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ORIGINAL PAPER

Phylogenetic position and description of *Rhytidocystis cyamus* sp. n. (Apicomplexa, Rhytidocystidae): a novel intestinal parasite of the north-eastern Pacific 'stink worm' (Polychaeta, Opheliidae, *Travisia pupa*)

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Abstract A new *Rhytidocystis* species is described from the north-eastern Pacific Coast. Trophozoites of *R. cyamus* sp. n. were isolated from the intestines of the opheliid polychaete *Travisia pupa*, collected from mud dredged at a depth of 80 m. The trophozoites of *R. cyamus* sp. n. were relatively small (40–64 μm long, 27–30 μm wide) and bean-shaped with a centrally located nucleus. The trophozoite surface was inscribed by 10–12 longitudinal rows of short transverse folds and less conspicuous grooves with an irregular pattern. Micropores were observed in association with the transverse folds. A mucron or apical complex was not observed with either light or scanning electron microscopy. The trophozoites did not show any degree of motility. The SSU rDNA sequence obtained from *R. cyamus* sp. n. clustered strongly with *R. polygordiae* within the rhytidocystid clade. Although the precise phylogenetic position of the rhytidocystid clade within the Apicomplexa remains uncertain, the rhytidocystid sequences diverged with a weak affinity to a terrestrial clade containing cryptosporidians, neogregarines and monocystids.

Keywords Alveolata · Apicomplexa · Agamococcidiorida · phylogeny · *Rhytidocystis*

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Introduction

Apicomplexans form a large and diverse group of unicellular parasites containing around 6,000 described species, and probably well over a million that still remain to be discovered (Adl et al. 2007; Hausmann et al. 2003). Despite the relatively well-studied intracellular pathogens of humans and livestock (e.g., *Plasmodium*—the causative agent of malaria—and *Toxoplasma*), the diversity of apicomplexans, especially those inhabiting the world's oceans, has just started to be explored with molecular phylogenetic approaches. Among the least understood apicomplexans are gregarines and several coccidian-like lineages with uncertain phylogenetic positions (Leander and Ramey 2006; Kopečná et al. 2006). A growing knowledge of these lineages should elucidate the diversity and early evolutionary history of apicomplexans (Leander 2008).

One of the poorly understood coccidian-like lineages are the so-called 'Agamococcidiorida' (Levine 1979), an ill-defined group of apicomplexans with eucoccidian-like cyst characteristics (e.g., oocysts containing sporocysts with two sporozoites each), but supposedly lacking gamonts and merogony (Cox 1994; Leander and Ramey 2006). Like many marine gregarines, most 'agamococcidians' possess relatively large extracellular trophozoites; however, unlike gregarines, 'agamococcidian' trophozoites show no motility and are embedded within host epithelial tissues. This mixture of unique features might provide important insights into deep relationships among gregarines, cryptosporidians, and eucoccidians (Leander and Ramey 2006; Barta and Thompson 2006). Initially, 'agamococcidians' were interpreted to be unusual gregarines (de Beauchamp 1912; Henneguy 1907), but subsequent researchers argued that

these parasites were more closely allied to coccidians than to gregarines per se (de Beauchamp 1913; Levine 1979; Porchet-Henneré 1972). Molecular phylogenetic data are only available from *R. polygordiae* and a poorly understood parasite from the giant clam *Tridacna crocea*; although these data have helped establish a rhytidocystid clade, the deep phylogenetic position of this clade relative to coccidians and gregarines remains uncertain (Leander and Ramey 2006).

