

Ultrastructural features of the tomont of *Cryptocaryon irritans* (Ciliophora: Prostomatea), a parasitic ciliate of marine fishes

RUI MA¹, XINPENG FAN^{1*}, FEI YIN^{2*}, BING NI¹ and FUKANG GU¹

¹ School of Life Sciences, East China Normal University, Shanghai, China

² Key Laboratory of East China Sea and Oceanic Fishery Resources Exploitation, Ministry of Agriculture, East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Shanghai, China

(Received 12 October 2016; revised 9 December 2016; accepted 14 December 2016; first published online 30 January 2017)

SUMMARY

Numerous studies have been conducted on the cellular morphology of *Cryptocaryon irritans*. However, details regarding the tomont stage of its life cycle remain lacking. In this study, we investigated the morphology of the tomont stage throughout encystment and cell division using light and electron microscopy. Results showed that there was no secretion of encystation-specific secretory vesicles or extrusomes during formation of the cyst wall. Instead, the synthesis and construction of the *C. irritans* cyst wall materials may involve molecular events at the pellicle. The somatic cilia and the cytosome were present during encystment and covered by the newly formed cyst wall. New somatic cilia were continuously created between old cilia and showed various lengths during cell division, a process that was similar to morphogenesis in many free-living ciliates. During cell division inside the tomont, dividing daughter cells formed temporary cell chains with no oral primordia before separating from each other into dissociative tomite precursors. The process of cell division may not be accompanied by stomatogenesis, and new oral primordia in offspring cells likely formed before the dividing cell chains split into dissociative spherical tomites. Mitochondrial autophagy was observed in encysting *C. irritans* cells. Numerous endosymbionts and Golgi structures were observed in the tomont cytoplasm. Cellular metabolic activity in the *C. irritans* tomont was quite high, with large amounts of materials or cellular organelles potentially being synthesized and prepared for the following infective theront stage.

Key words: *Cryptocaryon irritans*, tomont, electron microscopy, ultrastructure.

INTRODUCTION

The ciliated protozoan *Cryptocaryon irritans* Brown, 1951 is an obligate parasite that causes cryptocaryonosis in a wide range of marine teleosts. Over the years, this disease has had a significant negative impact on the marine aquaculture industry worldwide, resulting in large economic losses (Colorni and Burgess, 1997; Dickerson, 2006). Many chemical and physical methods have been used to control this parasite, and several biological substances have been found to be lethal to *C. irritans* or useful as potential antigens for vaccine development. These exhibit various degrees of efficacy and frequently accompany with various drawbacks (Pironet and Jones, 2000; Hirazawa *et al.* 2001, 2003; Hatanaka *et al.* 2007; Bai *et al.* 2008; Wang *et al.* 2010; Huang *et al.* 2012; Li *et al.* 2013; Niu *et al.* 2013; Rigos *et al.* 2013).

The difficulty in eradicating *C. irritans* from the mariculture system partially arises from the

complexity of its life cycle (Colorni and Burgess, 1997; Rigos *et al.* 2013). The life cycle of *C. irritans* is customarily divided into four stages, including the highly motile, infective theront; the parasitic and feeding trophont; the off-host and temporarily free-living protomont; and the reproductive cyst or tomont, which has a thick cyst wall (Yoshinaga and Dickerson 1994; Colorni and Burgess, 1997; Dickerson, 2006; Lokanathan *et al.* 2010). Treatment is particularly difficult owing to the existence of the thick cyst wall and asynchronous excystment of the infective theronts from tomites (Colorni, 1985; Burgess, 1992; Hirazawa *et al.* 2003; Dickerson, 2006). Studies aimed particularly at elucidating the tomont stage of *C. irritans* will therefore enhance our understanding of the basic features of the tomont, allowing for further exploration of targeted methods to prevent and control the parasitic disease.

Numerous studies have been conducted on the biology of *C. irritans*, including research into its life cycle, cellular morphology, proteome, transcriptome and immunogenicity (Brown, 1963; Nigrelli and Ruggieri, 1966; Cheung *et al.* 1981; Colorni and Diamant, 1993; Matthews *et al.* 1993; Colorni and Burgess, 1997; Diggles, 1997; Huang *et al.* 2005; Dickerson, 2006; Li *et al.* 2006; Ma

* Corresponding author. School of Life Sciences, East China Normal University, Shanghai 200241, China. E-mail: xpfan@bio.ecnu.edu.cn; Key Laboratory of East China Sea and Oceanic Fishery Resources Exploitation, Ministry of Agriculture, East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Room 316, Building 6, 300 Jungong Road, Shanghai 200090, China. E-mail: yinf@ecsf.ac.cn

et al. 2006; Bai *et al.* 2008; Lokanathan *et al.* 2010; Mai *et al.* 2015; Ma *et al.* 2016). However, information is still lacking with regards to the ultrastructural features of the tomont stage. While the fine structure of the cyst wall has been roughly described in several studies, very few details are available involving the formation of the cyst wall (Xu *et al.*, 1992; Colorni and Diamant, 1993; Matthews *et al.* 1993; Kesintepe, 1995; Huang *et al.* 2005). It is confused regarding whether somatic cilia are present during encystment (Nigrelli and Ruggieri, 1966; Cheung *et al.* 1981; Colorni and Diamant, 1993; Matthews *et al.* 1993; Huang *et al.* 2005). In addition, knowledge of the features of cell division in the tomont are restricted to the light microscopic level (Brown, 1963; Nigrelli and Ruggieri, 1966; Xu *et al.*, 1992), and there are few descriptions of cellular organelles in the cytoplasm of the tomont (Matthews *et al.* 1993; Huang *et al.* 2005).

In this study, the morphology of the tomont of *C. irritans* was investigated using light and electron microscopy. Detailed observations were made throughout encystment and subsequent cell-division progress.

MATERIALS AND METHODS

Culture and collection of C. irritans tomonts and theronts

Cryptocaryon irritans cells were isolated from a naturally infected host fish, *Larimichthys crocea*, in an aquaculture farm in Ningde, Fujian Province, China (Fufa Fisheries Co., Ltd.). Cells were propagated using a method modified from Yin *et al.* (2015) with a water temperature of 25 ± 1 °C and *L. crocea* as the host fish. Cells at different stages of the life cycle of *C. irritans* were collected as follows. Trophonts were scraped from the skin or gills of hosts, and then collected using a glass pipette. Protomonts and tomonts were collected from petri dishes placed on the bottom of the aquarium containing infected *L. crocea*. Tomonts were collected once an hour in the 15 h after protomont attachment. They need to be fixed using 2.5% glutaraldehyde at 4 °C for 1 h before being brushed off by an eyelash pencil and collected with a glass pipette. Theronts were obtained by incubating collected tomonts at 25 °C in well-oxygenated, fresh sea water.

Observation of living tomonts

Living tomonts were isolated and directly observed *in vivo* using differential interference contrast microscopy.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

Sample preparation for SEM was conducted according to methods by Gu and Ni (1993). Collected cells

were fixed in 2.5% glutaraldehyde at 4 °C for 24 h. Theronts were fixed in a 6:1 mixture of saturated HgCl₂ and 1% O₅O₄ at 4 °C for 10 min. All fixing solutions were diluted in sodium cacodylate buffer (pH 7.2). Fixed cells were washed with buffer, dehydrated in a graded ethanol series, critical-point dried, placed on aluminium stubs, and sputter coated with platinum. To observe the cells inside tomonts, tomonts pasted on a sample stage were lacerated with a blade, and the cyst wall was stripped off with a needle. Prepared samples were observed with a Hitachi S-4800 SEM.

