Development of the Macronucleus of Cryptocaryon irritans, a Parasitic Ciliate of Marine Teleosts, and its Ingestion and Digestion of Host Cells

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ABSTRACT—We investigated the development of the macronucleus of Cryptocaryon irritans, and the ingestion and digestion of host cells by the parasite. All developmental stages of the parasite except the theront (trophont, protomont and tomont) were examined with histological staining and/or whole-mount staining. The sections were also subjected to in situ hybridization targeting the 18S rRNA gene of the host fish. The macronucleus developed negligibly, maintaining its four-segmented shape in trophonts, elongated and formed a massive nucleus in tomonts before cell division, and subsequently underwent repeated divisions to generate theronts. Denatured host cells, examined in situ hybridization, were accumulated in trophonts, and disappeared in tomonts by the beginning of cell division. Denatured host cells were also observed in the host tissue surrounding trophonts. They had condensed nuclei. Protomonts filled with denatured host cells presented a 180-bp DNA ladder in gel electrophoresis, suggesting apoptosis of the host cells. These results indicate that DNA synthesis occurs exclusively in the early stage of the tomont and that host cells are fed and accumulated, probably as apoptotic cells, in the trophont and are digested in the early tomont stage.

Key words: Cryptocaryon irritans, development, nucleus, DNA synthesis, ingestion, digestion, host cell, apoptosis

Cryptocaryon irritans Brown 1951 is a parasitic ciliate of marine teleosts, which causes cryptocaryoniasis (‘white spot disease’), and was first described in the Aquarium of Tokyo Imperial University in Japan (Sikama, 1937). The parasite invades the epithelial layer of the skin and gills of marine teleosts and disturbs the osmotic control and respiratory activity of its host, often causing mass mortalities in heavily infected fish (Colorni and Burgess, 1997). This parasite is a major threat to marine aquaculture in tropical and subtropical waters (Colorni and Burgess, 1997).

The life cycle of C. irritans consists of four developmental stages, namely, theront, trophont, protomont, and tomont (Sikama, 1937; Brown, 1963; Wilkie and Gordin, 1969; Colorni, 1985, 1987). The theront is in the free-swimming stage, during which C. irritans invades the surface tissue of fish. Therein, it becomes a trophont, entering the parasitic stage. The trophont grows in the epithelium of the skin, fins, and gills of fish. Fully developed trophonts leaves the host as protomont. The protomont sinks, settles on the substrate, and encysts as tomont. The tomont stage is the phase of cytoplasmic division, in which daughter cells, or ‘tomites’, are produced by repeated binary cell division, or piniformy. Fully developed tomites are released into seawater as theronts.

The development of the trophont and tomont has not yet been clarified. Especially, little is known about the time course of the development of the nucleus in the trophont and tomont. This is partly because the internal structures of the trophont and tomont of C. irritans are not visible under conventional light microscopy (Dickerson and Dawe, 1995), and stained sections or transmission electron microscopy (TEM) is required to observe their internal structures, in contrast to its freshwater counterpart Ichthyophthirius multifilis, the trophont and tomont of which are transparent, and their internal structures are easily observed with light microscopy (Dickerson and Dawe, 1995). There is little detailed information also on the mechanism of trophont feeding and digestion of fish cells, although Brown (1963) suggested that the trophont of C. irritans feeds on the host cells and digests them during the cyst stage.
In this study, we investigated the development of *C. irritans* in detail, focusing on the time course of nuclear development with conventional histological stainings and a whole-body staining of the nucleus. Because we found apoptotic-body-like structures in the trophont and protomont and in the host tissues surrounding the trophont during the investigation, we also examined the possibility that apoptosis was involved in the feeding of the parasite with DNA electrophoresis profiling.

