

Fine structure of a septate gregarine trophozoite in the black tiger shrimp *Penaeus monodon*

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ABSTRACT: Gregarines are parasitic protozoa that occasionally parasitize the gut lumen of penaeid shrimp and other crustaceans. Here we describe the morphology of gregarine trophozoites found in the black tiger shrimp *Penaeus monodon* using light microscopy (LM) and scanning (SEM) and transmission (TEM) electron microscopy. Using LM with fresh preparations and with paraffin sectioning followed by hematoxylin-eosin staining, several trophozoites were discovered in the foregut and midgut, and gametocysts were found in the hindgut. Trophozoites existed both as solitary individuals and in association and exhibited either caudofrontal or lateral patterns. They were cylindrical in shape, 60 to 300 µm long by 10 to 60 µm wide, and consisted of 2 parts: a small anterior portion known as a protomerite, which was separated by a septum from a larger posterior portion known as a deutomerite. Under SEM, both the protomerite and deutomerite were found to be entirely covered with longitudinal, parallel pellicular folds 0.1 µm thick. Under TEM, the deutomerite was seen to contain a nucleus with a single eccentric nucleolus and several areas of peripherally located chromatin. The cytoplasm of both the protomerite and deutomerite contained abundant vesicles and granules of different sizes. The pellicle consisted of a double layer of electron-dense membranes separated by an electron-lucent area 30 nm wide and containing a microfilament. A few microfilaments were also observed in the cytoplasm underneath the pellicle, possibly serving as locomotive apparatus for the parasite. Based on its morphology, this gregarine appears similar to those of the genus *Nematopsis*.

KEY WORDS: Gregarine · Black tiger shrimp · *Penaeus monodon* · *Nematopsis* sp.

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INTRODUCTION

Four genera of gregarines (*Nematopsis*, *Cephalobus*, *Cephaloidophaidae*, and *Paraophioidina*) have been reported to infect penaeid shrimp (Couch 1978, Lightner 1993, Jones et al. 1994, Jimenez et al. 2002). Lightly infected shrimp may not show any clinical signs (Couch 1978), but severely infected shrimp may have reduced growth rates and yellow discoloration of the midgut, with perforation and hyperplasia of the midgut epithelium (Lightner 1993, 1996). The trophozoites are generally divided into septate and non-septate types, depending on the presence or absence of 'septa' dividing their bodies into different compartments known as the epimerite, protomerite, and deutomerite (Wallace & Taylor 1996). One of the 2 most economically valuable shrimp species, the Pacific white

leg shrimp *Litopenaeus vannamei*, has been reported to be infected both with septate (*Nematopsis penaeus*, *N. vannamei*, and *N. marinus*; Jimenez et al. 2002), and non-septate (*Paraophioidina scolecoides*) gregarines (Jones et al. 1994). The differences between septate and non-septate gregarines can be clearly illustrated in *N. marinus* and *P. scolecoides*. In the case of *N. marinus*, both immature and mature trophozoites were found in the midgut, and gametocysts and gymnosporos in the hindgut. The trophozoites consisted of a small hemispheric protomerite separated from a larger deutomerite containing a spherical or oval nucleus, and were either in gliding motion within the shrimp gut lumen or attached to the gut epithelium, and while some were solitary, others formed caudofrontal or lateral associations. Gametocysts were formed from curled-up trophozoites in the hindgut

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(Jimenez et al. 2002). In the case of *P. scolecooides*, only immature and mature trophozoites were observed in the midgut lumen of *L. vannamei* postlarvae, and no gametocysts were found. Trophozoites were found to be motile and form lateral but not caudofrontal associations (Jones et al. 1994).

The black tiger shrimp *Penaeus monodon*, another highly valuable species, has also been known to be infected by gregarines, especially during larval and postlarval stages (B. Withyachumnarnkul unpubl.). Recently, a gregarine infection was observed in the gut lumen of *P. monodon* juveniles with monodon slow-growth syndrome, without significant mortality or morbidity to the host (Chayaburakul et al. 2004). Since there have been no reports on the fine structure of gregarines infecting shrimp species thus far, we report here the morphological findings of gregarine trophozoites infecting the gut lumen of *P. monodon* under light (LM) and scanning (SEM) and transmission electron (TEM) microscopy.