To date, there are two subgroups described within the 'Agamococcidia': Rhytidocystidae and Gemmocystidae (Levine 1979; Upton and Peters 1986). The latter family was described by Upton and Peters (1986) and contains one monotypic genus/species, namely *Gemmocystis cylindrus* Upton and Peters, 1986, that was isolated from the gastrodermis of different Caribbean scleractinian corals. Trophozoites were not described from *Gemmocystis*. The Rhytidocystidae contains the genus *Rhytidocystis* and four described species. Two of these species, namely *R. opheliae* Henneguy, 1907 and *R. henneguyi* de Beauchamp, 1912, were described from opheliid polychaetes (de Beauchamp 1912; Henneguy 1907). The most recently described species, *R. polygordiae* Leander and Ramey, 2006, was isolated from a polygordiid polychaete (Leander and Ramey 2006), a host that is closely related to opheliids (Giard 1880; McIntosh 1875; Rouse and Pleijel 2001). The host of the fourth *Rhytidocystis* species, *R. sthenelais* (Porchet-Henneré 1972) Levine, 1979, is the polychaete *Sthenelais boa* (Levine 1979). The life cycle of *Rhytidocystis* is relatively streamlined and involves sporozoites that penetrate the host intestine and then persist in the connective tissue, gonads, and coelom. Trophozoites develop from the sporozoites and form numerous "sporoblasts" by budding from the cell surface (Perkins et al. 2000). Each of the released sporoblast cells is surrounded by a resistant wall and becomes a spore. The cell within the spore wall divides once to form two sporozoites (compare Perkins et al. 2000). The hosts of all four described species were collected from the Atlantic Ocean, three (*R. ophelia*, *R. henneguyi*, and *R. sthenelais*) on the Atlantic Ocean shore of France and one (*R. polygordiae*) from the western coast of the Atlantic Ocean near Beach Haven Ridge, NJ, USA.

In this study, we describe for the first time a novel rhytidocystid species from the north-eastern Pacific Ocean. The host organism, the so-called 'stink worm' (*Travisia pupa*), is a polychaete that also belongs to the family Opheliidae. Light and scanning electron microscopy enabled us to characterize the general morphological features of this species. We sequenced the small subunit rDNA of the new species in order to establish a DNA barcode for this lineage and to evaluate its phylogenetic position relative to *R. polygordiae* and other apicomplexans.

Materials and methods

Collection and isolation of organisms

A dredge haul was conducted in the Imperial Eagle Channel (48°54′N, 125°12′W) in July 2007 at a depth of 80 m during a trip on the research vessel MV 'Alta' from the Bamfield Marine Science Centre, British Columbia, Canada. The Pacific 'stink worm' *Travisia pupa* Moore, 1906, an opheliid polychaete, was collected from these samples.

Trophozoites of *Rhytidocystis cyamus* sp. n. were released from host tissue by teasing apart the intestines with fine-tipped forceps under a low magnification stereomicroscope (Leica MZ6). Gut contents containing rhytidocystids were examined with an inverted compound microscope (Zeiss Axiovert 200 or Leica DM IL), and individual trophozoites were isolated by micromanipulation and washed in filtered seawater before being prepared for microscopy and DNA extraction.

Light and scanning electron microscopy

Differential interference contrast (DIC) light micrographs of *Rhytidocystis cyamus* sp. n. were produced by securing parasites under a cover slip with Vaseline and viewing them with an imaging microscope (Zeiss Axioplan 2) connected to a color digital camera (Leica DC500).

Individual trophozoites of *Rhytidocystis cyamus* sp. n. (n= 34 and 30) were prepared for scanning electron microscopy (SEM). Isolated cells were deposited directly into the threaded hole of separate Swinnex filter holders, containing a 5-μm polycarbonate membrane filter (Millipore, Billerica, MA), that was submerged in 10 ml of seawater within a small canister (2 cm diameter and 3.5 cm tall). A piece of Whatman filter paper was mounted on the inside base of a beaker (4 cm diameter and 5 cm tall) that was slightly larger than the canister. The Whatman filter paper was saturated with 4% OsO_4 and the beaker was turned over the canister. The parasites were fixed by OsO_4 vapours for 30 min. Ten drops of 4% OsO_4 were added directly to the seawater and the parasites were fixed for an additional 30 min on ice. A 10-ml syringe filled with distilled water was screwed to the Swinnex filter holder and the entire apparatus was removed from the canister containing seawater and fixative. The parasites were washed then dehydrated with a graded series of ethyl alcohol and critical point dried with CO_2. Filters were mounted on stubs, sputter coated with 5 nm gold, and viewed under a scanning electron microscope (Hitachi S4700). Some SEM data were presented on a black background using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA).