**Materials and Methods**

**Parasite**

*Cryptocaryon irritans* was isolated from an ornamental fish, *Paracanthurus hepatus*, purchased in a local pet shop in Tokyo, and passaged on black molly *Pecilia* sp. (2–3 cm in standard length). The propagation and collection of the parasite were according to the protocol of Yoshinaga and Dickerson (1994), with some modifications. Briefly, protomonts leaving infected black mollies were allowed to settle in 1 L of seawater in plastic aquarium and transform into encysted tomonts on the bottom of the aquaria. The tomonts attached to the bottom of the aquaria were washed by rinsing the bottom with filtered seawater and incubated at 25°C in 50 mL of filtered seawater left in the aquaria. The seawater in the aquaria was replaced every day. Five to seven days after the collection of the protomonts, when the tomonts released theronts, the theronts were collected and placed in a 2 L plastic aquarium containing 10 black mollies, at 1,000 theronts/fish, for challenge. Fish exposed to the theronts for 6 h were transferred to a 60 L aquarium and maintained at 25°C. Three or four days after challenge, the protomonts were collected from the fish. This process was repeated to maintain *C. irritans* infection and to collect protomonts, tomonts, theronts, and infected fish.

**Observation of infected fish tissues and trophonts**

Ten naive black mollies were challenged with theronts (250 theronts/fish) for 6 h in 2 L of seawater in a plastic aquarium and then kept in 2 L of seawater in a new aquarium at 25°C. The fish were sampled immediately after challenge and at 1–3 days after challenge. They were killed with an excess of 2-phenoxyethanol. After we had confirmed the presence of trophonts with a stereomicroscope, the infected fins were removed, fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for about 24 h, decalcified in EDTA, embedded in paraffin wax, and sectioned at 5 μm thickness, according to conventional methods. The sections were stained with the Feulgen reaction to visualize the DNA in the nuclei or with hematoxylin-eosin (HE) for microscopic observation. Feulgen reaction reflects the amount of DNA in the cell nuclei and is often used for the semi-quantification of DNA.

Some of the fixed fins were also washed with PBS and stained without sectioning for 15 min in a solution of Hoechst 33258 (200 μM in PBS), which emits fluorescence when bound to double-stranded DNA. The samples were then mounted on slides, and observed under an ultraviolet (UV) microscope.

**Cross-sections of protomonts and tomonts**

Fifty protomonts were placed in seawater in 1.5-mL tubes for 24 h to allow them to stick together in a cluster. The clusters of encysted tomonts were transferred to seawater in Petri dishes and incubated at 25°C. Protomonts before encystment and the clusters of tomonts were sampled daily from the Petri dishes, fixed in 4% PFA for 24 h, washed in PBS, and pre-embedded in 2% agar gel. The pre-embedded protomonts and tomonts were embedded in paraffin wax, sectioned at 5 μm, and stained with Feulgen reaction and HE, according to conventional methods. They were then observed under a light microscope.

In situ hybridization

Sections of trophont, protomont, and tomont were prepared with the procedure described above. They were mounted on MAS-coated glass slides (MAS-GP type A, Matsunami, Japan). The sections were deparaffinized, hydrated, and washed in PBS.