MATERIALS AND METHODS

Shrimp samples. Seventy gregarine-infested *Penaeus monodon* juveniles, 10 to 20 g body weight, were obtained from commercial farms in Thailand. None showed any signs of lethargy or morbidity, and showed normal growth, with no significant mortality. The shrimp gut was isolated using fine forceps, placed on glass slides in normal seawater, and examined for the presence of gregarine trophozoites and gametocysts using a dissecting microscope, and the fresh specimens were photographed. Individual trophozoites and gut segments that contained trophozoites and gametocysts were removed and observed and these fresh preparations were examined by LM, as well as being processed for paraffin section, and for SEM and TEM studies.

Morphological studies. For LM studies, the tissues were fixed in Davidson's fixative, dehydrated through an ascending ethanol series, embedded in paraffin blocks, sectioned at 5 μm thickness, and stained in hematoxylin and eosin (H&E).

For SEM, individual trophozoites were deposited directly into the threaded hole of a filter holder containing a 5 μm polycarbonate membrane filter submerged in 10 ml of seawater within a small canister (2 cm diameter and 3.5 cm high). A piece of Whatman filter paper was mounted on the inside base of a beaker (4 cm diameter and 5 cm high) 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 vapors for 30 min. Ten drops of 4% OsO_4 were then

added directly to the seawater, and the parasites were fixed for an additional 30 min. A 10 ml syringe filled with distilled water was screwed to the filter holder, and the entire apparatus was removed from the canister containing seawater and fixative. The specimens were washed and dehydrated through a graded ethanol series and critical point dried with CO_2 . Filters were then mounted on stubs, sputter coated with 5 nm gold, and viewed under an SEM (Hitachi S4700).

For TEM, small tissue pieces (~2 to 3 mm^3) were fixed in 2.5% glutaraldehyde fixative for 12 h at room temperature, rinsed in 0.2 M cacodylate buffer at pH 7.2 for 48 h, and post-fixed in 1% OsO_4 using the same buffer. The specimens were dehydrated through an ascending ethanol series, cleared in propylene oxide, and embedded in Araldite 502 Epon resin. Blocks were cut on an LKB ultramicrotome. Most semi-thin sections (1 μm thick) were stained with 2.5% toluidine blue in 1% aqueous sodium borate solution, while a few semi-thin sections were additionally stained with periodic acid-Schiff (PAS), to identify any carbohydrate content in the parasite. Ultra-thin sections were collected on copper grids and double stained with uranyl acetate and lead citrate and examined by TEM at 120 kV.

RESULTS

Ten or more motile trophozoites were observed in the foregut and midgut, and several gametocysts were found in the hindgut of an infected *Penaeus monodon*. The fresh specimens showed 3 forms of trophozoites: solitary (Fig. 1a), caudofrontal association (Fig. 1b), and lateral association (Fig. 1c). The trophozoites were cylindrical, 60 to 300 μm long by 10 to 60 μm wide, and consisted of a hemispheric shaped protomerite separated from a longer deutomerite part by a septum; both parts contained numerous refractile granules. The deutomerite contained a distinctive nucleus, 10 to 12 μm in diameter, in the anterior third. A clear zone of about 5 μm in width was observed between the cytoplasm and the outer limit of the trophozoite (Fig. 1c); this zone corresponded to the pellicles observed under TEM (see Fig. 4). With H&E staining, the trophozoite nucleus and cytoplasm stained basophilically and were seen to contain refractile granules and vesicles (Fig. 2a), the vesicles being clearly observed in semi-thin sections with toluidine and PAS stains (Fig. 2b). Under PAS staining, both protomerite and deutomerite were seen to contain deep purple granules, suggesting that they were glycogenic in nature; they were distributed evenly in the protomerite and deutomerite of the parasite, but were clearly voided in a narrow space about 1 to 3 μm underneath the parasitic outer limit. It appeared that this narrow space repre-