Sample preparation for TEM was performed according to Gu *et al.* (2002). Collected cells were fixed in 3% glutaraldehyde at 4 °C for 24 h. After washing with buffer, samples were post-fixed in 1% O₅O₄ at 4 °C for 2 h. All fixing solutions were diluted in sodium cacodylate buffer (pH 7.2). Fixed cells were washed with buffer, dehydrated in a graded acetone series, embedded with Epon 812, and polymerized at 37 °C for 16 h, 45 °C for 24 h and 60 °C for 48 h. Ultrathin sections were created using a diamond knife and were then stained with uranyl acetate and lead citrate. Sections were examined with a Hitachi HT-7700 TEM.

RESULTS

Protomonts that were naturally shed by host tissues moved freely for <2 h. They then gradually began to crawl more slowly and attach to the underwater substrate. Secretion of cyst wall materials and encystment began concomitantly. A typical palintomic cell division later occurred inside the tomont, and scores of theronts were then released.

Structure of the cyst wall

Tomonts of *C. irritans* were generally spherical with a relatively flat attachment area (Fig. 1A and B). The external surfaces of tomonts were usually peppered with bacteria and even small, sessile ciliates occasionally (Fig. 1B and C). The mature cyst wall was ~4 μm thick and could be easily stripped off in SEM sample preparation (Fig. 1D–F). The cyst wall was a multilayered structure that varied in layer number across different tomonts (Fig. 1E and F). In general, the middle portion of the cyst wall was compact, while the outer and inner portions were loose (Fig. 1E and F). The compact middle portion, which accounted for half of the total thickness, was composed of alternately arranged thin, electron-dense layers and thick, electron-lucent layers. There was no obvious stratification in the outer portion, but the inner portion was stratified finely (Fig. 1E and F). The cyst wall contained abundant fibrous materials in certain layers, which were irregularly interwoven (Fig. 1G and H). Cyst wall layers were not always tightly joined to each

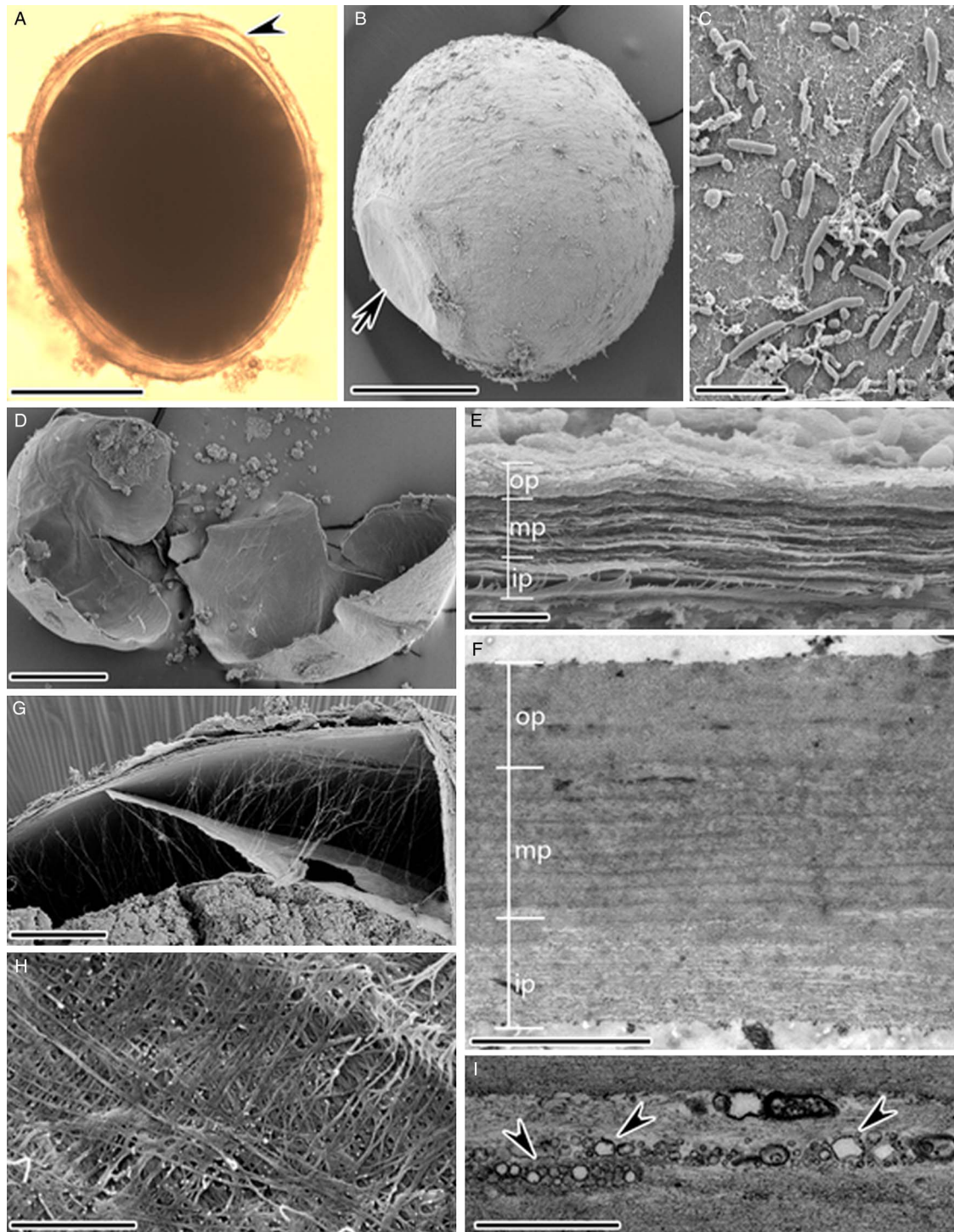


Fig. 1. Tomont of *Cryptocaryon irritans* viewed *in vivo* (A) and by SEM (B–E, G, H), TEM (F, I). (A, B) Holistic view of tomont. Arrowhead depicts the wide spaces between layers of cyst wall; arrow marks the relatively flat attachment area; (C) External surface of tomonts, showing bacterial attachments; (D) Cyst wall stripped off in SEM sample preparation; (E, F) Sectional view of cyst wall, showing the outer, middle and inner portion of the thick cyst wall; (G, H) Fibrous materials of the cyst wall. (I) Alveoli (arrowheads) present between layers of cyst wall. op, mp, ip, outer, middle and inner portion of cyst wall, respectively. Scale bars = 100 μm (A, B, D), 20 μm (G), 5 μm (C), 2 μm (E, F) and 1 μm (H, I).

other but sometimes were widely spaced (Fig. 1A). In fact, multiple layers were rarely appressed when observed from the TEM cross-section. Many alveoli of various sizes were occasionally present between layers (Fig. 1I).

Formation of the tomont

The secretion of sufficient cyst wall materials to form the thick cyst wall was a slow process, taking 14–15 h from the attachment of the protomont to the

formation of a mature cyst wall. Secretion began even before movement had stopped and the proto-mont had attached. Over time, larger amounts of semi-transparent mucus material could be observed surrounding the cell *in vivo*, and cell locomotion was slowed and finally halted owing to the accumulation of cyst wall materials (Fig. 2A). These materials were a mixture of mucus and fibrous materials (Fig. 2B and C).

