradinium* species in *O. similis* (as *O. helgolandica* Claus) was conspecific with the parasite found in *A. clausi*. We, therefore, find it justified to identify the species reported herein as *P. poucheti*.

Both morphology and SSU rDNA sequences indicate that *Paradinium* sp. (PaEu41) from *Euterpina acutifrons* is a species closely related to *P. poucheti* and, even though the two species exhibit some genetic dissimilarity, one may conclude that they are members of the same genus. The SSU rDNA sequence of an uncultured marine eukaryote (clone UEPAC05Hp2 from coastal waters of the Pacific Ocean) shows high similarity to *P. poucheti* (96% identical positions including introduced gaps) and is even more similar to *P. poucheti* than is *Paradinium* sp. (PaEu41). There is, therefore, little doubt that the uncultured clone UEPAC05Hp2 represents a *Paradinium* species. Due to limited material, thorough morphological studies of *Paradinium* sp. from *E. acutifrons* were not possible and we are, therefore, reluctant to describe it as a new species.

Originally, *Paradinium* was believed to be related to the syndinian dinoflagellates (Chatton 1910, 1920) due to its formation of a parasitic, multinucleate plasmodium and because it produces bi-flagellated spores. The flagellated spores of *Paradinium poucheti* have only been observed on a few occasions (Chatton 1920; Jepps 1937) and the spores do not possess any typical dinoflagellate characters. It has, therefore, not been possible to classify *Paradinium* with certainty despite *Paradinium* having been subject to several detailed morphological and ultrastructural studies (Chatton 1910, 1920; Cachon et al. 1968; Chatton and Soyer 1973). However, the mitotic processes in *Syndinium* and *Paradinium* are distinct, and this led Cachon et al. (1968) and Chatton and Soyer (1973) to draw the preliminary conclusion that *Paradinium* was affiliated with the Mycetozoa rather than with the dinoflagellates. Today, there is a large amount of genetic information available on mycetozoans (e.g. Baldauf and Doolittle 1997), and from SSU rDNA data alone one can argue that there is no close relationship between *Paradinium* spp. and the Mycetozoa. This is also reflected in Figure 5 in which *Paradinium* and Mycetozoa (included in Amoebozoa) branch distantly from each other.

Phylogenetic Position of *Paradinium*

The phylogenetic analyses indicated that both *Paradinium* species are related to the spot prawn parasite, SPP, of *Pandalus* spp. (Bower and Meyer 2002) and that the clade formed by these (Fig. 5) is basal to the Haplosporidia and the Foraminifera. SPP was, like *Paradinium poucheti*, originally considered a dinoflagellate-like organism due to the formation of a multinucleate plasmodium with some resemblance to the plasmodium formed by parasitic syndinian dinoflagellates (Meyers et al. 1994). However, molecular phylogeny based on both SSU rRNA and actin genes contradicted this and suggested SPP to be related to the haplosporidians (Reece et al. 2004). The present data corroborate the latter by suggesting a relationship between SPP, *Paradinium* spp., and the haplosporidians/Foraminifera. A phylogenetic study by Reece et al. (2004) demonstrated a close relationship between SPP and members of Haplosporidia, but it may have been premature when these authors concluded SPP to be a "haplosporidian parasite" because the Foraminifera may be even more closely related to the Haplosporidia (Fig. 5). A close relationship between Foraminifera and Haplosporidia has been shown before in an analysis based on both SSU rRNA and actin gene sequences (Nikolaev et al. 2004). It has also been demonstrated that the Foraminifera are related to Cercozoa in SSU rRNA single gene phylogeny as long as other fast-evolving sequences are omitted from the analysis (Berney and Pawlowski 2003). The present study is the first to include *Paradinium* in a phylogenetic analysis and when these sequences are included in the dataset, SPP does not group among the haplosporidians. This finding is, however, not controversial, considering that there are no morphological data supporting that SPP (or *Paradinium*) should belong to the Haplosporidia. They are all parasites of aquatic metazoans, but this gives little support for a phylogenetic relationship, since parasitism is common in many, if not most, groups of organisms. Distinctive morphological characters of Haplosporidia are the presence of haplosporosomes and the formation of a spore with an anterior pore with a hinged lid (Perkins 1990). The study by Cachon et al. (1968) revealed no haplosporosomes in *Paradinium* and no formation of the typical haplosporidian spore has been reported for *Paradinium*. These two observations also apply for

SPP (Bower and Meyer 2002). The conclusion at this stage must be that neither *Paradinium* nor SPP are haplosporidians. Based on SSU rDNA phylogeny, they have a strong affiliation to each other and they are members of the Rhizaria.