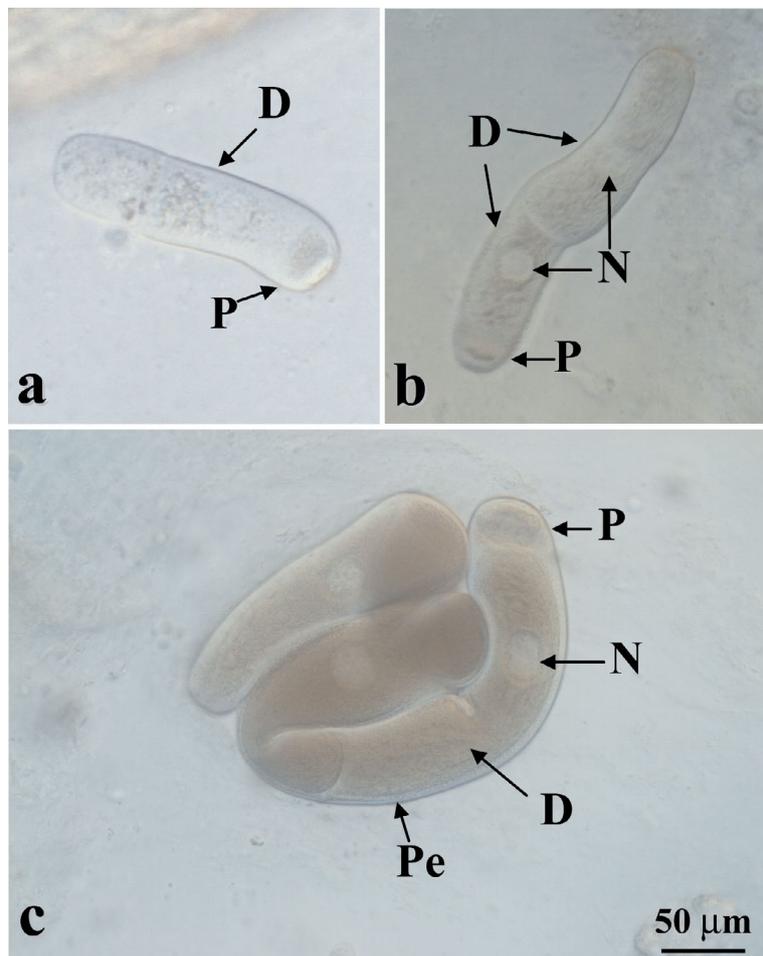


Fig. 1. Fresh preparation of the gregarine infecting *Penaeus monodon* showing (a) a solitary trophozoite, (b) a trophozoite in caudofrontal association and (c) a trophozoite in lateral association. Magnification (scale bar) is the same for all 3 images. D: deutomerite; N: nucleus; P: protomerite; Pe: pellicle

sented the pellicle (see Fig. 4), which contained no glycogen granules. There were several large clear vesicles (Fig. 2b), predominantly in the protomerite, which were probably lipid in nature, as the lipid was dissolved away by solvent under the TEM procedure. The gametocysts were spherical and of variable diameter within the range of 20 to 80 μm (Fig. 2c), some of them with a clear gelatinous wall (Fig. 2c, inset).

Under SEM, the body surface of the trophozoite had a striated appearance with several 'pits' distributed all over the surface (Fig. 3a). The protomerite was located on the anterior tapering end, separated from the deutomerite by a slightly raised ridge. About one-third from the anterior end of the deutomerite, a bump on the surface was observed in the region where the nucleus was located ('N' in Fig. 3a). With higher magnification the striation was clearly observed as being formed by longitudinal epicytic folds of about 0.1 μm in thickness (Fig. 3b–d). The folds were parallel and ran

along the trophozoite's longitudinal axis, and were open at several points, creating pits 1 to 2 μm wide by 5 to 10 μm long, as observed under low-power magnification. The pits were wider at the middle than at the tail of the deutomerite (cf. Fig. 3c,d), and the tail of the deutomerite had a rather blunt end (Fig. 3c) compared to the tapering shape of the protomerite.