One hour after the attachment of encysting cells, the cyst wall was quite thin (Fig. 2D and G), and 2 h later, it had thickened slightly (Fig. 2H). Four hours after attachment, the cyst wall was $\sim 1 \mu\text{m}$ thick, and the formation of the outer portion was completed (Fig. 2E and I). Ten hours after attachment, the cyst wall was $\sim 3 \mu\text{m}$ thick, and the distinctly layered middle portion had formed completely (Fig. 2F and J). The formation of the inner portion took additional 4–5 h. Throughout the encysting process, there were no prepackaged secretory cytoplasmic vesicles or specific extrusomes observed under the pellicle accompanying the accumulation of the cyst wall materials (Fig. 2G–J). In addition, no foci of secretion or exocytosis were visible during SEM or TEM observations (Fig. 2G–J, K and M).

The close-set pellicular swells, which cover the cell surface of the trophont and protomont, dwindled in size and were immersed in the pellicle after the start of encystment (Fig. 2G–J). When the cyst wall was stripped off, swells were compressed and the pellicle was flat (Fig. 2K and M). The cytostome was present during encystment (Fig. 2C and M). Both circumoral and somatic cilia, with their basal bodies, existed during encystment. These were overwhelmed by abundant cyst wall materials and clung to the pellicle, all of which were covered by the newly formed cyst wall (Fig. 2B, C and K–N).

Palintomic division inside the tomont

In most cases, the typical palintomic cell division in the *C. irritans* tomont began 1–2 days after cyst wall formation and was completed in 2–4 days. The metrocyte experienced continuous asymmetrical cleavage inside the tomont, and the daughter cells did not separate from each other immediately, but formed a long, coiled cell chain of variously sized daughter cells. At the end of this division process, 200–400 small, dissociative tomites had been produced. The overall process involved the following stages. (1) One metrocyte first divided into a large daughter cell and a small daughter cell (Fig. 3A and J); the large cell then successively divided out several smaller daughter cells (Fig. 3B and K). (2) More small daughter cells were generated, about 20–30 of which were stacked into 2–3 spirals. The large daughter cell diminished further as it divided (Fig. 3C and L). (3) A large cell was no longer visible, and small daughter cells similar in size

accumulated as a cell chain (Fig. 3D and M). (4) These daughter cells continued to divide, and 200–400 dissociative spherical tomites were produced. They moved slowly in the tomont (Fig. 3E and N). These tomites were obviously more and smaller than the former daughter cells, but we failed to recognize the cleavage during this process was asymmetrical or symmetrical (Fig. 3D, E, M and N). (5) Small, spherical tomites developed further and subsequently transformed into ellipsoidal theront precursors (Fig. 3F and O). The motion of the theront precursors was so rapid that it was difficult to clearly recognize cell profiles (Fig. 3F and G). The fast-moving theront precursors broke through the cyst wall and exited the tomont (Fig. 3G), leaving behind an empty cyst wall (Fig. 3H). At times, a few small cells, which divided or matured abnormally, remained in the empty cyst wall (Fig. 3I).

Cilia were present in the tomont throughout the cell division process. During cell division, cilia on the daughter cells were of various lengths, with the longest ones 7–8 μm long (Fig. 3P). Cilia beat actively when observed *in vivo*. Cilia on the dissociative spherical tomites and theront precursors were all 7–8 μm in length, which is equal to the length of theront cilia (Fig. 3Q). The cleavage rings, which did not contain cilia, ran perpendicular to the ciliary rows of the dividing cells (Fig. 3R). No oral primordia were observed on the dividing cell chains, appearing on the dissociative tomites only after cytokinesis (Fig. 3S).

Specific activities of some tomont cellular organelles

In protomonts and encysting cells, we observed numerous nascent mitochondrial autophagosomes, in which the mitochondria began to be fused and digested by the primary lysosomes (Fig. 4A). The arrangement of the mitochondrial cristae of the dividing cells inside the tomont was less compact than those of trophonts and theronts (Fig. 4B–D).

Mucocysts were not observed in the trophonts or protomonts. However, numerous mucocyst precursors appeared as electron-dense pyriform structures bounded by a single membrane in the cytoplasm of the dividing cells inside the tomont (Fig. 4E and F). These matured in the theronts, appearing as elliptical or elongated saccular structures regularly located beneath the pellicle (Fig. 4G and H).

Endosymbionts were present in the cytoplasm at all stages of the *C. irritans* life cycle; however, numbers clearly increased in tomonts, with endosymbionts even clustering in groups (Fig. 4I). Endosymbionts were oblong or rod-shaped and contained a granular cytoplasm, inner cytoplasmic membrane and wavy outer envelope (Fig. 4I–L). They were always surrounded by a prominent electron-lucent halo and were sometimes individually contained in membranous vacuoles (Fig. 4I and J). Endosymbionts were

