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In vitro culture technique for *Cryptocaryon irritans*, a parasitic ciliate of marine teleosts

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ABSTRACT: A medium for the *in vitro* culture of *Cryptocaryon irritans*, which is an obligatorily parasitic ciliate of marine teleosts and causes 'white spot disease', was developed. The medium consisted of a layer of cultured fish cells (FHM), with an agarose gel layer covering the cell layer. The agarose gel contained 0.22% agarose, 10% fetal calf serum, 100 I.U. ml^{-1} Penicillin G potassium and 100 µg ml^{-1} streptomycin sulphate. Theronts of *C. irritans* transformed to trophonts and grew to 180 µm in mean length in the medium, although they gradually decreased in number. When trophonts fully developed in medium were transferred into seawater 4 d after inoculation, approximately 70% of them transformed to encysted tomonts and released theronts. When fish were challenged with theronts obtained from *in vitro*-raised parasites, approximately 40% of the theronts were recovered from fish, indicating comparative infectivity of *in vitro*-raised theronts to those of *in vivo*-raised theronts. This is the first report that *C. irritans* fully developed *in vitro* and its entire life cycle was completed without a host fish.

KEY WORDS: *Cryptocaryon irritans* · Ciliophora · *In vitro* culture · White spot disease · Marine fish

INTRODUCTION

Cryptocaryon irritans is an obligatorily parasitic ciliate of marine teleosts and causes 'white spot disease'. The life cycle consists of 4 developmental stages: parasitic trophonts growing in size without cell division within the epithelial layer of the skin and gills of the host fish, protomonts leaving hosts and later being encysted in seawater, encysted tomonts in which repeated cell fissions occur, and theronts released from tomonts into seawater (Colorni 1985, Colorni & Burgess 1997, Dickerson 2006). This parasite is cosmopolitan in distribution, ranging from tropical to subtropical waters, and is very common in public or private aquaria and mariculture facilities (Colorni & Burgess 1997). Mass mortalities of fishes caused by the parasite often result in large economic losses in mariculture.

Despite economic losses and stock mortality caused by *Cryptocaryon irritans*, few effective treatments or control measures have been developed for the parasite, especially for food fish cultured in open waters, although several chemotherapeutic agents are known to be effective for ornamental fish in closed environments (Colorni & Burgess 1997, Dickerson 2006). For net cage culture situations, the transfer of net cages containing infected fish to more open waters is mainly used and indeed is the only method currently available (Yoshinaga 2001). However, difficulties in the early detection of the parasite and in keeping areas pristine prior to transfer often make the therapy ineffective and unfeasible.

The absence of an *in vitro* culture technique is a major obstacle in studies aimed at developing treatments and control measures for *Cryptocaryon irritans*. In most experiments, the parasite has to be maintained

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and propagated *in vivo* using live fish, as for *Ichthyophthirius multifiliis*, the freshwater counterpart of *C. irritans*, which has a life cycle very similar to that of *C. irritans*. *In vivo* propagation of the 2 parasitic ciliates is labour intensive and can be unreliable. Development of *in vitro* propagation techniques for both parasites has, therefore, been sought. Ekless & Matthews (1993), Nielsen & Buchmann (2000), Xu et al. (2000) and Yambot & Song (2004) have tried to develop propagation techniques for *C. irritans* and *I. multifiliis*. Some of these authors have succeeded in transforming theronts into trophonts and some have succeeded in growing them to a certain extent in artificial media, but development to the tomont and theront stages has not been accomplished under *in vitro* conditions for either species.

In the present study, we developed a medium for the *in vitro* culture of *Cryptocaryon irritans* and examined the survival and growth of the parasite in the new medium. The development of *in vitro*-raised parasites in seawater and the infectivity of theronts derived from the *in vitro*-raised parasites were also examined.