DNA isolation, PCR amplification, cloning, and sequencing

Twenty-six individual trophozoites were manually isolated from dissected hosts, washed three times in filtered seawater, and deposited into a 1.5-ml Eppendorf tube. Genomic DNA was extracted from the cells using the MasterPure complete DNA and RNA purification Kit (EPICENTRE, Madison, WI, USA). Small subunit rDNA sequences were PCR amplified using puReTaq Ready-to-go PCR beads (GE Healthcare, Quebec, Canada) and the following eukaryotic PCR primers: F1 5′-GCGCTACCTGGTTGATCCTGCC-3′ and R1 5′-GATCCTTCTGCAGGTTCACCTAC-3′ (Leander et al. 2003). The following internal primers, designed to match existing eukaryotic SSU sequences, were also used: F2 5′-AAGTCTGGTGCCAGCAGCC-3′, F3 5′-TGCGCTACCTGGTTGATCC-3′ and R2 5′-GCCTYGC GACCATACTCC-3′. PCR products corresponding to the expected size were gel isolated and cloned into the pCR 2.1 vector using the TOPO TA cloning kit (Invitrogen, Frederick, MD). Eight cloned plasmids, for each PCR product, were digested with *Eco*RI, and inserts were screened for size using gel electrophoresis. Two identical clones were sequenced with ABI Big-dye reaction mix using vector primers and internal primers oriented in both directions. The SSU rDNA sequences were identified by BLAST analysis and molecular phylogenetic analyses (GenBank Accession number GQ149767).

Molecular phylogenetic analysis

The new SSU rDNA sequence was aligned with 53 additional alveolate sequences using MacClade 4 (Maddison and Maddison 2000) and visual fine-tuning. The program PhyML (Guindon and Gascuel 2003; Guindon et al. 2005) was used to analyze the 54-sequence alignment (1178 unambiguously aligned positions; gaps excluded) with maximum-likelihood (ML) using a general-time reversible (GTR) model of nucleotide substitutions (Posada and Crandall 1998) that incorporated invariable sites and a discrete gamma distribution (eight categories) (GTR + I + Γ + 8 model: α=0.420 and fraction of invariable sites = 0.062). ML bootstrap analyses were performed on 100 re-sampled datasets using the same program set to the GTR model + Γ + 8 rate categories + invariable sites. Bayesian analysis of the 54-sequence dataset was performed using the program MrBayes 3.0 (Huelsenbeck and Ronquist 2001). The program was set to operate with GTR, a gamma-distribution, and four Monte Carlo Markov chains (MCMC; default temperature = 0.2). A total of 2,000,000 generations were calculated with trees sampled every 50 generations and with a prior burn-in of 100,000 generations (2,000 sampled trees were discarded; burn-in/convergence was checked manually). A majority rule consensus tree was constructed from 38,001 post-burn-in trees. Posterior probabilities correspond to the frequency at which a given node was found in the post-burn-in trees. Independent Bayesian runs on each alignment yielded the same results.