Using TEM, the flat sheets observed under SEM appeared as pellicular folds each 0.1 μm wide and 0.1 to 5–6 μm long (Fig. 4a), the length probably depending on the angle of the sections. At the anterior end of the protomerite, the pellicular folds were in close contact with microvilli of the shrimp gut epithelial cells (Fig. 4a,c). Each fold was lined with double electron-dense membranes separated by an electron-lucent area 30 nm wide; a microfilament was occasionally observed within the electron-lucent area (Fig. 4b). The 2 parts of the trophozoite were separated by a septum comprising an amorphous electron-dense band 250 nm

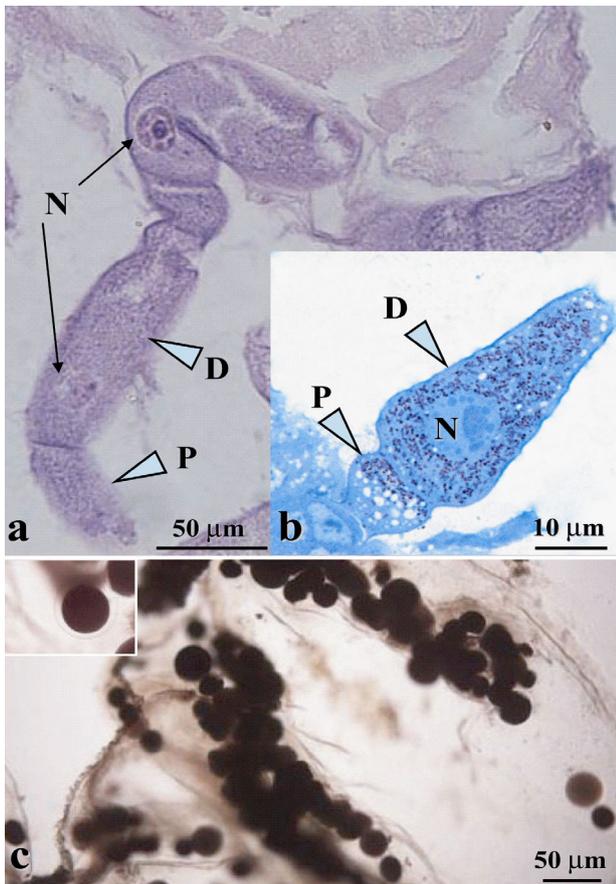


Fig. 2. (a) Mature trophozoites in the gut lumen of *Penaeus monodon* in caudofrontal association, paraffin section, H&E stain, and (b) solitary trophozoite shown by semi-thin section with toluidine and periodic acid-Schiff stain. (c) Fresh preparations of gametocysts obtained from the hindgut were of different sizes; inset shows a gametocyst with a gelatinous wall (same magnification). D: deutomerite; N: nucleus; P: protomerite

thick (Fig. 4c,d). Both the protomerite and deutomerite contained abundant vesicles and electron-dense granules. The vesicles were round or oval, of variable size between 0.1 and 0.8 μm in diameter, and contained electron-lucent material. Many of the vesicles had a clear space at the center of the electron-lucent material, while others were entirely occupied by clear space. Vesicles were more abundant in the protomerite and could be observed clearly under LM (Fig. 2b). The electron-dense granules were divided into small (10 to 20 nm) and large (0.1 to 0.3 μm) granules (Fig. 4a,d). The larger granules were also observed in the periphery of the parasite underneath the surface, had different levels of electron-density and appearance, and were similar to those located on the external surface of the parasite, especially between the pellicular folds (Fig. 4a,b,f), indicating that they might be structures