MATERIALS AND METHODS

Fish and parasites. A porcupine puffer *Diodon holocanthus* infected with *Cryptocaryon irritans* was obtained from a local pet shop and used as a source of *C. irritans*. The parasite was propagated *in vivo* using black mollies (*Poecilia* sp. hybrid, probably *P. latipinna*, 3 to 4 cm in body length) as hosts, and protomonts were collected from infected black mollies following Yoshinaga & Dickerson (1994). Collected protomonts were washed 5 times with seawater, and incubated in seawater in Petri dishes (10 cm^2) at 25°C; 500 to 1000 protomonts were placed in 4 ml seawater in each dish, and seawater was replaced daily with fresh seawater. Seawater, filtered with 0.45 µm filters and supplemented with 100 I.U. ml^{-1} Penicillin G potassium and 100 µg ml^{-1} streptomycin sulphate, was used for washing and incubation of protomonts and tomonts throughout this study, unless otherwise stated. When placed in seawater, protomonts transformed to encysted tomonts within several hours and subsequently began to release theronts in 4 to 6 d. These theronts were used for the *in vitro* culture.

***In vitro* culture.** A double-layered medium consisting of a cell layer and an agarose gel layer was developed and used regularly for the *in vitro* culture of *Cryptocaryon irritans*. The medium mimics the epithelial layer of fish skin, which harbours the parasite. FHM cells, an epithelial cell line originating from the fat head minnow *Pimephales promelas*, cultured in Leibovits L-15 medium supplemented with 10% fetal calf serum (FCS), 100 I.U. ml^{-1} Penicillin G potassium and 100 µg ml^{-1} streptomycin sulphate, were inoculated in cell culture dishes (10 cm^2) at a density of 2.5×10^5 cells cm^{-2}. After the cells settled on dish bottoms, forming cell layers, a 4 ml solution of ultra-low-melting-point agarose (Sigma, Catalogue No. A-0701) dissolved in L-15 medium supplemented with FCS and antibiotics was distributed on the cell layer in each dish and gelatinized at 4°C; the final concentrations of the constituents were 0.22% agarose, 9% FCS, 90 I.U. ml^{-1} Penicillin G potassium and 90 µg ml^{-1} streptomycin sulphate (0.5% agarose solution, 50 parts; double-strength L-15 medium, 50 parts; FCS, 10 parts; 100-fold strength of Penicillin G potassium and streptomycin sulphate dissolved in 0.85% NaCl solution, 1 part). The concentrations of agarose were optimized by our preliminary experiments (data not shown). A theront suspension containing 100 to 300 theronts in 15 µl of seawater was inoculated at the centre of the cell layer beneath the gel with 200 µl micropipette tips and incubated at 25°C. Parasites in the medium were observed and counted with an inverted phase-contrast microscope at Hour 4 PI (post-inoculation) and subsequently every 24 h, and photographed for further measurements of their size. This experiment was carried out in triplicate. In total, 20 to 30 trophonts were measured from each dish, and the measurements from 3 dishes were pooled to obtain the mean and frequency distribution in parasite length.

Trophonts and tomont-like cysts that transformed from inoculated theronts in the medium were recovered from the medium using pipettes on Day 2 to 7 PI; gel layers were detached from dish bottoms by putting sterile seawater below the gels and removed using pipettes, prior to the recovery. The trophonts and tomont-like cysts obtained from the medium were washed, incubated in 2 ml of seawater supplemented with antibiotics in Petri dishes at 25°C, and examined with an inverted phase-contrast microscope daily for 10 d to observe release of theronts. The experiments were carried out in triplicate. For comparison, protomonts obtained from 3 mollies infected in the propagation aquaria were pooled, washed and incubated in seawater in Petri dishes in the same way. Tomonts and theronts derived from parasites recovered from *in vitro* cultures and experimentally infected fish were photographed for further measurements; theronts were fixed in 1% formalin before measurement to stop their movement.

Large-scale *in vitro* culture. For large-scale cultures, medium was constituted in large culture dishes (57 cm^2), in which 25 ml of the same L-15 medium (supplemented with agarose, FCS and antibiotics) as that used in regular-scale culture was layered on FHM cell layers (2.5×10^5 cells cm^{-2}). Theronts released from

tomonts that were obtained from the fish were concentrated by centrifugation (2000 *g*, 10 s), and a theront suspension was inoculated at 6 points evenly distributed in each dish and incubated at 25°C. This experiment was carried out twice using different batches and volumes of the theront suspension; 15 µl of suspension containing 2700 theronts was inoculated at 6 points in each dish (16 200 theronts dish^{-1} or 280 theronts cm^{-2}) in the first experiment, and 30 µl of suspension containing 1280 theronts was inoculated in each of 2 dishes (7700 theronts dish^{-1} or 140 theronts cm^{-2}) in the second experiment. Trophonts raised in the 2 dishes in the second experiment were collected and pooled on Day 4 PI, and incubated in seawater at 25°C to obtain tomonts and theronts. Theronts released from tomonts obtained in the second experiment were used for experimental infection of fish.