The general procedure for in situ hybridization followed that of Stokes and Berreson (1995) originally developed to detect *Haplorosporidium nelsoni*. We replaced the *H. nelsoni*-specific probes with probes targeting the 18S rRNA gene of the black molly in this study. We designed four probes sufficient to discriminate black molly cells from *C. irritans* cells by comparing 18S rRNA gene sequences of the two organisms: molly 18S-1 (5′-TTCTCTCTGCATGAGGCGGA-3′), molly 18S-2 (5′-CTCGATGTCTTAGCTGAGTGTCT-3′), molly 18S-3 (5′-ATTGGATGTGTTGATGCTCGG-3′), and molly 18S-4 (5′-TTGAGGCCCTGTAATTTGA-3′). The probes were labeled with digoxigenin using the DIG Oligonucleotide Tailing Kit, 2nd generation (Roche, Switzerland), according to the manufacturer's instructions. The sections were treated for 10–15 min with 100 μg/mL proteinase K dissolved in PBS at 37°C. The treated sections were washed with PBS containing 0.2% glycine for 5 min, postfixed with 4% PFA for 10 min, placed in 2 X standard saline citrate (SSC) for 10 min, and prehybridized at 42°C for 30 min in prehybridization solution (4 X SSC, 50% formamide, 5 x Denhardt’s solution, 0.5 mg/mL heat-denatured fish sperm DNA) in a moist chamber. The sections on the slide were hybridized in 200 μL prehybridization solution pre-mixed with the four digoxigenin-labeled black molly probes (2.5 pmol/mL for each probe). The slides were covered with Parafilm M (Bemis Company Inc., U.S.), and hybridized on a heat block at 100°C for 1 min, placed on ice for 1
min, and incubated overnight at 42°C in a moist chamber. The sections were washed twice with 4 × SSC for 5 min each, twice in 2 × SSC for 5 min each, twice in 1 × SSC for 5 min each, and twice in 0.5 × SSC for 10 min each at 42°C. All washes were performed at room temperature except those in 0.5 × SSC. After treatment with Buffer 1 (100 mM Tris [pH 7.5], 150 mM NaCl) for 1–2 min and with 1% blocking reagent (Roche, Switzerland) in Buffer 1 for 30 min, the sections were covered with Parafilm M and reacted with an alkaline-phosphatase-conjugated anti-digoxigenin antibody diluted 1/500 in Buffer 1 containing 1% blocking reagent in a moist chamber at room temperature for 2 h. The sections were then washed twice with Buffer 1 and then twice with Buffer 2 (100 mM Tris [pH 9.5], 100 mM NaCl, 50 mM MgCl₂), and placed in NBT/BCIP solution (Roche, Switzerland), diluted with Buffer 2, for 1.5 h in the dark. To stop the color reaction, the sections were washed with TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) and rinsed in distilled water. Finally, the sections were counterstained with either 0.5% Bismarck Brown Y in distilled water or Hoechst 33258 (200 μM) in PBS and mounted with Pristine Mount (Falma).

Whole-mount staining of tomonts with acetoarmine
We prepared eight 0.35-mm culture dishes with 14-mm glass bottom (Glass Bottom Culture Dish, MatTek Corp.). Approximately 100–150 protomonts were placed in seawater in each dish, allowed to become encysted on the bottom glass, and incubated at 25°C. The bottom glass cover slip was removed daily from a dish, and the tomonts attached to the glass cover slip were stained with acetoarmine solution (1% carmine solution in 45% acetic acid solution supplemented with several drops of 4% iron alum solution) for 10 min. After staining, the tomonts were decolorized with 70% ethanol supplemented with 1% HCl, dehydrated in a graded alcohol series, cleared in xylene, and mounted on slide with Canada balsam. The macronuclei of the tomonts attached to the glass cover slip were observed under a light microscope. This experiment was repeated three times using different batches of protomonts at different opportunities.

Electrophoresis profile of protomont DNA
About 300 protomonts were collected in a 1.5-mL tube containing 50 μL of seawater, in which the protomonts were ruptured by pipetting. Guanidine hydrochloride solution (450 μL; 6 M guanidine hydrochloride, 0.1 M sodium acetate [pH 5.5]) was added and the mixture was incubated for 1 h at room temperature. After ethanol precipitation, the pellet DNA was dissolved in 50 μL of TE buffer. An aliquot (30 μL) of the solution was mixed with GR Green 6× DNA Loading Buffer (Labgene Scientific Suisse), loaded onto a 1.5% agarose gel, and electrophoresed at 100 V for 30 min. The DNA bands were visualized under a UV transilluminator.