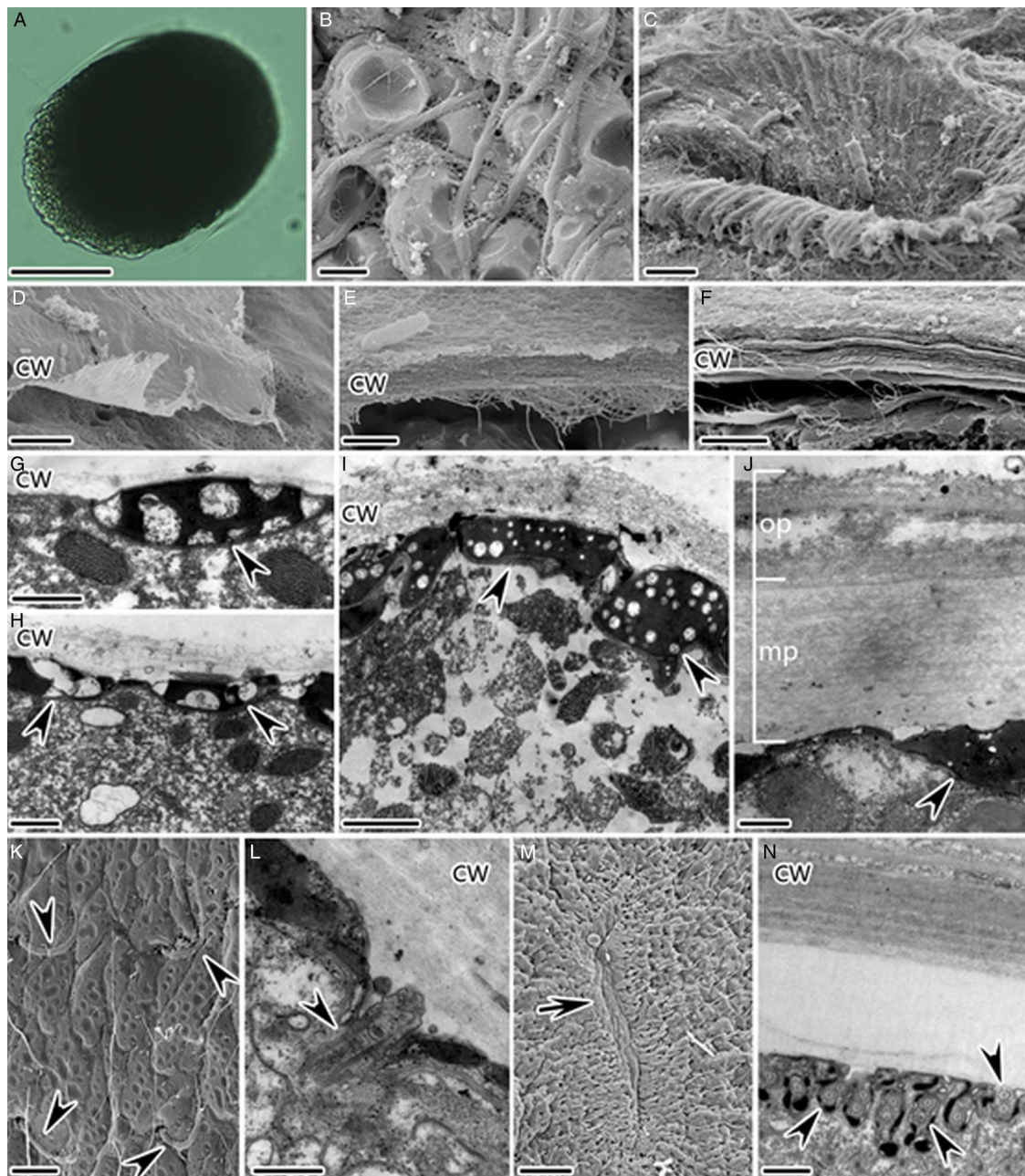


Fig. 2. Cells during encystment of *C. irritans* viewed *in vivo* (A) and by SEM (B–F, K, M), TEM (G–J, L, N). (A–C) Protomonts slowed down before attachment, with initial cyst wall materials surrounding the cell. (D–J) Cells during encystment showing the gradually thickened cyst wall and the cytoplasm underneath the pellicle at 1 h (D, G), 2 h (H), 4 h (E, I) and 10 h (F, J) after attachment of the encysting cells. Arrowheads indicate pellicular swells; (K, M) The flat cell surface after the cyst wall was stripped. Arrowheads mark the somatic cilia clinging to the pellicle, and arrow depicts the cytostome under the newly formed cyst wall; (L) Basal bodies of somatic cilia are present during encystment (arrowhead); (N) Circumoral cilia are also present (arrowheads). CW, cyst wall; op, mp, outer and middle portions of cyst wall, respectively. Scale bars = 100 μm (A), 5 μm (D, F, M), 2 μm (C, K), 1 μm (B, E, G–J, N) and 0.5 μm (L).

also commonly observed in the macronuclei of tomonts; these were never surrounded by membrane vacuoles, however, and only some were surrounded by an electron-lucent halo (Fig. 4K and L). Moreover, nuclear endosymbionts exhibited circular electron-lucent patches according to observations of transverse sections (Fig. 4L).

There were many more Golgi-like cisternae scattered throughout the cytoplasm of the dividing

cells inside the tomont than in other life cycle stages (Fig. 4E and M). These appeared as flattened, membrane-bound sacs and several to dozens were stacked in parallel arrays (Fig. 4M–O). In cross-section, most were banded in shape, while some were semi-circular to circular (Fig. 4N and O). Sometimes an endosymbiont was surrounded by several Golgi-like sacs arranged in a monolayer (Fig. 4P).

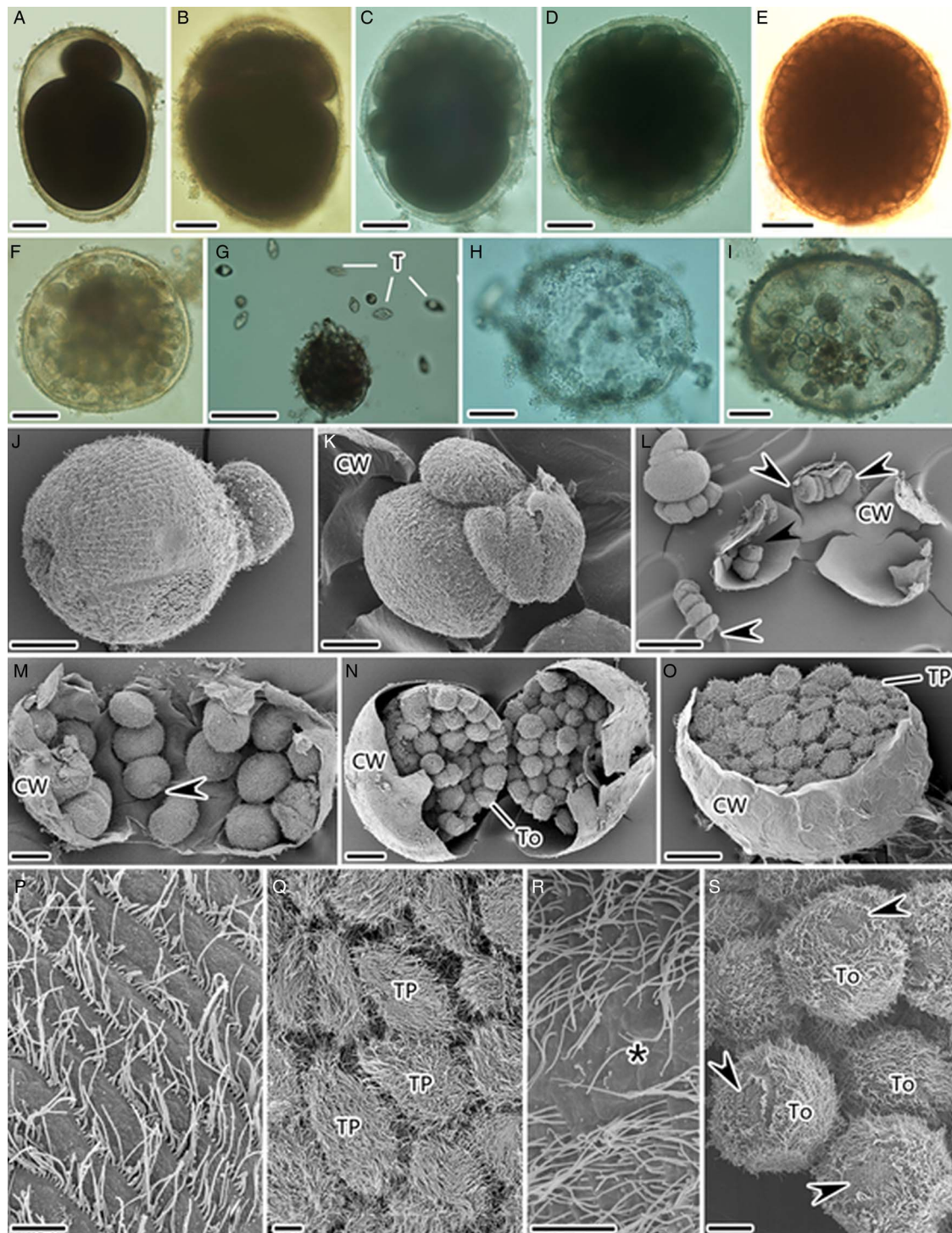


Fig. 3. Cell division inside the *C. irritans* tomont viewed *in vivo* (A–I) and by SEM (J–O). Arrowheads indicate artificial broken joints between dividing daughter cells; (P) Cilia on the dividing daughter cells are of various lengths; (Q) Theront precursors; (R) Cleavage ring (asterisk) between dividing cells; (S) Dissociative spherical tomite with oral primordia (arrowheads). CW, cyst wall; T, Theront; TP, theront precursor; To, dissociative spherical tomite. Scale bars = 200 μm (G, L), 50 μm (A–F, H–K, M–O), 10 μm (Q, S) and 5 μm (P, R).

DISCUSSION

No secretion of encystation-specific secretory vesicles or extrusomes during formation of C. irritans cyst wall

Protist cyst walls exhibit a great deal of morphological diversity. Many are composed of two or more distinct layers with different compositions.