GenBank accession numbers

(AF494059) *Adelina bambarooniae*, (AJ415519) *Amoebophrya* sp. ex. *Prorocentrum micans*, (DQ462456) *Ascogregarina culicis*, (DQ462455) *Ascogregarina taiwanensis*, (AY603402) *Babesia bigemina*, (AY078092) *Colpodella pontica*, (AF330214) *Colpodella tetrahymenae*, (L19068) *Cryptosporidium baileyi*, (AF093489) *Cryptosporidium parvum*, (AF093502) *Cryptosporidium serpentis*, (AF39993) *Cytauxzoon felis*, (U67121) *Eimeria tenella*, (AB191437, AF372779, AF372780, AF372786, AY179975, AY179976, AY179977, AY179988) Environmental sequences, (FJ832163) *Filipodium phascolosomae*, (AF129882) *Gregarina niphandrodes*, (FJ832159) Gregarine from *Paranemertes peregrina*, (FJ832156) Gregarine from *Phyllochaetopterum prolifica*, (FJ832160) Gregarine from *Tubulanus polymorpha*, (AF022194) *Gymnodinium fuscum*, (AF286023) *Hematodinium* sp., (AF130361) *Hepatozoon catesbianae*, (DQ093796) *Lankesteria abbotti*, (EU670240) *Lankesteria chelyosomae*, (EU670241) *Lankesteria cystodytae*, (AF080611) *Lankesterella minima*, (FJ832157) *Lecudina longissima*, (AF457128) *Lecudina tuzetae*, (AF457130) *Leidyana migrator*, (DQ093795) *Lithocystis* sp., (AB000912) Marine parasite from *Tridacna crocea*, (AY334568) *Mattesia geminata*, (AF457127) *Monocystis agilis*, (AJ271354) *Neospora caninum*, (AF129883) *Ophryocystis elektroscirrha*, (AY196708) *Platyproteus vivax* ex. *Selenidium vivax*, (DQ093794) *Pterospora floridiensis*, (DQ093793) *Pterospora schizosoma*, (GQ149767) *Rhytidocystis cyamus*, (DQ273988) *Rhytidocystis polygordiae*, (M64244) *Sarcocystis muris*, (FJ832161) *Selenidium orientale*, (FJ832162) *Selenidium pisinnus*, (DQ683562) *Selenidium serpulae*, (AY196709) *Selenidium terebellae*, (DQ176427) *Syncystis mirabilis*, (AF013418) *Theileria parva* and (M97703) *Toxoplasma gondii*.

Results

The trophozoites of *Rhytidocystis cyamus* sp. n. were around 56 μm in length (40–64 μm, n=9) and 26.4 μm in width (27–30 μm, n=9). The cells were non-motile and were bean-shaped with a concave ventral and convex dorsal side. Neither a mucron nor an apical complex was observed

in the trophozoites (Fig. 1a–c, e). The trophozoites were symmetrical along the mid transverse planes with rounded ends at both sides (i.e., there was no distinction between an anterior end and a posterior end). The spherical to ellipsoidal nucleus was situated in the middle of the cell and measured 13.6 (12–15) μm in diameter (n=8). In some trophozoites, a large nucleolus about 6 (5–7) μm in diameter was visible within the nucleus (Fig. 1d). The cytoplasm had a granular appearance. Neither sporozoites nor oocysts were observed.

The trophozoite surface contained 5–6 longitudinal rows of short transverse folds per visible side (Fig. 1e–g); an extrapolated 10–12 longitudinal rows of short transverse folds was present in each trophozoite. The longitudinal rows of transverse folds did not follow a straight path and were more wave-like in organization. Some longitudinal rows were interconnected by a series of short folds that were oriented perpendicular to the longitudinal axis of the cell (Fig. 1e). The surface was also inscribed by relatively inconspicuous interconnecting grooves (Fig. 1e–f) that were distributed without any obvious pattern. Minuscule pores were also observed, mostly in association with the longitudinal rows of transverse folds (Fig. 1g).

Molecular phylogenetic analyses

Phylogenetic analyses of the 54-taxon data set resulted in a poorly resolved backbone that gave rise to a strongly