Results
Observation of trophont, protomont, and tomont nuclei
In the early-stage trophonts (day 0), the macronucleus was stained densely with the Feulgen reaction (Fig. 1A) and Hoechst 33258 (Fig. 2A). On day 1 post-challenge, the macronucleus was slightly enlarged but its stainability had not altered. Feulgen-positive granules appeared in the trophont (Fig. 1B). In the middle stage (day 2), whereas the size of the trophonts and the number of Feulgen-positive granules increased, the stainability of macronucleus decreased. Although the shape of the macronucleus in the trophonts on day 2 seemed club-like in sections (Fig. 1C), the staining of the whole trophonts with Hoechst 33258 clearly showed the four-segmented structure in the macronucleus on day 2 (Fig. 2B). In the late stage (day 3), although the trophonts grew considerably, the macronucleus remained small and stained much more weakly with the Feulgen reaction than in previous days (Fig. 1D). The trophonts were filled with Feulgen-positive granules, which increased markedly from day 2. HE staining frequently detected denatured cells with condensed nuclei, which appeared to be host cells, in the trophont (Fig. 3A). The Feulgen-positive granules appeared to be the condensed nuclei of the denatured cells. Host cells with condensed nuclei, which were similar to the denatured cells in the trophonts, were found in the host tissues surrounding the trophonts (Fig. 1C and Fig. 3B). The presence of host cells with condensed nuclei was confirmed both in the trophonts and in the host tissue surrounding the trophonts by staining with Hoechst 33258 (Fig. 2B).

The protomonts showed weakly stained macronucleus and were filled with numerous Feulgen-positive granules, similar to the trophont on day 3 (Fig. 1E).

The tomonts showed macronucleus weakly stained with the Feulgen reaction just after encystment and were filled with Feulgen-positive granules, similar to the fully developed trophont and protomont (Fig. 1F). The macronucleus then became very elongated and was found to be coiled in the observations of serial sections (Fig. 1G), and its stainability increased. The coil-shaped macronucleus later aggregated to form a massive macronucleus. The tomonts started unequal cell division. Repeated cell division produced tomonts with four-segmented macronuclei, which were released as theronts (Fig. 1H and 1I). While the macronucleus became elongated and increased in stainability with the Feulgen reaction, the number of Feulgen-positive granules decreased and had almost disappeared by the beginning of cell division (Fig. 1G, H, I). The Feulgen-positive granules prevented the observation of the micronucleus; as such, the micronuclei were only visible in the early stages of the trophonts (Fig. 1A–D) and
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**Fig. 1.** Histological sections of trophonts, protomonts and tomonts, stained with the Feulgen reaction. A: trophont immediately after infection (day 0); B: trophont on day 1 post-infection; C: trophont on day 2 post-infection; D: trophont on day 3 post-infection; E: protomont; F: tomont immediately after encystment; G: tomont with an elongated and densely stained macronucleus; H: tomont soon after the start of cell division; I: tomont containing tomites. In D, two trophonts are tightly attached to each other (arrowhead). ma = macronucleus. m = cell membrane of *C. irritans*. fg = Feulgen-positive granules. ht = host tissue. dn = probable denatured host cell. Scale bars = 50 μm.

**Fig. 2.** Whole-mount staining of trophonts in infected fish fins with Hoechst 33258. A: a trophont on day 0 exhibiting a macronucleus composed of four segments; B: a trophont on day 2 post-infection. Cells with condensed nuclei were observed in the tissue (arrow) surrounding the trophont and within the trophont (arrowhead). The macronucleus comprising four segments was still present in the trophont on day 2. Scale bars = 50 μm.

**Fig. 3.** Histological sections of trophonts in the skin epithelium of black molly (hematoxylin and eosin stain). A: trophont on day 2 containing probable host cells with a condensed nucleus; B: another trophont on day 2. In B, probable host cells with a condensed nucleus are visible both in the trophont and in host tissue surrounding the trophont. ma = macronucleus. hc = host cell. Scale bars = 50 μm.
tomites (Fig. 1I).

In situ hybridization

In the in situ hybridization, positive reaction with probes designed to detect the 18S rRNA coding a species specific region of the black molly were found in granules in trophonts, protomonts, and early-stage tomonts of C. irritans (Fig. 4A–C) as well as in the nuclei of host cells (Fig. 4A). The probes did not hybridize with the nucleus, cytoplasm or cell membrane of C. irritans, confirming the specificity of the probes to black molly cells. Although the granules filled the protomonts, they decreased in number as the protomonts developed into tomonts (Fig. 4B, C) and finally disappeared by the beginning of cell division in the tomonts (Fig. 4E, F).