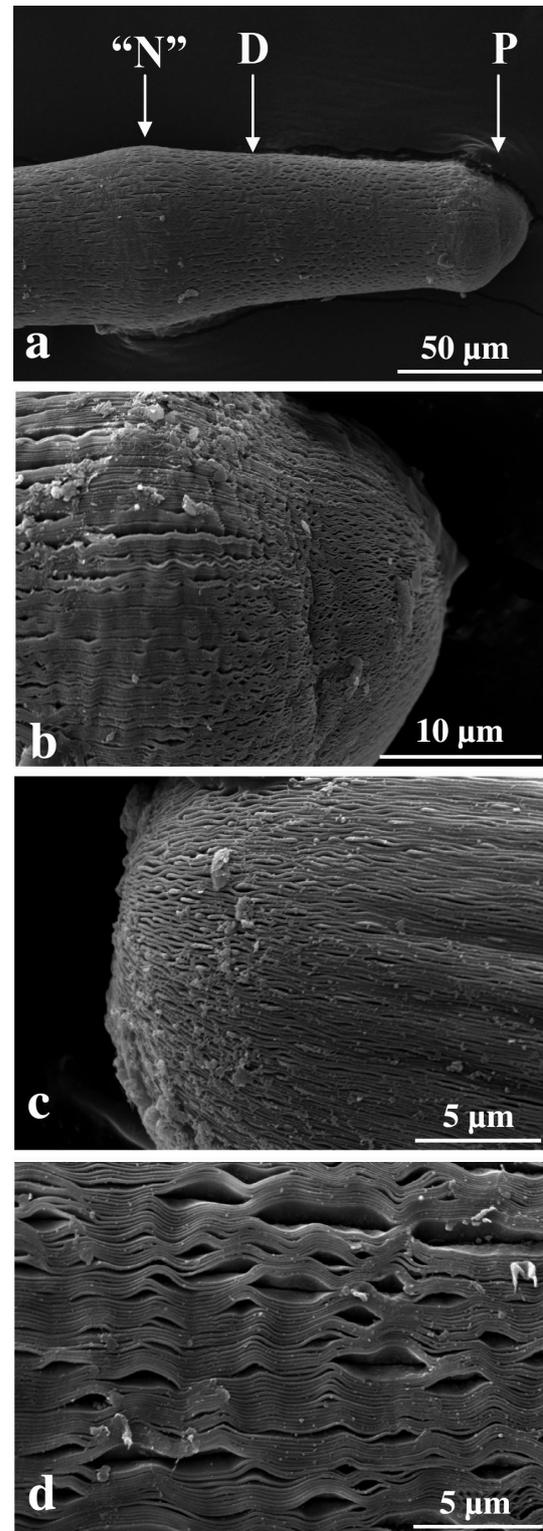


Fig. 3. SEM of the surface of a mature trophozoite, showing (a) low magnification of the protomerite, where 'N' represents a bulging mark on the surface thought to be the location of the nucleus, (b) high magnification of the protomerite, (c) the end and (d) the middle parts of the deutomerite. D: deutomerite; P: protomerite