Experimental infection of fish with theronts produced by *in vitro* culture. Black mollies were experimentally infected with theronts that were released from tomonts obtained in the second large-scale *in vitro* culture experiment to investigate the infectivity of theronts produced by *in vitro* culture. Three back mollies were exposed to 2600 theronts within 3 h after their release, in 300 ml of seawater without antibiotics, in a beaker for 1 h. Subsequently,

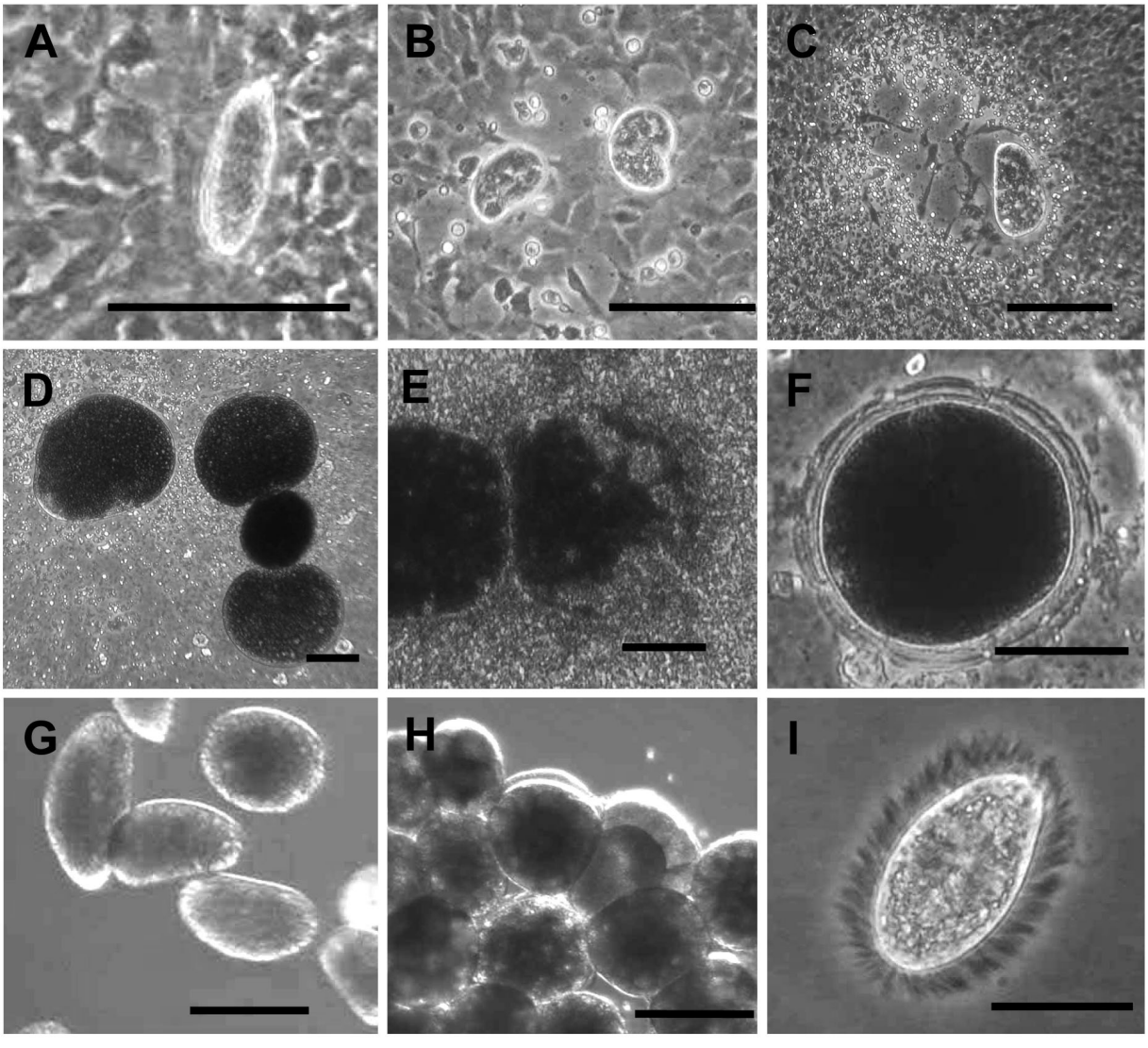

Fig. 1. *Cryptocaryon irritans*. Illustrations in the double-layered medium (A to F) and further development after transfer to seawater (G to I). (A) Theront just after inoculation, (B) trophont at Hour 4 PI, (C) trophont in a hollow-like empty space, (D) fully developed trophonts, (E) ruptured trophont (right), (F) tomont-like cyst formed in a medium, (G) protomonts just after transfer from medium to seawater, (H) encysted tomonts produced with the *in vitro* culture technique and (I) theront released from a tomont produced by the *in vitro* culture technique. Scale bars = 100 µm (A, B, F), 200 µm (C, D, E, G, H), and 25 µm (I)

they were individually transferred and reared at 25°C in 450 ml of seawater with gentle aeration; seawater was replaced daily with fresh seawater. Parasites that left the fish 3 to 4 d after exposure were then found at the bottom of the water column, collected and counted.

Image analysis. Photo-images of parasites were taken with a CCD camera attached to an inverted microscope and analyzed with Scion Image (Scion) image analysis software for measurements.