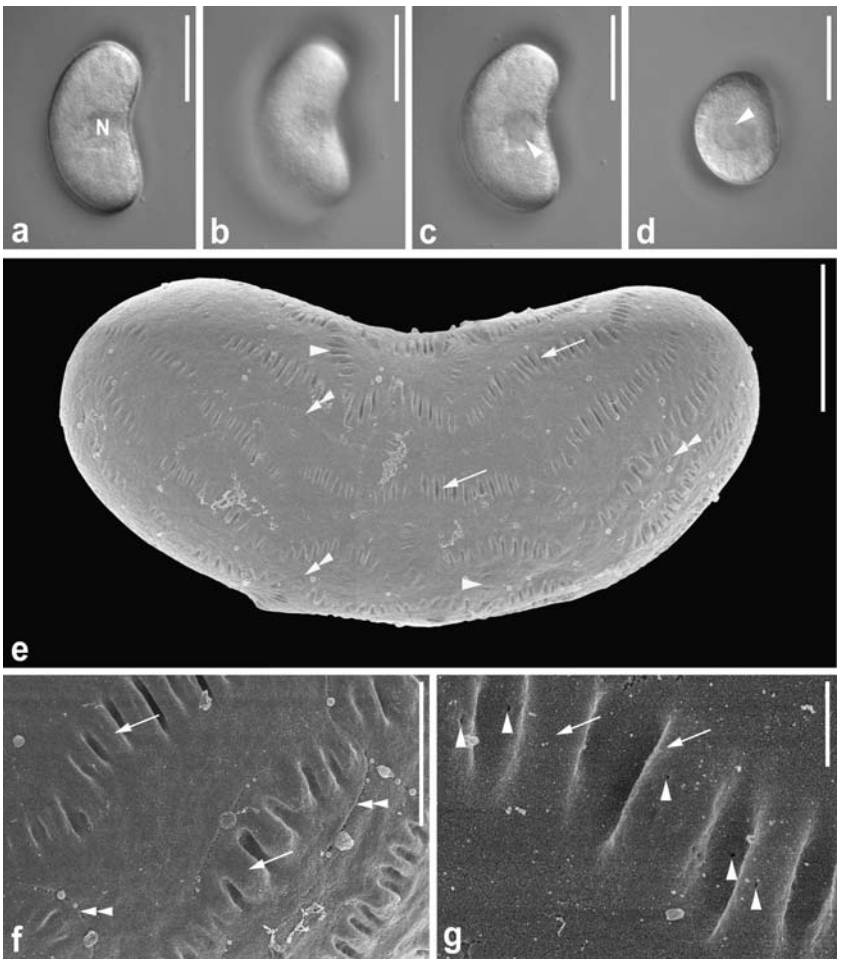

Fig. 1 Differential interference contrast (DIC) light micrographs and scanning electron micrographs (SEM) showing the general morphology and surface ultrastructure of the trophozoites of *Rhytidocystis cyamus* sp. n. **a–c** Trophozoites in different focal planes showing the bean-shaped cells and granular cytoplasm. The anterior and posterior ends are both rounded. A spherical nucleus (*N*) is situated in the middle of the cell and contains a prominent nucleolus (*arrowhead*). **d** Trophozoite in dorsal view showing the nucleus and nucleolus (*arrowhead*). **e** SEM showing a bean-shaped trophozoite in lateral view with longitudinal rows of short transverse folds (*arrows*); some longitudinal rows were interconnected by a series of short folds that were oriented perpendicular to the longitudinal axis of the cell (*arrowhead*). An unordered arrangement of inconspicuous grooves (*double arrowheads*) was also visible on the cell surface. **f** High magnification SEM of the short transverse folds (*arrows*) and the inconspicuous grooves (*double arrowheads*). **g** High magnification SEM of the short transverse folds (*arrows*) showing an irregular pattern of micropores (*arrowheads*). *Scale bars* **a–d** 30 μm, **e** 13.5 μm, **f** 5 μm, **g** 1 μm

supported clade of dinoflagellates (outgroup), a moderately supported clade of colpodellids, and a weakly supported clade of apicomplexans (Fig. 2). Within the apicomplexan clade, coccidians formed a paraphyletic group that gave rise to a strongly supported clade of piroplasmids. The apicomplexan backbone also gave rise to a strongly supported cryptosporidian clade that clustered with a strongly supported clade of neogregarines and monocystid-eugregarines; however, this clade of mainly terrestrial parasites received only weak statistical support. The SSU rDNA sequences from archigregarines, marine eugregarines and septate eugregarines formed a weakly supported clade, within which archigregarines formed the paraphyletic stem group. A clade of terrestrial septate eugregarines was strongly supported and formed the nearest sister group to a strongly supported clade of marine eugregarines, consisting of urosporids (*Pterospora* and *Lithocystis*) and lecudinids (*Lecudina* and *Lankesteria*).