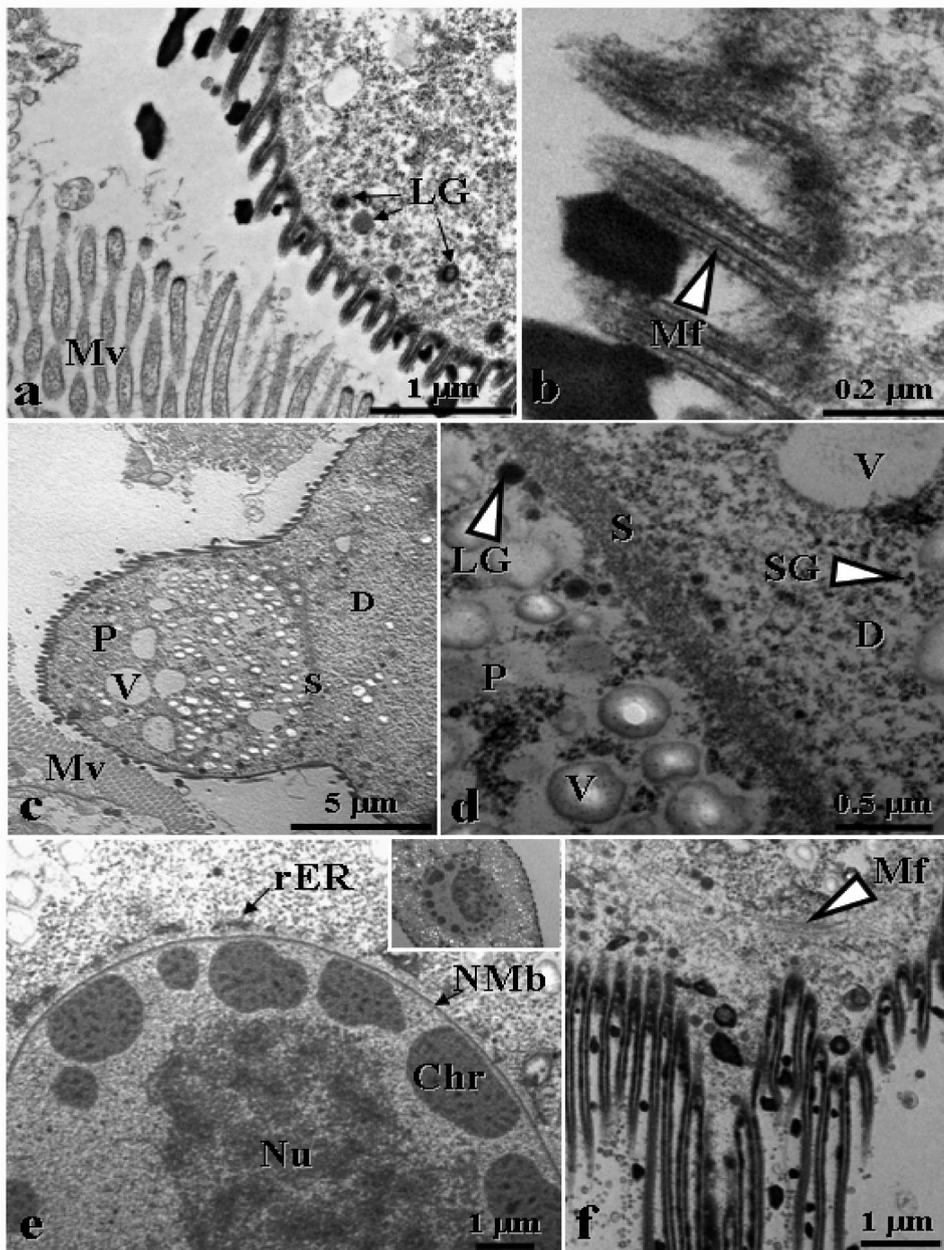


Fig. 4. TEM of mature trophozoites. (a) The trophozoite pellicular folds are in close approximation to the microvilli of the shrimp gut epithelium. (b) High magnification of the pellicular fold shows detail of the pellicle with microfilament in the electron-lucent area between 2 electron-dense membranes. (c) The protomerite is separated by a septum from the deutomerite; both parts contain cytoplasmic vesicles and granules. (d) At high magnification, the septum is an amorphous structure containing no organelles. (e) The nucleus of the trophozoite, with inset showing the entire nucleus, contains a large eccentric nucleolus and several peripheral patches of chromatin. (f) The tail of the deutomerite contains microfilaments in the cytoplasm. Chr: chromatin body; D: deutomerite; LG: large electron-dense granule; Mf: microfilament; Mv: microvilli; NMb: nuclear membrane; P: protomerite; Nu: nucleolus; rER: rough endoplasmic reticulum; S: septum; SG: small electron-dense granule; V: vesicle

with secretory/excretory functions or for uptake of materials from the exterior milieu.

The nucleus contained a fairly uniform, fine granular nucleoplasm, a relatively large and eccentrically located nucleolus, and peripheral patches of chromatin

along the nuclear membrane (Fig. 4e). Rough endoplasmic reticula (rER) were observed close to the nuclear membrane. Bundles of 20–30 nm microfilaments, possibly myonemes, were occasionally detected beneath the pellicle (Fig. 4f).

DISCUSSION

The morphology of this septate-type gregarine as observed under LM was similar to the gregarine *Nematopsis marinus* found in *Litopenaeus vannamei* (Jimenez et al. 2002). Both the size and structure under fresh preparation, together with the size and shape of gametocysts as described by the authors, were similar to the gregarine found in the present study, including the association characteristics and striate appearance of the surface of the trophozoite. Under LM, the shape and size of the nucleus of *N. marinus* in the study by Jimenez et al. (2002) was similar to that in the present study, although without ultra-structural features for comparison, it is impossible to indicate if the gregarine species in this study is actually *N. marinus*. Jimenez et al. (2002) also reported a longitudinally striated appearance in *N. marinus*, which could be interpreted as the pellicular folds of the gregarine as observed in our study, but since Jimenez et al. (2002) did not include SEM features in their study this cannot be stated with certainty.