RP Parasite

The parasite mass that developed inside the tissue of copepods infected with the RP parasite had some resemblance to the plasmodium of *Paradinium* spp., and both parasites left their host through the digestive tract and subsequently adhered to the host's urosome. Based on gross morphology, the RP parasite was similar to the organism observed and identified as *Paradinium* by Jepps (1937) in the Clyde Sea area, since the organisms were similar with respect to the size, color, pathology, and manner of expulsion from their host. However, there are considerable morphological discrepancies between the RP parasite and *P. poucheti*, and this was also noted by Jepps (1937): the plasmodium of the RP parasite had a conspicuous reddish color; upon expulsion from its host it formed a cell mass that was considerably larger than the gonosphere of *P. poucheti* and this cell mass was amorphous, variable in size, and not bounded by a membrane with a reticulate surface pattern. While these characters apply for both the observations on the RP parasite in the present study and with those of Jepps (1937), they differ from the well-defined morphology of the *Paradinium* spp. gonosphere (Fig. 1, Cachon et al. 1968). Based solely on the morphology of the infectious stage, one must thus conclude that the RP parasite is not a *Paradinium* species.

In the present study morphological observations of unicellular stages of the RP parasite were made only of the specimen collected in the North Atlantic Ocean. Both SSU rDNA sequences, on the other hand, originated from isolates RPP1 and RPP2 collected in the Mediterranean Sea. Without having morphological observations and gene sequences from the same specimen, it may not be possible to state conclusively that all isolates belonged to the same species, but based on gross morphology and pathology it is to be expected that the North Atlantic isolate (Fig. 2B—D) is an organism identical, or at least closely related, to RPP1 and RPP2. The unicellular stage observed (Fig. 4) was different from the *Paradinium* "bodonispores" observed by Chatton (1920) and Cachon et al. (1968). However, Jepps (1937) observed the presence of both flagellated "bodonispore"-like cells and non-flagellated cells in material from RP parasites. Any presence or absence of flagella could not be concluded upon in the material of the present study. Some cells seemed to be bearing appendices (Fig. 4), but the morphology of these was variable and not consistent with the typical morphology of flagella. It is possible that the spherical cells observed were not fully developed at the time of fixation and that they would produce flagella at a later stage. While this is merely speculation, it would concur with the observations made by Jepps (1937).

Phylogenetic Position of the RP Parasite

Molecular data corroborated that the RP parasite is not related to *Paradinium*. The two SSU rDNA sequences obtained from individuals of the RP parasite are clearly different from those of *Paradinium* spp.; SSU rDNA sequences of the RP parasite consist of less than 1800 bp whereas SSU rDNA sequences of *Paradinium* spp. comprise more than 2000 bp. In the phylogenetic analyses (Fig. 5) the RP parasites formed a clade which included the sequence from an uncultured eukaryote, clone SCM37C34 sampled in the Sargasso Sea. The similarity between these sequences and the dissimilarity between these and other known sequences suggest that the sequence SCM37C34 originates from an organism closely related to the RP parasite. All three sequences branched as fast-evolving members of MAGI, the so-called "Marine alveolate Group I" (López-García et al. 2001; Moreira and López-García 2002), with high support in both BI and NJ analyses (Fig. 5). The monophyletic origins of both MAGI and the Syndiniales were originally established solely on the basis of SSU rRNA gene sequences from environmental samples (López-García et al. 2001, Moon-van der Staay et al. 2001). At the time of establishment of these groups, they were considered alveolates affiliated with the dinoflagellates, and the groups were tentatively named "Marine alveolate Groups I and II" because no morphological or ecological data were available. Marine alveolate Group II was subsequently demonstrated to represent Syndiniales, an order of parasites closely related to the dinoflagellates (Moreira and López-García 2002; Saldarriaga et al. 2004; Skovgaard et al. 2005). Interestingly, the parasitic dinoflagellate *Ichthyodinium* sp., which is presently classified among Syndiniales (Cachon and Cachon 1987), also branched with sequences from MAGI (Fig. 5), thereby supporting the recent finding by Mori et al. (2007). Another parasitic dinoflagellate, *Duboscquella* sp., has recently also been shown to

Table 1. List of hosts observed infected with *Paradinium* spp. or RP parasite.