RESULTS

Immediately after the inoculation into medium, theronts of *Cryptocaryon irritans* showed a teardrop-like or spindle-like appearance and were swimming actively between the cell layer and gel layer (Fig. 1A). By Hour 4 PI, most of the theronts transformed to trophonts (Fig. 2); they became rounder and began to slowly crawl between the layers (Fig. 1B). After transformation, trophonts grew gradually. As trophonts grew, FTM cells around them detached from the dish bottom and empty hollows were sometimes formed around trophonts (Fig. 1C). Structures very similar to the detached FTM cells were frequently observed within trophonts, suggesting that the parasites were eating detached FTM cells. Although trophonts decreased in number day by day (Fig. 2), they showed a clear increase in size until Day 4 PI. Trophonts grew as large as 180 µm in mean length, and several >300 µm in length appeared (Figs. 1D & 3). Subsequently, many of the trophonts ruptured in the medium (Fig. 1E). Simultaneously, a small number of trophonts transformed in medium to tomont-like cysts, having a cyst wall around a cell body (Figs. 1F & 2). Most of the trophonts and cysts died in the medium by Day 10 PI (Fig. 2).

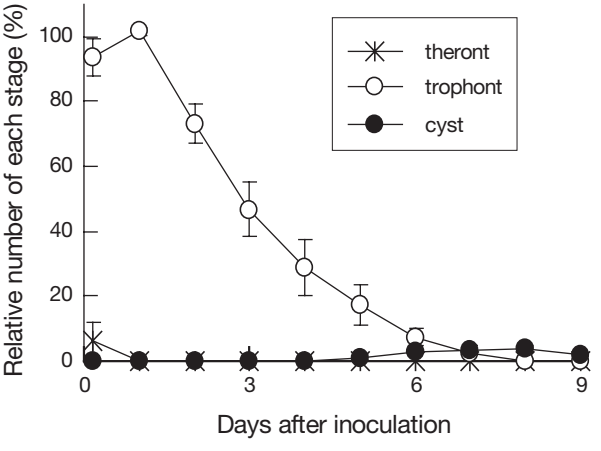

Fig. 2. *Cryptocaryon irritans*. Transformation and survival in the double-layered medium. Mean percentages of theronts, trophonts and tomont-like cysts in triplicates are shown with standard deviations (vertical bars). The total number of parasites in each culture dish at Hour 4 post inoculation is set at 100%