Time course of tomont development

In the whole-mounting staining with aceticarmine, 50–100 of tomonts were recovered from each dish and could be microscopically observed. Using the staining, which differentiates the cell nucleus well, the nuclear development of the tomonts was observed clearly, without sectioning. The tomonts were filled with aceticarmine-positive granules in the early stage and their macronucleus was not clearly visible (Fig. 5A). As shown in the sections stained with the Feulgen reaction, the macronucleus had become elongated and coiled (Fig. 5B) and later aggregated to form a massive nucleus (Fig. 5C). The numbers of aceticarmine-positive granules decreased and disappeared as the macronucleus elongated. The tomonts then commenced unequal cell division (Fig. 5D). Repeated cell divisions produced tomites with macronuclei composed of four segments in the cysts (Fig. 5E), which were released as theronts (Fig. 5F). Dead cyst lacking nuclear structures, which was evenly stained, was frequently observed, especially

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Fig. 4. In situ hybridization with probes designed for detection of the 18S rRNA gene of the black molly. A: trophont in the skin epithelium of black molly; B: protomont; C: tomont immediately after encystment; D: tomont with an elongated and coiled macronucleus; E: tomont shortly after the start of cell division; F: tomont producing tomites. The sections were counterstained with Bismarck Brown Y and Hoechst 33258 in A and B–F, respectively. The images of B–F were created by merging light microscopy and UV microscopy images. The small, dark stained particles result from the reaction of the probes with black molly DNA. ma = macronucleus. hD = host DNA. Scale bars = 50 μm.

Fig. 5. Tomonts stained with aceticarmine. A: tomont containing numerous aceticarmine positive granules (Stage 1); B: tomont with a coiled macronucleus (Stage 2); C: tomont with a massive nucleus (Stage 3); D: tomont undergoing cell division (Stage 4); E: tomont filled with tomites, with a segmented macronucleus (Stage 5); F: empty cyst after encystment; G: A dead cyst. ma = macronucleus.-ag = aceticarmine positive granule. Scale bars = 100 μm in A–G Scale bar in A2 and B2 = 50 μm.
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The time course of tomont development is shown in Fig. 6, in which the tomonts were classified into five stages. The mean percentages of each stage at three repetitions at different days were given in the figure. According to their development, the macronuclei were still weakly stained and could not be seen clearly one day after encystment. On day 2, macronuclear elongation (41% of tomonts) and cytoplasmic division (21%) commenced. On day 3, tomites appeared in 24% of tomonts. The percentage of tomonts that had not begun nuclear development decreased (to 15%). On day 4, empty tomonts, indicating the release of theronts, began to appear, and tomonts showing no development from stage 1 constituted only 5%.

Electrophoresis profiles of protomont DNA

The DNA obtained from the protomonts produced an electrophoretic profile consisting of bands forming an approximately 180-bp ladder (Fig. 7).

Discussion

The gradual loss of the stainability with Feulgen reaction and the only slight enlargement of the macronucleus during the trophont stage (Fig. 1A–D) suggested that DNA in the macronucleus little increased, considering that the Feulgen reaction reflects the amount of DNA in cell nuclei and is often used for the semiquantification of DNA. The result is consistent with Coloni and Diamant (1993) demonstrating that the chromatin network in the early-stage trophont was extremely dense, but became loose and thin as the trophont grew, based on TEM observations of the macronucleus of *C. irritans*. In contrast, the extensive elongation and increase of stainability with Feulgen reaction of the macronucleus in tomonts followed by formation of a massive nucleus and repeated cell divisions indicated that DNA was synthesized in the macronucleus during this period and palintomy, or repeated cell divisions, occurred after the formation of the massive nucleus (Fig. 1E–I), as have also been described by Brown (1963).