The apicomplexan backbone also gave rise to a moderately supported clade of rhytidocystids, which included a poorly characterized parasite of giant clams (*Tridacna corcea*) and two *Rhytidocystis* species: *R. cyamus* sp. n. and *R. polygordiae* (Fig. 2). A total of 1,672 base pairs were compared between the three species in the rhytidocystid clade: 161 nucleotide differences and 9 gaps existed between the two *Rhytidocystis* species; 277 nucleotide differences and 34 gaps existed between *R. cyamus* sp. n. and the *Tridacna* parasite; and 270 nucleotide differences and 41 gaps existed between *R. polygordiae* and the *Tridacna* parasite. A pair-wise

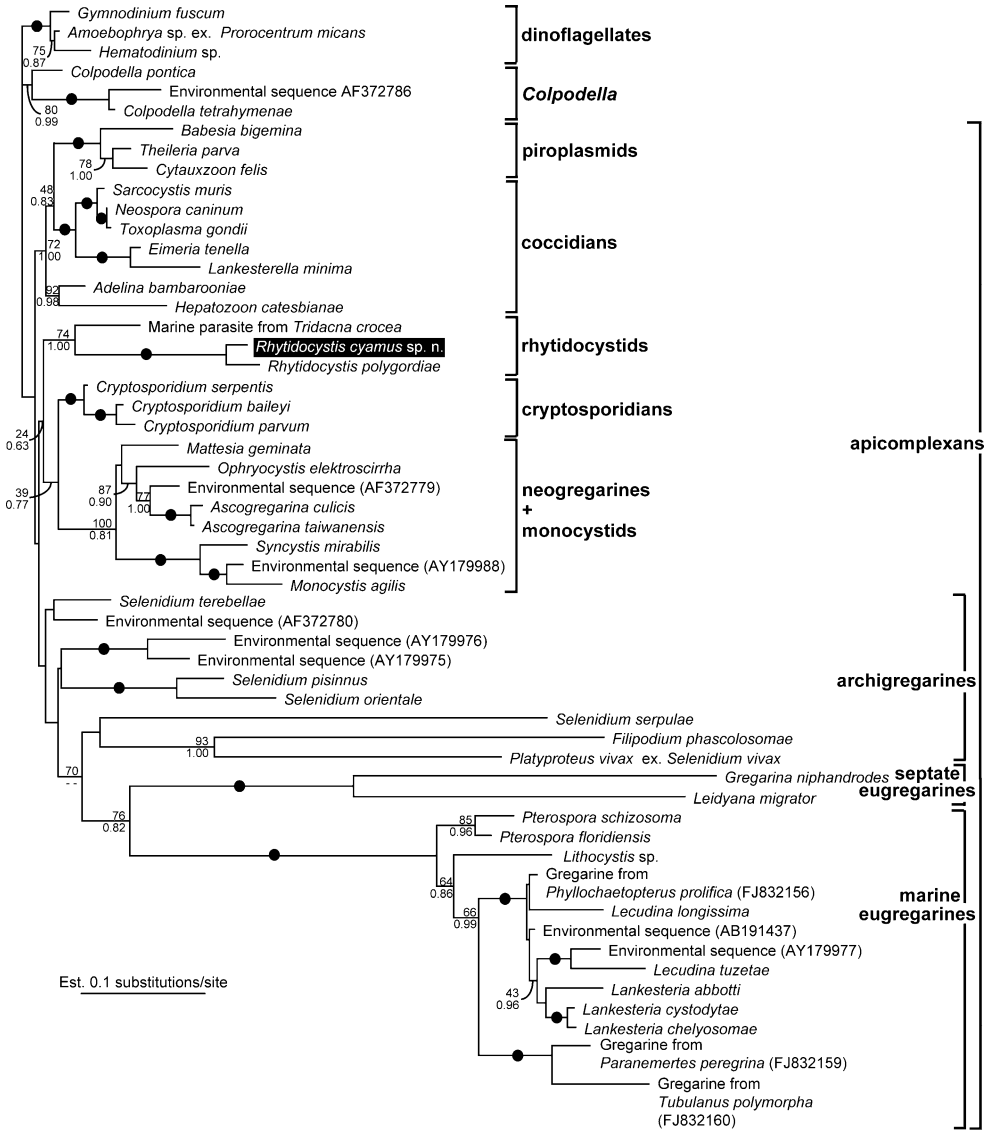

Fig. 2 Maximum-likelihood tree as inferred using the GTR model of nucleotide substitutions, a Γ-distribution and invariable sites on an alignment of 54 SSU rDNA sequences and 1178 unambiguously aligned sites (-ln L=157,58.58044, α=0.420, fraction of invariable sites = 0.062, eight rate categories). *Numbers at the branches* denote maximum likelihood (ML) bootstrap percentages (*top*) and Bayesian posterior probabilities (*bottom*). *Black dots on branches* denote Bayesian posterior probabilities and ML bootstrap percentages of 95% or higher. The sequence derived from this study is highlighted in the *shaded boxes*

distance calculation based on the Kimura 2-parameter model (Kimura 1980) of 1,672 base pairs (excluding the indels) resulted in a 10.6–19.3% sequence divergence between the three species.

Taxonomy

Apicomplexa Levine 1970
Agamococcidiorida Levine 1979
Rhytidocystidae Levine 1979