For example, the resting cyst wall of *Histiculus muscorum* consists of four layers: the ectocyst, mesocyst, endocyst and granular layer (Matsusaka and Hongo, 1984; Lynn, 2008; Aguilar-Díaz *et al.* 2011; Verni and Rosati, 2011). However, the three portions of the *C. irritans* cyst wall could not be distinguished to several nature distinct layers and might be of

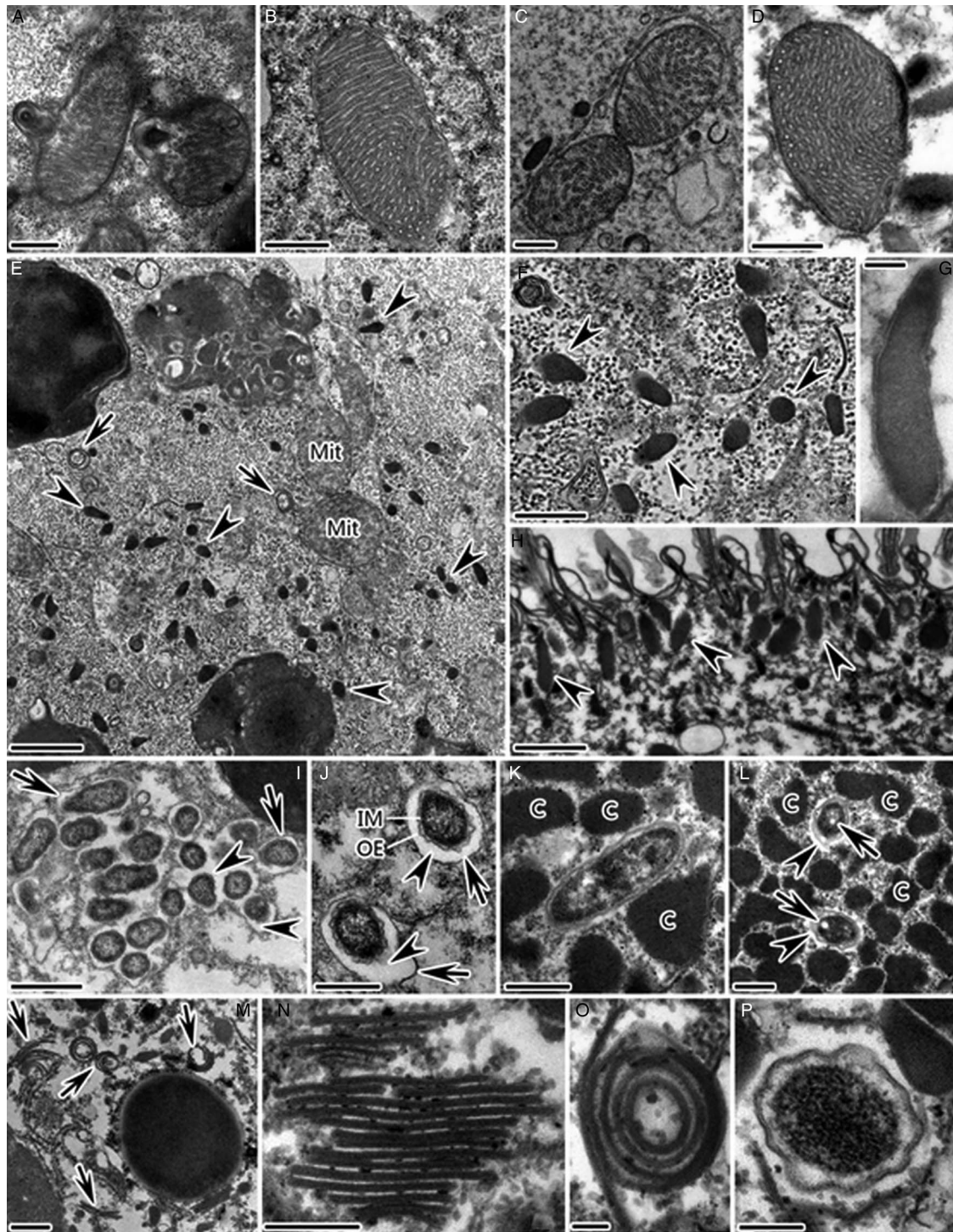


Fig. 4. TEM images of some cellular organelles in the cytoplasm of *C. irritans* tomonts. (A) Nascent mitochondrial autophagosomes in the encysting cells; (B) Trophont mitochondria; (C) Mitochondria in tomont dividing cells, (D) Theront mitochondria showing different arrangements of cristae; (E, F) Appearance of cytoplasm in the dividing cells inside the tomont. Arrowheads indicate mucocyst precursors; (G, H) Mucocysts beneath the pellicle of theronts (arrowheads); (I, J) Endosymbionts in the cytoplasm of tomont. Arrowheads indicate electron-lucent halos. Arrows mark membranous vacuoles; (K, L) Endosymbionts in the macronucleus of tomont. Arrowheads depict electron-lucent halos. Arrows indicate circular, electron-lucent patches; (M, N) Golgi-like cisternae scattered throughout the cytoplasm of dividing cells inside tomont (arrows); (O) Semi-circular to circular Golgi-like cisternae; (P) Endosymbiont surrounded by several Golgi-like sacs arranged in a monolayer. Mit, mitochondria; IM, inner cytoplasmic membrane of endosymbionts; OE, outer envelope of endosymbionts; C, chromatin patches. Scale bars = 1 μm (E, H, I), 0.5 μm (A–D, F, J–M), 0.2 μm (P, N) and 0.1 μm (G, O).

similar composition. Since the three portions exhibit similar morphology with the exception of electron-dense, thin layers in the middle portion.

During encystment, many protists secrete cyst wall materials via exocytosis of encystation-specific secretory vesicles from the cytoplasm (Ruthmann and Kuck, 1985; Greco *et al.* 1990; Chávez-Munguía *et al.* 2004, 2007; Lynn, 2008; Aguilar-Díaz *et al.* 2011). In other protists, the cyst wall is formed by secretion of specific extrusomes, such as mucocysts or clathrocysts (Holt and Chapman, 1971; Hausmann, 1978; McArdle *et al.* 1980; Ewing and Ewing, 1983; Lynn, 2008). During encystment of *C. irritans*, however, there were no prepackaged secretory cytoplasmic vesicles or visible foci of secretion observed, even though cells were sampled every hour throughout the entire encystment process. Moreover, there were no mucocysts or other extrusomes observed during development of the mature trophont, protomont, or earlier tomont, suggesting that cyst wall formation in *C. irritans* does not involve secretory vesicles or extrusome secretion.

Instead, the mechanism of cyst wall formation in *C. irritans* may be similar to that of some apotome, peritrich and hypotrich ciliates, and some flagellates. The cyst wall materials of these protozoa originate in the cytosol and polymerize at the plasma membrane without formation or secretion of membrane-limited cyst wall precursors (Herth *et al.* 1986; Greco *et al.* 1990; Landers, 1991; Calvo *et al.* 2003). The synthesis and construction of the cyst wall materials of *C. irritans* may also involve molecular events in the pellicle, which are difficult to observe during routine electron microscopy.