Species	Host species	Locality	References
Paradinium poucheti Chatton	Calanoida: *Acartia clausi*	North Atlantic Ocean (French coast)	Pouchet (1890)[a]
		Mediterranean Sea (French coast)	Chatton and Soyer (1973), Chatton (1910, 1920, 1927)
	Cyclopoida: *Oithona similis*	Mediterranean Sea (French and Spanish coasts)	Cachon et al. (1968), Skovgaard and Saiz (2006), present study
		Godthåbsfjord (Greenland)	Present study
Paradinium caulleryi Chatton and Soyer	Cyclopoida: *Oncaea media*	Mediterranean Sea (French coast)	Chatton and Soyer (1973)
Paradinium mesnii Chatton and Soyer	Cyclopoida: *Oncaea conifera*	Mediterranean Sea (French coast)	Chatton and Soyer (1973)
Paradinium sp. (Parasite21)	Cyclopoida: *Oithona* sp.	North Sea	Apstein (1911)
Paradinium sp.	Harpacticoida: *Euterpina acutifrons*	Mediterranean Sea (Spanish coast)	Skovgaard and Saiz (2006), present study
"Paradinid"	Calanoida: *Centropages typicus*	Mediterranean Sea (French coast)	Chatton (1920)
	Calanoida: *Clausocalanus arcuicornis*	Mediterranean Sea (French coast)	Chatton (1920)
	Cyclopoida: *Oithona plumifera*	Mediterranean Sea (French coast)	Chatton (1920)
Atelodinium spp.[b]	Calanoida: *Paracalanus parvus*	Mediterranean Sea (French coast)	Chatton (1920)
RP parasite	Calanoida: *Calanus finmarchicus*	Clyde Sea, Scotland	Jepps (1937)
	Calanoida: *Pseudocalanus elongates*	Clyde Sea, Scotland	Jepps (1937)
	Calanoida, unidentified	North Atlantic Ocean	Present study
	Calanoida: *Clausocalanus* sp.	Mediterranean Sea (Spanish coast)	Present study

[a]"*Dias longiremis*" (presumably = *A. clausi*, Chatton 1920)
[b]Presumably *Paradinium* sp. (Chatton and Soyer 1973)

be a member of MAGI (Harada et al. 2007) and this is, likewise, corroborated in Figure 5. The inclusion of the RP parasite, *Ichthyodinium*, and *Duboscquella* in MAGI and the discovery of Dolven et al. (2007) that SSU rRNA gene sequences from a number of supposed parasites of marine Radiolaria group within MAGI (uncultured marine alveolate isolates 11, 21, 29, 37, 65, and 68; Fig. 5) suggest that all members of MAGI are most likely heterotrophic parasites, just as it appears to be the case for the related Syndiniales. There are still very little morphological data available on members of MAGI. Parasitic stages and free-swimming spores have been observed for *Duboscquella* spp. and *Ichthyodinium chabelardi*, and a few observations on the morphology of the RP parasite are reported in the present paper. However, the data on the RP parasite are not yet sufficient for a formal description of the genus, and it may not be a trivial task to obtain enough material for such a description, considering that until now only a few specimens of this parasite have ever been observed. In addition to the 3 specimens studied in the present paper, the RP parasite has thus far been observed only by Jepps (1937).

Occurrence of *Paradinium* spp. and the RP Parasite

Table 1 summarizes reports of *Paradinium* and the RP parasite from the literature. *Paradinium* spp. have been found off the Atlantic coast of France, in the NW Mediterranean Sea, in the North Sea, and now also in Greenland coastal waters (Table 1). This wide geographical distribution suggests that *Paradinium* spp. are ubiquitous in coastal waters. However, *Paradinium* spp. are only conspicuous when live gonosphere-bearing hosts are observed, since gonospheres detach during fixation of a sample (Skovgaard and Saiz 2006). Prior to the production of gonospheres, an infection of *Paradinium* spp. is only discernable after thorough microscopic studies (Chatton and Soyer 1973). Infection by *P. poucheti* can, however, reach a considerable prevalence. Chatton and Soyer (1973) found that up to 35% of an *Acartia clausi* population was infected with this parasite and these authors obtained a large number of gonospheres by incubating potentially infected hosts under laboratory conditions. There is little doubt that *Paradinium* spp. are common, but overlooked, components of neritic zooplankton communities.