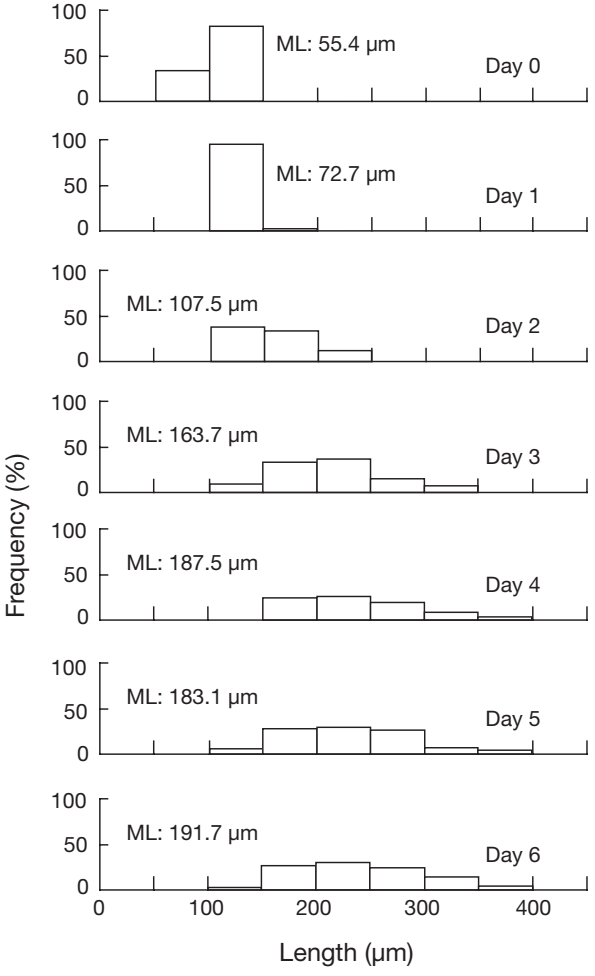

Fig. 3. *Cryptocaryon irritans*. Growth in the double-layered medium. Mean length (ML) and length-frequency distributions of trophonts are shown for Day 0 to 6 post inoculation

When trophonts were transferred into seawater from the medium, some of them crawled on the substrate as subspherical protomonts (Fig. 1G) and subsequently transformed to tomonts encysted in the cyst wall (Fig. 1H), from which theronts were released (Fig. 1I). The ratios of transformation to tomonts and that of release of theronts were closely associated with the timing of the transfer into seawater. The highest ratios were obtained in trophonts transferred on Day 4 PI; 84% of trophonts recovered on Day 4 PI transformed to tomonts and, on average, 66.5% of the trophonts released theronts (Table 1). Tomont-like cysts formed in the medium also released theronts at high ratios, when they were transferred into seawater on Days 4 and 5 PI (Table 1).

The sizes of tomonts derived from trophonts recovered from *in vitro* culture medium were smaller than those obtained from the experimentally infected fish; the mean length of the former was approximately 55 to 66% of that of the latter (Table 1). However, the percentages of theront release were comparable between tomonts that were derived from trophonts recovered from *in vitro* culture on Day 4 PI and those recovered from infected fish; the percentages of theront release were 66.5% in the former and 59.0% in the latter. Moreover, the mean length (±SD) was almost identical between theronts derived from *in vitro*-raised parasites and those derived from parasites recovered from infected fish; they were 50.2 ± 3.7 µm (n = 45) and 52.4 ± 4.0 µm (n = 45), respectively (Student's *t*-test, $p > 0.05$, no significant difference).

In the large-scale culture, inoculated theronts transformed to trophonts and subsequently became encysted as tomonts when transferred into seawater, similar to those from regular-scale cultures. In the first and second experiments, percentages of transformation to tomonts were 11 and 5%; 1736 and 825 tomonts were obtained from 16 200 and 15 400 theronts, respectively.

In the experimental infection of fish, where black mollies *Poecilia* sp. were exposed to theronts released from tomonts produced with the *in vitro* culture technique, 347 and 335 parasites were recovered from 2 of 3 challenged fish, and 1 fish accidentally escaped from its tank and died. The percentages of parasite recovery were 41 and 39%, respectively, for the 2 fish.

DISCUSSION

In the present study, *Cryptocaryon irritans* fully developed *in vitro*, and, for the first time, its entire life cycle could be followed in the absence of a host fish; theronts transformed to trophonts and grew in double-layered medium, and, when transferred to seawater, trophonts recovered from medium transformed to encysted tomonts and subsequently released theronts. Moreover, theronts produced with the *in vitro* culture technique were infective to fish. Infection ratios of theronts produced with the *in vitro* culture technique were approximately 40%, which compares well with the infection ratio of 40.4% in a previous experimental infection, in which black mollies *Poecilia* sp. were challenged by a method similar to the present one (Yoshinaga & Dickerson 1994)