Cilia are present during encystment and cell division of the tomont

It is well known that *C. irritans* trophonts, protomonts and theronts have somatic cilia (Cheung *et al.* 1981; Colorni and Diamant, 1993; Matthews *et al.* 1993; Diggles, 1997; Huang *et al.* 2005; Li *et al.* 2006; Ma *et al.* 2006, 2016). Previous studies reported that the cilia were also present on the dividing cells inside the tomont (Colorni and Diamant, 1993; Huang *et al.* 2005; Ma *et al.* 2016), and our study confirms this. However, there has been some confusion in the literature regarding whether somatic cilia are present during encystment. Theories have suggested that they are absorbed (Nigrelli and Ruggieri, 1966), gradually internalized (Cheung *et al.* 1981), mostly shed (Matthews *et al.* 1993), embedded in the thickened cyst wall with bacteria and cellular debris (Colorni and Diamant, 1993) or lost (Huang *et al.* 2005). In the present study, we found that the somatic cilia are never shed and do not disappear during encystment; instead, they are present under the cyst wall materials.

During subsequent cell divisions in the tomont, numerous new, somatic cilia of various lengths are constantly being created between old cilia; this is similar to what happens in morphogenesis in many free-living ciliates (Lynn, 2008). During encystment, the retained, old somatic cilia may be resorbed or distributed to daughter cells intact; in the latter case, they may play an important role in determining the locations of new somatic cilia, as in hypotrich ciliates (Gu and Zhou, 1990).

Huang *et al.* (2005) briefly mentioned that the buccal apparatus was lost in the early stage of tomont development. In this study, however, we found that both the cytostome and its circumoral cilia were present under the newly formed cyst wall during encystment. These may subsequently be disintegrated and resorbed, as the dividing cell chains exhibited no oral primordia. Tuffrau (1952) reported that in dividing colpodids, all parental oral structures dedifferentiated, and the ciliates were astomatus during palintomy. A previous study claimed that in *C. irritans*, oral primordia were apparent until after cytokinesis of the cell chains during telophase of cell division (Ma *et al.* 2016). This was not accompanied by stomatogenesis during cell division in the tomont. New oral primordia in the offspring likely formed before the dividing cell chains split into dissociative spherical tomites.

Cilia are also present during encystment and cell division inside the reproductive cysts of *Hyalophysa chattoni* and *Ichthyophthirius multifiliis* (Landers, 1991; Matthews, 2005; Dickerson, 2006). We speculate therefore that this may represent a common feature in these typical histophagous ciliates.

Dividing daughter cells in the tomont form temporary cell chains before separation into dissociative spherical tomite precursors

The phenomenon of reproduction and cell division inside a cyst has been reported in numerous ciliates, including some free-living ciliates [tetrahymenids, hypotrichs and colpodids (Williams, 1960; Foissner, 1993; Benčatová *et al.* 2016)] and some histophagous ciliates [apostomes, ophryoglenids and prostomes (Savoie, 1962a, 1962b; Bradbury, 1966; Hiller and Bardele, 1988; Leipe, 1989; Bradbury *et al.* 1997; Molloy *et al.* 2005)]. It is worth noting that in these cases, two to eight tomites or filial products are produced. In contrast, the two parasitic ciliates *C. irritans* and *I. multifiliis* produce up to 200 and 1000 tomites, respectively, from a single cyst (Ewing and Ewing, 1983; Diggles and Lester, 1996; Colorni and Burgess, 1997; Lynn, 2008). It may be that the number of filial products created by a reproductive cyst is related to the feeding habits of the ciliate, with parasitic ciliates able to produce many more tomites.

Previous studies have indicated that *C. irritans* tomonts undergo asymmetric palintomic division,

culminating in numerous daughter tomites (Colorni and Burgess, 1997; Huang *et al.* 2005; Dickerson, 2006). Beyond this, our study found that the dividing daughter cells form temporary cell chains in the tomont, rather than being pinched off or separated from each other with each binary fission. In certain species of astomes, apostomes, hymenostomes and scuticociliates, chains of daughter cells are also formed during the reproduction process (Molloy *et al.* 2005; Lynn, 2008; Long and Zufall, 2010). Our findings indicate a similar characteristic in prostomes. This suggests that the formation of cell chains may represent either an ancestral feature in these groups or a convergent trait that has arisen multiple times independently. Other ciliates that have not been intensively studied, especially those with a polymorphic life cycle, may also share such a trait.

Activities of some cellular organelles in the C. irritans tomont cytoplasm are not identical to those in resting cysts

Autophagic activity, in which numerous autophagic vacuoles containing membranous materials and some cytoplasmic organelles are generated, is a common phenomenon during encystment for many resting cysts (McArdle *et al.* 1980; Gutierrez and Perez-Silva, 1983). Mitochondrial autophagy was also observed in encysting cells of *C. irritans*; this process may reflect lower-energy requirements when protomonts stop moving and begin to encyst.

The activities of some cellular organelles in the cytoplasm of *C. irritans* tomont were clearly different from those in resting cysts of other protists. Similar to the development of toxicysts (Ma *et al.* 2016), mucocysts matured underneath the pellicle in theronts, with mucocyst precursors first developing in the dividing cells inside the tomont. These organelles may contribute to the processes of theronts escaping from the tomont or invading host tissues. In addition, there were numerous endosymbionts and Golgi structures in the cytoplasm of dividing cells inside the tomont, with some Golgi structures associated with endosymbionts. *Cryptocaryon irritans* may provide a suitable environment as a host for endosymbiont growth and proliferation, and our findings implied an active interaction between endosymbionts and their host cells in this stage. The Golgi apparatus is the central sorting organelle in eukaryotic cells and is involved in many important cell activities (Farquhar and Palade, 1981; Rothman, 1981; Kurz and Tiedtke, 1993; Morgado-Diaz *et al.* 2001). The appearance of additional Golgi-like cisternae indicates increased cell activity and an increase in material synthesis in the tomont. Yin *et al.* (2016) reported that numerous genes were activated before the tomont began cell division. In conclusion, unlike a

resting cyst, cellular metabolic activity in the *C. irritans* tomont was quite high, with large amounts of materials or cellular organelles synthesized and prepared for the subsequent infective theront stage.

FINANCIAL SUPPORT

This work was funded by the National Natural Science Foundation of China (grant numbers. 31101932 and 31572223) and the Special Scientific Research Funds for Central Non-profit Institutes of China (grant no. 2014M01, East China Sea Fisheries Research Institute; grant no. 2015B05XK01, Chinese Academy of Fishery Sciences).