It is apparent that the Feulgen-positive granules in
the trophonts, protomonts and early stage tomonts, as well as acetocarmine-positive granules in the tomonts, were the nucleus of black molly cells, because they showed positive reaction in the in situ hybridization (Fig. 4). The morphology of the nuclei indicated the cells were denatured. As the denatured host cells increased in the trophonts, filled the protomonts and disappeared by the commencement of cell division during tomonts (Fig. 1E-I), it is likely that the host cells were ingested and accumulated during the trophont stage, and were digested in the tomont stage, indicating the trophont of Cryptocaryon irritans feeds on host cells.

The present study was consistent with the conclusion by Diggles and Adlard (1997) that the trophont feed on host cells. On the other hand, Coloni (1987) and Coloni and Diamond (1993) suggested that the trophont feeds on tissue debris and body fluids. The present study could not clarify whether tissue debris and body fluids play important roles as food for the trophont.

The condensation of the nucleus, which was observed in the denatured host cells in trophont (Fig. 2B, 3B), is a morphological characteristic of apoptotic bodies (Kerr et al., 1972). The DNA extracted from protomonts filled with denatured host cells appeared as a ladder of 180-bp bands on gel electrophoresis (Fig. 7), which is the characteristic pattern of the DNA in apoptotic bodies (Wyllie, 1980). The denatured host cells appear to have been apoptotic bodies derived from the host cells and that they were stored as apoptotic bodies in the trophont for subsequent digestion during the encystment of the tomont. Host cells with condensed nuclei were also observed in the host tissues surrounding the trophonts, as well as in Cryptocaryon irritans. The denatured host cells with condensed nuclei were observed when host tissues were stained with Hoechst 33258 (Fig. 2B), indicating that the apoptotic bodies were generated within the tissues, and had not dispersed from the trophont after mechanical breakage when the trophont was sectioned and stained. As apoptotic cells are generally disassociated from tissues (Kerr et al., 1972). Trophont of Cryptocaryon irritans may have induced apoptosis and facilitated the ingestion of host cells. Alternatively, apoptosis may have been predominantly induced in the host cells after they were ingested by the trophont. Further studies are required to clarify how apoptosis is involved in the feeding and development of Cryptocaryon irritans.

From the results of this study, the development of the macronucleus and the nutrient intake of Cryptocaryon irritans can be summarized as follows (Fig. 8). Trophont feeds and grows in size in the host epithelial tissues. It

Fig. 8. The life cycle of Cryptocaryon irritans.
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Ingests host cells detached from host tissues, which accumulate as denatured cells, or apoptotic bodies, although it remains unclear whether all of the host cells are ingested after apoptosis or whether some of them undergo apoptosis after ingestion. Little DNA synthesis occurs in the macronucleus in the trophont stage. Protomont is filled with denatured host cells and has small macronucleus. The early-stage tomont, before cell division, is the stage in which DNA synthesis occurs in the macronucleus. The digestion of the denatured host cells occurs mainly in this stage. In the early-stage tomont, the macronucleus becomes elongated, with intensive DNA synthesis, and subsequently forms a massive macronucleus. Once the tomont begins to divide, the cell undergoes palintomy, or a series of rapid cell fissions with little growth, and finally generates and releases infective theronts into the environmental water for the invasion of new hosts.