The RP parasite is considerably easier to spot than *Paradinium* thanks to its dark reddish-brownish plasmodium occupying a great part of the infected host's body cavity. Nevertheless, a plasmodium of an RP parasite in, e.g. *Clausocalanus* spp. may be mistaken as being egg masses that often have a comparable dark tint. The parasite is, therefore, easily overlooked in both live and fixed zooplankton samples. The RP parasite has so far only been observed on a few occasions and exclusively by researchers who were searching specifically for parasites in planktonic copepods (Table 1). However, the finding sites for this organism cover a large geographical area, and it is possible that the RP parasite has a wide distribution.

Methods

Sampling: Live copepods were collected in the NW Mediterranean off Port Olímpic, Barcelona, Spain (41°22′77″ N, 02°13′15″ E), in the Atlantic Ocean north of the Azores (42°58′75″ N, 30°23′21″ W), and in the Godhåbsfjord, Greenland (64°10′23″ N, 51°47′36″ W). Samples were collected using a 100 μm mesh size plankton net hauled vertically from depths of 35—50 m to the surface. Animals were kept and examined at ambient seawater temperature (13—23 °C). Infected *Oithona similis* were collected on August 6, 2003, April 20, 2004, June 22, 2004 (NW Mediterranean), and September 12, 2006 (Godthåbsfjord). Infected *Euterpina acutifrons* were collected on March 23 and October 10, 2004 (NW Mediterranean). Infected *Clausocalanus* sp. were collected on September 1 and 22, 2004 (NW Mediterranean), and the infected calanoid was collected on September 16, 2006 (North Atlantic Ocean).

Light microscopy: Infected copepods were placed individually in 3 ml of GF/F-filtered Seawater contained in 3 ml Falcon™ multiwell cell culture plates (BD Biosciences). Live parasites and hosts were then observed and photographed while still contained in the cell culture plate using an Olympus SZX16 stereomicroscope. In addition, *P. poucheti* gonospheres were placed on microscope slides and observed at a higher magnification using a Nikon Diaphot inverted microscope. Gonospheres were delicate and disintegrated if a cover slip was placed on the slide, making more detailed microscopy troublesome.

SEM: Cells produced by an RP parasite from the unidentified calanoid were left in the filtered seawater for 4 d and then collected and mixed with an equal volume of filtered seawater containing 2% glutaraldehyde. This sample was then retained on a 5 μm pore size Isopore membrane filter (Millipore Corporation, Billerica, MA, USA), dehydrated through a graded ethanol series, and critical point dried (BAL-TEC CPD 030 Critical Point Dryer, BAL-TEC AG, BALZERS, Liechtenstein). The filter was then sputter-coated and viewed in a field emission scanning electron microscope (JEOL JSM-6335F, JEOL Ltd., Tokyo, Japan).

DNA extraction and amplification: Live copepods that had a *Paradinium* sp. gonosphere attached or were visibly infected with the RP parasite were isolated and rinsed in GF/F-filtered seawater. *Paradinium* spp. gonospheres were carefully removed by use of dissection needles, placed in 0.75 ml lysis buffer (40 mM EDTA, 50 mM Tris—HCl, 0.75 M sucrose) and stored at −70 °C. Copepods infected with the RP parasite were incubated for 1 d in 3 ml of filtered seawater prior to collecting the cell mass, which was then treated like the *Paradinium* gonospheres. Nucleic acids were extracted twice with phenol-chloroform-isoamyl alcohol as described in Skovgaard et al. (2005), recovered in 50 μl of sterile MilliQ water (Millipore Corporation), and stored at −70 °C until analysis. SSU rRNA genes were amplified by polymerase chain reaction (PCR) with the eukaryotic primers EukA and EukB (Medlin et al. 1988). The 50-μl PCR mixture contained 5 μl DNA extract as a template, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM of each primer, 1.25 units of Taq DNA polymerase (Promega Corporation, Madison, WI, USA), and the PCR buffer supplied with the enzyme. The PCR cycle, run in an automated thermocycler (MJ Mini cycler, Bio-Rad Laboratories, Hercules, CA, USA), was as follows: an initial denaturing step at 94 °C for 3 min, 30 cycles of denaturing at 94 °C for 45 s, annealing at 55 °C for 1 min and extension at 72 °C for 3 min, and a final extension at 72 °C for 10 min. In cases when no or very little PCR product was visible on an EtBr-stained agarose gel, a pair of semi-nested PCRs was performed using primers Euk A and Euk B together with ND7R and ND3F (Ekelund et al. 2004), respectively. 1 μl of the original PCR product was used as a template for the semi-nested PCRs that were run under the same conditions as the first reaction, except that the number of cycles was lowered to 20.