REFERENCES

- Aguilar-Diaz, H., Carrero, J. C., Argüello-García, R., Laclette, J. P. and Morales-Montor, J. (2011). Cyst and encystment in protozoan parasites: optimal targets for new life-cycle interrupting strategies? *Trends in Parasitology* **27**, 450–458.
- Bai, J., Xie, M., Zhu, X., Dan, X. and Li, A. (2008). Comparative studies on the immunogenicity of theronts, tomonts and trophonts of *Cryptocaryon irritans* in grouper. *Parasitology Research* **102**, 307–313.
- Benčáfová, S., Tirjaková, E. and Vďačný, P. (2016). Resting cysts of *Parentocirrus hortualis* Voß, 1997 (Ciliophora, Hypotrichia), with preliminary notes on encystation and various types of excystation. *European Journal of Protistology* **53**, 45–60.
- Bradbury, P. C. (1966). The life cycle and morphology of the apostomatous ciliate, *Hyalophysa chattoni* n. g., n. sp. *Journal of Protozoology* **13**, 209–225.
- Bradbury, P. C., Song, W. and Zhang, L. (1997). Stomatogenesis during the formation of the tomite of *Hyalophysa chattoni* (Hymenostomatida: Ciliophora). *European Journal of Protistology* **33**, 409–419.
- Brown, E. M. (1963). Studies on *Cryptocaryon irritans* Brown. In *Progress in Protozoology, Proceedings of the 1st International Congress on Protozoology*, Prague (ed. Ludvík, J., Lom, J., Vavra, J.), pp. 284–287. Czechoslovak Academy of Sciences, Prague, 21–23 August 1961.
- Burgess, P. J. (1992). *Cryptocaryon irritans* Brown, 1951 (Ciliophora): transmission and immune response in the mullet *Chelon labrosus* (Risso, 1826). PhD thesis. University of Plymouth, Plymouth, U.K..
- Calvo, P., Fernandez-Aliseda, M. C., Garrido, J. and Torres, A. (2003). Ultrastructure, encystment and cyst wall composition of the resting cyst of the peritrich ciliate *Opisthonecta heneguyi*. *Journal of Eukaryotic Microbiology* **50**, 49–56.
- Chávez-Munguía, B., Cedillo-Rivera, R. and Martínez-Palomo, A. (2004). The ultrastructure of the cyst wall of *Giardia lamblia*. *Journal of Eukaryotic Microbiology* **51**, 220–226.
- Chávez-Munguía, B., Omaña-Molina, M., González-Lázaro, M., González-Robles, A., Cedillo-Rivera, R., Bonilla, P. and Martínez-Palomo, A. (2007). Ultrastructure of cyst differentiation in parasitic protozoa. *Parasitology Research* **100**, 1169–1175.
- Cheung, P. F., Nigrelli, R. F. and Ruggieri, G. D. (1981). Scanning electron microscopy on *Cryptocaryon irritans* Brown, 1951, a parasitic ciliate in marine fish. *Journal of Aquaculture* **2**, 70–72.
- Colorni, A. (1985). Aspects of the biology of *Cryptocaryon irritans*, and hyposalinity as gilt-head sea bream *Sparus aurata*. *Diseases of Aquatic Organisms* **22**, 19–22.
- Colorni, A. and Burgess, P. (1997). *Cryptocaryon irritans* Brown 1951, the cause of 'white spot disease' in marine fish: an update. *Aquarium Sciences and Conservation* **1**, 217–238.
- Colorni, A. and Diamant, A. (1993). Ultrastructural features of *Cryptocaryon irritans*, a ciliate parasite of marine fish. *European Journal of Protistology* **29**, 425–434.
- Dickerson, H. W. (2006). *Ichthyophthirius multifiliis* and *Cryptocaryon irritans* (Phylum Ciliophora). In *Fish Diseases and Disorders*, Vol. 1, 2nd Edn, (ed. Woo, P. T. K. and Leatherland, J. F.), Protozoan and metazoan infections, pp. 116–153. CABI Publishing, Oxfordshire.
- Diggles, B. K. (1997). Some information on the morphology of *Cryptocaryon irritans* from South-East Queensland, Australia. *European Journal of Protistology* **33**, 200–210.
- Diggles, B. K. and Lester, R. J. G. (1996). Influence of temperature and host species on the development of *Cryptocaryon irritans*. *Journal of Parasitology* **82**, 45–51.