The surface details of the gregarine described herein have not previously been reported for any gregarine species. The clear peripheral zone of the parasite observed in fresh preparation may represent the pellicular folds observed under SEM. These pellicular folds may protect the parasite against enzymatic digestion in the shrimp gut lumen, and may also have a function in parasitic movement as supported by the presence of microfilaments within the pellicular folds. Separation of adjacent sheets to form pits may result from a longitudinal contraction of the deutomerite during peristaltic movements, which in turn may be produced by the contraction of microfilaments underneath the pellicle, similar to the myonemes described for the gregarine *Pyxinooides balani*, which infects the barnacle *Balanus tintinabulum* (Reger 1967). Fewer smaller pits were found at the tail of the deutomerite, suggesting that it may be less involved in gregarine motility than the middle part of the deutomerite.

Specialized structures covering gregarine bodies have previously been described. The gregarine *Urospora chiridotae*, which infects the sea cucumber *Chiridota laevis*, was shown to be covered by hair-like projections known as cytopili when viewed under SEM (Dyakin & Simdyanov 2005). Several microtubules were observed inside these cytopili, and it was proposed that the cytopili have a motility function in the parasite. Another gregarine species that has a specialized surface is *Gregarina niphandrodes* infecting the yellow mealworm beetle *Tenebrio molitor* (Toso & Omoto 2007). Its surface as observed under TEM is covered by folding structures called epicytic folds, 0.3 to 0.8 μm long, which is in the same range as the pellicular folds in our study; however, without SEM data it is not

known whether these epicytic folds are the same as the pellicular folds in the present study, or whether they are similar to cytopili as in the gregarine *U. chiridotae*. In 2 gregarines infecting the polychaete *AxiotHELLa mucosa* (*Pterospora floridiensis* and *P. schizosoma*), the surfaces were found to have several short parallel ridges forming lines connected in a web-like fashion or swelling rosette pattern, respectively, which are believed to aid movement inside the host coelom (Landers & Leander 2005). Lastly, the archigregarine *Selenidium orientale*, which infects the sipunculid *Themiste pyroides*, has a surface composed of 15 broad-based and relatively short pellicular ridges (Simdyanov & Kuvardina 2007). The surface structures of gregarines described thus far are similar to those of the gregarine reported in the present study.

Parasitic movement requires energy. Glycogen or another carbohydrate substance observed using the PAS stain could be a source of energy using the glycolytic pathway. The PAS-positive granules observed under LM could correspond to the small dense granules observed under TEM, and may be comparable to 'paraglycogen' granules observed in the gregarine *Haemogregarine* sp. from the frog *Rana berlandieri* (Desser & Weller 1973) and in *Lankesteria culicis* from the mosquito *Aedes aegypti* (Walsh & Callaway 1969). No typical mitochondria were observed in this gregarine species, but it is possible that they may exist in other forms (Landers 2002) or in other stages of development (Chen et al. 1997). As regards the vesicles, features of their contents were similar to amylopectin as previously described in the gregarine *Hemolivia mariae* infecting the lizard *Tiligua rugosa*, and the clear feature in the vesicles may represent fat droplets.

The ultra-structure of the pellicle in the present study, with a double layer of electron-dense membrane separated by an electron-lucent area, was also observed in the gregarine *Zeylanocystis burti* Dis-sanaike (Sathananthan 1977). These electron-dense membranes suggest the presence of abundant protein molecules on the lipid membrane, which could function as channel proteins to regulate the flow of substances during secretion/excretion or absorption of nutrients from the host gut environment.

The nucleus of the gregarine in this study was similar to those of the gregarines *Pterospora floridiensis* (Landers 2002) and *Haemogregarine* sp. (Desser & Weller 1973). A relatively large nucleolus in the nucleus suggests active protein production. A lack of Golgi complexes suggests the proteins produced could be peptides without glycosylation, in contrast to features observed in the gregarine *P. balani*, where Golgi complexes were oriented along the protomerite face of the septum and gave rise to cisternae constituting part of the septum (Reger 1967).

In conclusion, we have described the morphological features of a gregarine species infecting *Penaeus monodon*, including ultra-structural features showing both surface and cytoplasmic details of the parasite. Under LM, the gregarine appears similar to *Nematopsis marinus*, but without ultra-structural features for *N. marinus* with which to compare it, it is not known whether this is the same or a new species. Further studies on the ultra-structure of *N. marinus* and genomic DNA sequences of the gregarines infecting the 2 shrimp species concerned (*P. monodon* and *Litopenaeus vannamei*) should help answer this question.

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