There have been several trials of *in vitro* cultures of *Cryptocaryon irritans* and *Ichthyophthirius multifiliis*, which is the freshwater counterpart of *C. irritans* and has been much more intensively studied. Theronts of *C. irritans* were recently transformed *in vitro* to trophonts on tryptic soy agar blocks in a mixture of seawater, L-15 medium and FCS (Yambot & Song 2004). Theronts of *I. multifiliis* developed to some extent in tissues excised from fish (Xu et al. 2000),

Table 1. *Cryptocaryon irritans.* Comparisons of percentages of transformation to tomonts and theront release, and of tomont size of parasitic ciliates raised in *in vitro* culture medium or recovered from experimentally infected fish. n.m.: not measured. Different letters represent significant differences ($p < 0.05$) between parasites obtained at different days post-inoculation (PI). For statistical analyses of ratios (percentages), ratios obtained from 3 repetitions were subjected to arcsine root transformation and subsequently to ANOVA and post hoc Tukey's multiple comparison test. For analyses of tomont length, lengths of tomonts obtained in the 3 repetitions were pooled and subjected to ANOVA and post hoc Tukey's multiple comparison test

Parasite stage and origin	Days PI	Trophonts that transformed to encysted tomonts (%) Mean (range)*	Length of encysted tomonts (µm) N	Mean ± SD	Trophonts or tomont-like cysts that released theronts (%) Mean (range)*
Trophonts recovered from medium	2	0‡			0‡
	3	8.6 (2.4–19.3)^{a}	14	149 ± 27.0^{a}	5.6 (2.0–12.3)^{a}
	4	84.0 (81.5–85.9)^{b}	164	185 ± 35.9^{b}	66.5 (60.2–70.4)^{b}
	5	50.2 (27.9–70.6)^{c}	65	151 ± 37.4^{a}	28.4 (11.5–41.2)^{a}
Tomont–like cysts formed in medium	4		13	270 ± 59.8^{ab}	84.6‡
	5		32	255 ± 66.3^{a}	68.2 (56.3–85.7)^{a}
	7		111	288 ± 61.4^{b}	21.2 (12.5–28.3)^{b}
Trophonts recovered from experimentally infected fish		n.m.	42	270 ± 27.7	59.0‡

*Means and ranges in 3 repetitions
‡Data not statistically analyzed because of no variance
‡Data from a single trial

survived longer in Eagle's minimum essential medium than in freshwater (Elkless & Matthews 1993) and transformed to trophonts when given an Epitheliosa Papulosum Cyprini (EPC) cell line as a substrate (Nielsen & Buchmann 2000). However, these accomplishments in methods for the culture of both *C. irritans* and *I. multifiliis* were limited to incomplete growth and transformation of parasites under *in vitro* conditions.

Although the survival rate of parasites in the present *in vitro* culture medium was not high, roughly 30% of the inoculated parasites were still alive on Day 4 PI (Fig. 1), 66.5% of the parasites recovered on Day 4 PI released theronts (Table 1), and the number of theronts released from tomonts is known to be up to 200 theronts per tomont (Colorni 1985). If the value of 100 theronts per tomont is given as the mean number of released theronts, total yield of theronts from 1 theront in the present technique is estimated as ca. 20 by a simple arithmetic calculation. However, we have not succeeded in stably executing the parasite life cycle *in vitro* using the present technique: parasites were damaged when collected from the medium and the concentration of theronts by centrifugation and filtration decreased their viability (data not shown). Live fish are currently necessary to produce theronts to be inoculated into medium. However, our new technique is an important advance. Even the present *in vitro* culture technique enables direct and continuous observation of the development and behaviour of *Cryptocaryon irritans* using an inverted microscope. This will be of much help in studies of the biology and host–parasite interaction of *C. irritans*. Most chemotherapies against *C. irritans* target the free-living stages, namely, protomonts, tomonts and theronts. Chemotherapy targeting the parasitic stage has not been developed. This is partly because live fish infected with the parasite must be used for primary drug screening. The present culture technique may enable *in vitro* screening and assist with future drug development for *C. irritans*.

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Acquired immunity of fish against *Cryptocaryon irritans* has been demonstrated (Yoshinaga & Nakazoe 1997). If the mass production of theronts of the parasite were possible, it could lead to the development of an inactivated vaccine against the parasite.

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