- Ewing, M. S. and Ewing, S. A. (1983). *Ichthyophthirius multifiliis*: morphology of the cyst wall. *Transactions of the American Microscopical Society* **102**, 122–128.
- Farquhar, M. G. and Palade, G. E. (1981). The Golgi apparatus (complex)-(1954–1981)-from artifact to center stage. *Journal of Cell Biology* **91**, 77s–103s.
- Foissner, W. (1993). Colpodea (Ciliophora). In *Protozoenfauna*, Vol. 4/1. (ed. Matthes, D.), Gustav Fischer Verlag, Stuttgart, Jena, New York.
- Greco, N., Bussers, J. C., Van Daele, Y. and Goffinet, G. (1990). Ultrastructural localization of chitin in the cystic wall of *Euplotes muscicola* Kahl (Ciliata, Hypotrichia). *European Journal of Protistology* **26**, 75–80.
- Gu, F. and Ni, B. (1993). The exploration of preparing protozoan specimen for scanning electron microscopy. *Journal of Chinese Electron Microscopy Society* **12**, 525–529.
- Gu, F. and Zhou, Z. (1990). Study on morphogenesis of Hypotrich ciliates during asexual reproduction. *Journal of East China Normal University (Natural Science)* **2**, 84–93.
- Gu, F., Chen, L., Ni, B. and Zhang, X. (2002). A comparative study on the electron microscopic enzyme-cytochemistry of *Paramecium bursaria* from light and dark cultures. *European Journal of Protistology* **38**, 267–278.
- Gutiérrez, J. C. and Perez-Silva, J. (1983). Ultrastructural aspects of the precystic and cystic cytoplasm of the hypotrichous ciliate, *Laurentiella acuminata*. *Acta Protozoologica* **22**, 203–210.
- Hatanaka, A., Umeda, N., Yamashita, S. and Hirazawa, N. (2007). Identification and characterization of a putative agglutination/immobilization antigen on the surface of *Cryptocaryon irritans*. *Parasitology* **134**, 1163–1174.
- Hausmann, K. (1978). Extrusive organelles in protists. *International Review of Cytology* **52**, 197–276.
- Herth, W., Mulisch, M. and Zugenmaier, P. (1986). Comparison of chitin fibril structure and assembly in three unicellular organisms. In *Chitin in Nature and Technology* (ed. Muzzarelli, R., Jeuniaux, C. and Gooday, G. W.), pp. 107–120. Springer, New York, USA.
- Hiller, S. and Bardele, C. F. (1988). *Prorodon aklitlophon*, n. spec. and the “dorsal brush” as a character to identify certain subgroups in the genus *prorodon*. *Archiv Für Protistenkunde* **136**, 213–236.
- Hirazawa, N., Oshima, S., Hara, T., Mitsuboshi, T. and Hata, K. (2001). Antiparasitic effect of medium-chain fatty acids against the ciliate *Cryptocaryon irritans* infestation in the Red Sea bream *Pagrus major*. *Aquaculture* **198**, 219–228.
- Hirazawa, N., Goto, T. and Shirasu, K. (2003). Killing effect of various treatments on the monogenean *Heterobothrium okamotoi* eggs and oncomiracidia and the ciliate *Cryptocaryon irritans* cysts and theronts. *Aquaculture* **223**, 1–13.
- Holt, P. A. and Chapman, G. B. (1971). The fine structure of the cyst wall of the ciliated protozoan *Didinium nasutum*. *Journal of Protozoology* **18**, 604–614.
- Huang, W., Ma, Y. and Li, A. (2005). Ultrastructural study on *Cryptocaryon irritans* throughout the life cycle of artificially infecting *Trachinotus blochi*. *Journal of Fisheries of China* **29**, 635–642.
- Huang, X., Sun, Z., Guo, G., Zheng, C., Xu, Y., Yuan, L. and Liu, C. (2012). Cloning and characterization of a surface antigen CiSA-32.6 from *Cryptocaryon irritans*. *Experimental Parasitology* **130**, 189–194.
- Kesintep, M. (1995). Light and electron microscopic observations on the ciliate *Cryptocaryon irritans* (Brown 1951) throughout the life cycle. PhD thesis. University of Georgia, Athens, Georgia.
- Kurz, S. and Tiedtke, A. (1993). The Golgi apparatus of *Tetrahymena thermophila*. *Journal of Eukaryotic Microbiology* **40**, 10–13.
- Landers, S. C. (1991). Secretion of the reproductive cyst wall by the aposome ciliate *Hyalophysa chattoni*. *European Journal of Protistology* **27**, 160–167.
- Leipe, D. (1989). *Prorodon spirogyrophagus*, nov. spec. (Ciliophora, Prostomatea): morphology, infraciliature and food-uptake. *European Journal of Protistology* **24**, 392–401.
- Li, A., Huang, W., Ma, Y. and Xie, M. (2006). Cytostome ultrastructure of *Cryptocaryon irritans* GD1 strain from experimental host-snubnose pompano (*Trachinotus blochi*). *Acta Zootaxonomica Sinica* **31**, 256–263.
- Li, R., Dan, X. and Li, A. (2013). *Siganus oramin* recombinant L-amino acid oxidase is lethal to *Cryptocaryon irritans*. *Fish and Shellfish Immunology* **35**, 1867–1873.
- Lokanathan, Y., Mohd-Adnan, A., Wan, K. L. and Nathan, S. (2010). Transcriptome analysis of the *Cryptocaryon irritans* tomont stage identifies potential genes for the detection and control of cryptocaryonosis. *BMC Genomics* **11**, 76.
- Long, H. and Zufall, R. A. (2010). Diverse modes of reproduction in the marine free-living ciliate *Glauconema trihymene*. *BMC Microbiology* **10**, 108.
- Lynn, D. H. (2008). *The Ciliated Protozoa: Characterization, Classification, and Guide to the Literature*, 3rd Edn. Springer, Dordrecht.
- Ma, Y., Li, A., Xie, M. and Huang, W. (2006). Cortical ultrastructures of *Cryptocaryon irritans* GD1 strain infecting *Trachinotus blochi* experimentally. *Acta Zoologica Sinica* **52**, 396–405.
- Ma, R., Ni, B., Fan, X., Warren, A., Yin, F. and Gu, F. (2016). Ultrastructure observation on the cells at different life history stages of *Cryptocaryon irritans* (Ciliophora: Prostomatea), a parasitic ciliate of marine fishes. *Parasitology* **143**, 1479–1489.
- Mai, Y., Li, Y., Li, R., Li, W., Huang, X., Mo, Z. and Li, A. (2015). Proteomic analysis of differentially expressed proteins in the marine fish parasitic ciliate *Cryptocaryon irritans*. *Veterinary Parasitology* **211**, 1–11.
- Matthews, R. A. (2005). *Ichthyophthirius multifiliis* Fouquet and ichthyophthiriosis in freshwater teleosts. *Advances in Parasitology* **59**, 159–241.
- Matthews, B. F., Matthews, R. A. and Burgess, P. J. (1993). *Cryptocaryon irritans* Brown, 1951 (Ichthyophthiriidae): the ultrastructure of the somatic cortex throughout the life cycle. *Journal of Fish Diseases* **16**, 339–349.
- McArdle, E. W., Berquist, B. L. and Ehret, C. F. (1980). Structural changes in *Tetrahymena rostrata* during induced encystment. *Journal of Protozoology* **27**, 388–397.
- Molloy, D. P., Lynn, D. H. and Giamberini, L. (2005). *Ophryoglena hemophaga* n. sp. (Ciliophora: Ophryoglenidae): a parasite of the digestive gland of zebra mussels *Dreissena polymorpha*. *Diseases of Aquatic Organisms* **65**, 237–243.
- Morgado-Diaz, J. A., Nakamura, C. V., Agrellos, O. A., Dias, W. B., Previato, J. O., Mendonca-Previato, L. and De Souza, W. (2001). Isolation and characterization of the Golgi complex of the protozoan *Trypanosoma cruzi*. *Parasitology* **123**, 33–43.
- Matsusaka, T. and Hongo, F. (1984). Cytochemical and electrophoretic studies on the cyst wall of a ciliate, *Histiculus muscorum* Kahl. *Journal of Protozoology* **31**, 471–475.
- Nigrelli, R. F. and Ruggieri, G. D. (1966). Enzootics in the New York Aquarium caused by *Cryptocaryon irritans* Brown, 1951, a histophagous ciliate in the skin, eyes and gills of marine fishes. *Zoologica* **51**, 97–102.
- Niu, S., Jin, Y., Xu, X., Qiao, Y., Wu, Y., Mao, Y., Su, Y. and Wang, J. (2013). Characterization of a novel piscidin-like antimicrobial peptide from *Pseudosciaena crocea* and its immune response to *Cryptocaryon irritans*. *Fish and Shellfish Immunology* **35**, 513–524.
- Pironet, F. N. and Jones, J. B. (2000). Treatments for ectoparasites and diseases in captive Western Australian dhufish. *Aquaculture International* **8**, 349–361.
- Rigos, G., Karagouni, E., Kyriazis, I., Athanasiou, E., Grigorakis, K., Kotou, E. and Katharios, P. (2013). *In vitro* and *in vivo* evaluation of quinine in gilthead sea bream, *Sparus aurata* naturally infected with the ciliate *Cryptocaryon irritans*. *Aquaculture* **416**, 185–191.
- Rothman, J. E. (1981). The Golgi apparatus: two organelles in tandem. *Science* **213**, 1212–1219.
- Ruthmann, A. and Kuck, A. (1985). Formation of the cyst wall of the ciliate *Colpoda steinii*. *Journal of Protozoology* **32**, 677–682.
- Savoie, A. (1962a). *Ophryoglena mugardi* n. sp. (Ciliata, Hymenostomatida, Ophryoglenidae). Cycles de reproduction. *Journal of Protozoology* **9**, 296–303.
- Savoie, A. (1962b). *Ophryoglena faurei* n. sp. (Ciliata Hymenostomatida Ophryoglenidae) cycles de reproduction. The Journal of Protozoology **9**, 427–434. doi: 10.1111/j.1550-7408.1962.tb02648.x.
- Tuffrau, M. (1952). La morphogenèse de division chez les Colpodiidae. *Bulletin Biologique de la France et de la Belgique* **86**, 309–320.
- Verni, F. and Rosati, G. (2011). Resting cysts: a survival strategy in protozoa ciliophora. *Italian Journal of Zoology* **78**, 134–145.
- Wang, F., Xie, M. and Li, A. (2010). A novel protein isolated from the serum of rabbitfish (*Siganus oramin*) is lethal to *Cryptocaryon irritans*. *Fish and Shellfish Immunology* **29**, 32–41.
- Williams, N. E. (1960). The polymorphic life history of *Tetrahymena patula*. *Journal of Protozoology* **7**, 10–17.
- Xu, R., Jiang, J. and Chen, B. (1992). Light microscopy observation on the life cycle of the *Cryptocaryon irritans*. *Marine Sciences* **3**, 42–43.
- Yin, F., Gong, H., Ke, Q. and Li, A. (2015). Stress, antioxidant defence and mucosal immune responses of the large yellow croaker *Pseudosciaena crocea* challenged with *Cryptocaryon irritans*. *Fish and Shellfish Immunology* **47**, 344–351.
- Yin, F., Sun, P., Wang, J. and Gao, Q. (2016). Transcriptome analysis of dormant tomonts of the marine fish ectoparasitic ciliate *Cryptocaryon irritans* under low temperature. *Parasites and Vectors* **9**, 280.
- Yoshinaga, T. and Dickerson, H. W. (1994). Laboratory propagation of *Cryptocaryon irritans* on a saltwater-adapted *Poecilia* hybrid, the black molly. *Journal of Aquatic Animal Health* **6**, 197–201.