In *I. multifiliis*, the freshwater counterpart of *C. irritans*, the trophont feeds on the host cells and cell debris, grows and enlarges the macronucleus in host's epithelial tissues (Dickerson and Dawe, 1995; Matthews, 2005). The trophont develops fully in 7 days and leaves the host at around 20°C. On detaching from host, the protomont becomes encysted and undergoes palintomy to release theronts within 24 h after encystment at favorable temperatures. In *I. multifiliis*, DNA synthesis begins in the trophont (Usenskaya and Ovchinnikova, 1966, from English summary). It is thought that the digestion of the host cells and DNA synthesis occur in the trophont stage. In contrast, *C. irritans* digests the host cells and synthesizes DNA mainly after encystment as tomonts, and cell fissions begin after DNA synthesis is complete. These differences in the timing of the digestion of host cells, DNA synthesis, and cell fission appear to be related to the differences in the developmental time courses of *C. irritans* and *I. multifiliis*. In *C. irritans*, the period of parasitism is short (about 3 days). It is unfeasible to digest host cells and synthesize DNA concurrently with feeding. Therefore, *C. irritans* exclusively ingests the host cells and accumulates them during the trophont stage, and begins to digest them and synthesize DNA after it detaches from host in the early-stage tomont. After completing DNA synthesis, it then begins cell division in the late-stage tomont. The period of parasitism in *I. multifiliis* (about 7 days) is longer than that in *C. irritans*, and *I. multifiliis* concurrently completes the ingestion and digestion of the host cells and DNA synthesis in the macronucleus during the trophont stage. When the protomont leaves the host, it is ready for palintomic cell division and can generate numerous theronts within 24 h. Although the life cycle of *C. irritans* seems very similar to that of *I. multifiliis*, the details (the timing of host cells digestion and nuclear development) are quite different.

During the observation of trophont sections on day 3 post-challenge, two trophonts that were tightly attached to each other were found by chance (Fig. 1D). Although there has been no description of conjugation, or a sexual reproduction process, in *C. irritans*, Matthews et al. (1996) suggested that opportunities for conjugation might exist during the infection of the host tissue in *I. multifiliis*. Like *I. multifiliis*, it is possible that *C. irritans* conjugates in the trophont stage. However, further study of the conjugation of both *C. irritans* and *I. multifiliis* is required.

In the histological sections conventionally used to observe trophont in fish tissues, including in this study, the macronucleus has been described as having a club-like shape (Brown, 1963). However, on day 2, the trophont stained with Hoechst 33258 showed macronuclei composed of four bead-like segments (Fig. 2B). This observation indicates that the macronucleus maintains its four-segment structure in the trophont until they detach from the host.

It is well known that the development of the tomont does not progress synchronously in *C. irritans* (Coloni, 1985; Coloni and Burgess, 1997). Theronts are released from day 3 to day 35 or 38 after encystment, even when the tomonts are incubated under similar conditions. The development of the tomont is affected by temperature (Wilkie and Gordin, 1969), dissolved oxygen, and salinity (Yoshinaga, 2001; Coloni, 1985; Cheung et al., 1979). Strain-specific differences in the periods required for the release of theronts have also been reported (Diggles and Adiard, 1997; Jee et al., 2000). To clarify how environmental factors affect the development of tomont and the periods required for the release of theront may allow the prediction and prevention of outbreaks of cryptocaryoniasis in aquaculture facilities, such as marine net cages and land-based tanks. However, the effects of environmental factors on the release of theront have only been estimated until now, because sectioning has been required to observe the internal structures of the tomont. However, the whole-mount staining of tomont with acetocarmine used in the present study allows its internal structures to be observed and should permit the effects of environmental factors on tomont development to be examined. In this study, the timing of macronuclear elongation, the release of theront, and the death of tomont were clarified with this method. It also showed that the development of *C. irritans* was already asynchronous as early as 2 days after encystment.

More than half the tomonts died within 8 days of encystment at 25°C, indicating that only a few tomonts live longer than 8 days. Because the release of theronts has been reported to exceed 35 days after encystment (Coloni and Burgess, 1997), tomonts were considered to live more than several weeks, and tomonts that release theronts periodically appear for a long period. However, the present results indicate that not
all tomonts are long-lived. The parasite used in this study had been propagated in fish in our laboratory for more than one year, and we have found that *C. irritans* isolates propagated for long periods often lose their infectivity for fish and become difficult to maintain under laboratory conditions. The unexpected death of a large percentage of tomonts may have resulted from their long propagation. Further research is required to clarify the mortality during the tomont stage.

**References**