Rhytidocystis Henneguy 1907

Rhytidocystis cyamus Rueckert & Leander sp. n. (Fig. 1)

Description Trophozoites around 56 (40–64) μm long and 26.4 (27–30) μm wide. Trophozoites bean-shaped with rounded ends, without distinct mucron area or apical complex, and symmetrical along the mid-transverse plane. Spherical to ellipsoidal nucleus with large nucleolus positioned centrally in the cell. Trophozoites with 5–6 longitudinal wavelike rows of short transverse folds per side; 10–12 longitudinal rows of short transverse folds per cell. Longitudinal rows of short transverse folds sometimes connected by a series of short longitudinal folds oriented perpendicularly to the longitudinal axis of the cell. Relatively inconpsicuous grooves inscribe the cell surface without any obvious pattern. Micropores associated with the longitudinal rows of short transverse folds. No motility. Small subunit rDNA sequence (GenBank Accession number GQ149767).

Types

Iconotype Figures 1a, e.

Hapantotype Parasites on gold sputter-coated SEM stubs have been deposited in the Beaty Biodiversity Museum (Marine Invertebrate Collection) at the University of British Columbia, Vancouver, Canada.

Type locality Imperial Eagle Channel (48°54'N, 125°12'W) near Bamfield Marine Sciences Centre, Vancouver Island, Canada; muddy sediments at a depth of 80 m.

Habitat of the host Marine.

Etymology The species name *cyamus* (Greek) means bean (Borror 1960) and refers to the bean-shaped trophozoites of this species.

Type host Travisia pupa (Metazoa, Annelida, Polychaeta, Opheliidae).

Location in host Extracellular matrix of the intestinal epithelium.