Sequencing: Amplified PCR products were purified with the Qiagen PCR purification kit (Qiagen, Venlo, The Netherlands) and sequenced with a Big Dye Terminator Cycle Sequencing kit (v.3) (PE Biosystems) and an ABI PRISM model 3130 XL automated sequencer (Applied Biosystems,

Foster City, CA, USA). A combination of the PCR primers and the internal, eukaryote-specific primers 516r (Amann et al. 1990), 528f (Elwood et al. 1985), 1209f (Giovannoni et al. 1988) and ND4F, ND7R, ND8R, and ND9R (Ekelund et al. 2004) was used to sequence the complete SSU rRNA gene in both directions. Sequence reads were aligned and assembled using ChromasPro software (Technelysium Pty Ltd, Tewantin, Australia). Sequences were submitted to GenBank under the accession numbers EU189027, EU189028 (RP parasite), and EU189029—EU189032 (*Paradinium* spp.).

Sequence alignments: BLAST searches revealed SSU rDNA sequences of haplosporidians and haplosporidian-like organisms as closest matches to *Paradinium* spp. and revealed alveolate sequences as closest matches to SSU rDNA sequences from the RP parasite. When exploring the phylogenetic position of *Paradinium* spp. and the RP parasite, a number of sequences were, therefore, assembled among which sequences from members of Haplosporidia and Alveolata were particularly well represented. This sequence selection encompassed, first of all, the two different complete sequences of *P. poucheti* and *Paradinium* sp. (PaOi21 and PaEu41) and the two sequences from the RP parasite: RPP1 and RPP2. Sequences that came out as closest matches in BLAST searches made on *Paradinium* spp. or RP parasite sequences were then included together with a broad selection of alveolate, haplosporidian, and haplosporidian-like sequences. It was, furthermore, attempted to include sequences from representatives of the main eukaryotic lineages, but most sequences that are thought to represent particularly fast-evolving taxa (e.g. Microsporidia, Entamoebidae, and Euglenozoa) were excluded, because these may erroneously be attracted to each other and/or the base of the phylogenetic tree due to the long-branch attraction phenomenon (Embley and Hirt 1998). Some authors have suggested *Paradinium* to be affiliated with the Mycetozoa (Cachon et al. 1968) and three mycetozoan sequences were, therefore, included in the alignment, even though these typically result in long branches and thereby tend to be placed erroneously near the root of rDNA trees (Philippe and Adoutte 1998). Also three sequences of the assumed fast-evolving Foraminifera were included, because Foraminifera have been shown to be closely affiliated with Haplosporidia (Berney and Pawlowski 2003; Nikolaev et al. 2004). The final selection contained 101 sequences, which were aligned using Clustal X 1.83 (Thompson et al. 1997) with default settings. Very variable regions of the alignment were located and removed using Gblocks (Castresana 2000) with parameters optimized for rDNA alignments (minimum length of a block: 5; allow gaps in half positions), leaving 1283 positions (included inserted gaps).

Phylogenetic analysis: Bayesian (BI) analyses were performed with MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). For the BI analyses, a GTR substitution model was used with gamma-distributed rate variation across sites and further model settings being defaults of the program. Four simultaneous Monte Carlo Markov chains were run from random trees for a total of 4.5×10^6 generations in two parallel runs. A tree was sampled every 50 generations, and a total of 5.000 trees were discarded as "burn-in" upon checking for stationarity by examination of the log-likelihood curves over generations. Based on the post-burn-in trees, a consensus tree (50% majority rule) was constructed and PP were calculated.

Modeltest (ver. 3.7) by Posada and Crandall (1998) was used to find among the 56 predefined models the one which best fitted the SSU rDNA sequences by hierarchical likelihood ratio tests. The best-fit model was TrN+I+G by Tamura and Nei (1993), where among sites rate heterogeneity was $\alpha = 0.5646$, an estimated proportion of invariable sites was $I = 0.0574$, and two substitution rate categories were A−G = 2.5891 and C−T = 4.1838. Base frequencies were set as follows A = 0.3008, C = 0.1886, G = 0.2372, and T = 0.2734. The Tamura and Nei model was used to compute dissimilarity values and the resulting distance matrix was applied to Neighbor-joining bootstrap with a total of 1000 replications. PAUP* (ver. 4b10) by Swofford (2003) was used for MP bootstrap analyses with a total of 1000 replications.

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