Discussion

'Agamococcidians' are an inadequately defined group based on the absence of features, namely gamonts and merogony (Levine 1979); nonetheless, the group consists of five described species, four of which are described within *Rhytidocystis* from Atlantic polychaete hosts and one species falls within *Gemmocystis* from Caribbean scleractinian corals (Levine 1979; Upton and Peters 1986). The trophozoite morphology of *Rhytidocystis cyamus* sp. n. was concordant with that of the formerly described species (compare de Beauchamp 1913; Henneguy 1907; Levine 1979; Leander and Ramey 2006); however, trophozoites were not described in *R. sthenelais* (Porchet-Henneré 1972). *Rhytidocystis cyamus* sp. n. (40–64 μm long and 27–30 μm wide) is smaller than the two previously described species from Atlantic opheliid polychaetes (*R. opheliae* is 400 μm long and 300 μm wide, *R. henneguyi* 310 μm long and 220 wide), but was roughly the same size as *R. polygordiae* (50 μm long and 20 μm wide) from an Atlantic polygordiid polychaete. The bean-like shape of the trophozoites is different from all three species, which are either oblong or flat and oval (compare Leander and Ramey 2006). All *Rhytidocystis* species have a centrally located nucleus and a granular cytoplasm that is similar in appearance to the paraglycogen (syn. amylopectin) granules observed in gregarines (Landers 2002; Leander and Ramey 2006). A common and synapomorphic feature of rhytidocystids is longitudinal rows of short transverse folds. There are 5–6 rows per side (or 10–12 longitudinal rows per cell) in *R. cyamus* sp. n., 6–8 rows per cell in *R. polygordiae* (Leander and Ramey 2006) and 28–30 rows per cell in *R. opheliae* (de Beauchamp 1913). At this time, it is not known whether or not *R. henneguyi* also possesses these longitudinal rows of short transverse folds. Nonetheless, the trophozoite surface in *R. cyamus* sp. n. is also inscribed by irregular occurring rows of short longitudinal folds oriented perpendicular to the longitudinal axis of the cell and relatively inconspicuous grooves. These surface features, along with the novel host and the Pacific habitat, helped justify the establishment of the new species.

Molecular phylogeny and systematics of *Rhytidocystis cyamus* sp. n.

The establishment of the new *Rhytidocystis* species was also supported by the molecular phylogenetic analyses of

the 54-taxon SSU rDNA sequence dataset consisting of dinoflagellates, colpodellids, and apicomplexans. The new sequence of *R. cyamus* sp. n. clustered within the moderately supported clade of rhytodcystids and formed a strongly supported clade with *R. polygordiae*. Leander and Ramey (2006) first recognized the rhytidocystid clade as containing the marine parasite from *Tridacna*, *R. polygordiae* and an environmental sequence (AF372780); however, this particular environmental sequence no longer clustered with the rhytidocystid clade in our expanded phylogenetic analyses including *R. cyamus* sp. n. and several newly published gregarine sequences (Rueckert and Leander, unpublished data). Leander and Ramey (2006) also suggested that the parasite of *Tridacna* could be either another *Rhytidocystis* species or a closer relative to *Gemmocystis*, a parasite in the mesenterial filaments of corals (Upton and Peters 1986). Like in *Gemmocystis*, only the sporozoites of the *Tridacna* parasite have been described, which precludes comparative morphological analyses of the trophozoites (Upton and Peters 1986); whether or not trophozoite stages even exist in these two species remains uncertain. Nonetheless, a pair-wise comparison of the SSU rDNA sequences of both *Rhytidocystis* species (resulting in 161 differences and 9 gaps = 10.6% dissimilarity) strongly reinforces the morphological data and supports the establishment of a new *Rhytidocystis* species.

The precise phylogenetic position of the rhytidocystid clade in relation to eucoccidians and gregarines is still unclear. Morphologically, rhytidocystids share characteristics with both eucoccidians (e.g., cyst-related features) and gregarines (e.g., trophozoite features); in fact, rhytidocystids were first thought to be gregarines before subsequently interpreted to be more closely allied with coccidians (de Beauchamp 1913; Henneguy 1907; Levine 1979; Porchet-Henneré 1972). Although the molecular phylogenetic data are still relatively silent on this question, our analyses do show that the rhytidocystid clade forms a sister group to a clade consisting of cryptosporidians, neogregarines and monocystids, albeit with very weak support. Although these preliminary data are consistent with those presented by Leander and Ramey (2006) in supporting the possibility that rhytidocystids are most closely related to cryptosporidians and perhaps some gregarine lineages, additional data from different genetic markers are required for establishing more confident inferences about the early evolutionary history of rhytidocystid parasites.

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