A NOVEL ASSOCIATION BETWEEN AN ENDEMIC STICKLEBACK AND A PARASITIC DINOFLAGELLATE. 2. MORPHOLOGY AND LIFE CYCLE

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ABSTRACT

An unusual dinoflagellate has been discovered in association with an endemic population of stickleback, Gasterosteus (L.), from the Queen Charlotte Islands, Canada. The dinoflagellate spends most of its life cycle as a coccoid vegetative cyst, not as a parasitic trophont. The vegetative cyst is unique in containing a rigid fenestrated matrix, which is penetrated by cytoplasmic processes that emanate from a central area containing the dinokaryotic nucleus and associated chloroplasts. Some pores in the matrix are filled by oil droplets or starch granules. Intracellular bacteria are found throughout the cyst, sometimes in association with the nucleus. The cytoplasm contains accumulation bodies, microbodies, polyhedral crystals, chloroplasts and polyvesicular bodies. The encysted dinoflagellate has several potential strategies. It can 1) shed its wall and become amoeboid; 2) undergo sporogenesis and give rise to both regular and resistant spores; 3) divide mitotically, with a gradual reduction in the size of daughter cells down to 20 μm; and 4) apparently form a resting cyst, during which it secretes a thick outer wall composed of five layers. Taxonomically, this unusual dinoflagellate appears to be a new member of the Blastodiniales, although its position will become clearer when details of the motile stage are known.

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An endemic population of three-spine stickleback, Gasterosteus (L.), from Rouge Lake on the Queen Charlotte Islands, Canada, is infected seasonally by an ectoparasitic dinoflagellate unlike any previously described in fish (Reimchen and Buckland-Nicks 1989). The prevalence of photosynthetic, vegetative cysts on the stickleback rather than invasive parasitic feeding stages (termed "trophonts" by zoologists) substantially reduces the pathogenic effects that characterize most dinoflagellate infections (Lom and Schubert 1983, Cachon and Cachon 1987).

In this report we describe aspects of the morphology and life cycle of this first known ectoparasitic dinoflagellate of stickleback. Furthermore, we discuss general taxonomic affinities and a possible classification for this new species.

MATERIALS AND METHODS

Infected Gasterosteus (L.) were collected from Rouge Lake, Queen Charlotte Islands, British Columbia, in June 1987. The gelatinous epithelial covering containing cysts was dissected from the body and gills of infected fish and placed directly in 2% glutaraldehyde in filtered (2 μm pore size) lake water for 30 min. Some cysts were removed from the epithelial covering and gently fractured in a Radnoti tissue homogenizer (Fisher) to facilitate penetration of the embedding medium. The tissues were then transferred to 2% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 for 3 h. Post-fixation was with 2% OsO₄ in 0.1 M sodium cacodylate buffer at pH 7.4 for 1 h. The tissues were transferred to 70% ethanol and kept in this solution until our return to Edmonton two weeks later. Dehydration was then completed in an ethanol series, and the tissues were embedded under vacuum in Ultra Low Viscosity Resin (Polysci.). For light microscopy 0.5 μm sections were cut with glass knives, stained with Richardson’s stain (Richardson et al. 1960) and viewed with a Zeiss photomicroscope II equipped with Nomarski optics. For transmission electron microscopy (TEM) silver sections were cut with a diamond knife (Diatome), mounted on cooper grids, stained with alcoholic uranyl acetate followed by aqueous lead citrate, and examined in a Philips 201, 401 or a Hitachi 7000 transmission electron microscope. In spite of the above precautions to ensure that tissues in the cyst would be fixed and embedded adequately, we still had many problems, particularly with sectioning the brittle fenestrated matrix, which sometimes remained separate from the plastic and broke apart under the electron beam. Satisfactory solutions to these problems are still needed.

For scanning electron microscopy (SEM), individual cysts and gill filaments containing cysts in various stages of development were dissected and fixed separately as above. In the Edmonton laboratory specimens were dehydrated in an ethanol series and either air dried or passed through an amyl acetate series to pure amyl acetate and then critical point dried. Dried specimens were mounted on aluminum stubs with double-sided sticky tape. Some cysts were rolled on the sticky tape with a fine needle to remove the cyst wall and expose the contents. The specimens were sputter-coated with gold and examined in a Cambridge S250 stereoscanner microscope.

RESULTS

The stickleback response to dinoflagellate infection is epithelial hyperplasia, during which superficial epithelial cells are mobilized to form an envelope that overgrows and encloses the dinoflagellate cysts (Fig. 1). Macrophages and goblet cells can migrate between the epithelial cells and tend to aggregate at sites of infection (Fig. 1).

The infective stage has not been observed in detail and attempts to culture it from vegetative cysts have failed thus far. In numerous detailed examinations of infected skin and gills we have not observed a trophont stage and, if it exists, it is probably short-lived. The smallest cysts are less than 20 μm in diameter. Cysts were identified as dinoflagellate in origin, based on the presence of a typical dinokaryotic nucleus with continuously condensed, banded chromosomes. Two kinds of cysts were found, a thin-walled greenish-brown vegetative cyst, and a rarer thick-walled red resting cyst, which is probably a hypnozygote. We cannot yet exclude the possibility that the latter cyst belongs to a different dinoflagellate since only a few were observed.

Vegetative Cyst

The vegetative cyst is usually smooth-walled, slightly ovoid, with no obvious external pores (Fig. 17). A small indentation is sometimes visible. Fixatives and embedding media penetrated the intact cyst very slowly or else not at all. A fenestrated matrix, which in both fresh and fixed materials is hard and brittle and shatters under pressure, fills the interior of the cyst (Figs. 1, 3, 11). This matrix is a cytoplasmic separation that is poorly penetrated by embedding media, thus making electron microscopy extremely difficult.

The nucleus is located near the center of the cyst (Fig. 1) within an enlarged space in the fenestrated matrix (Fig. 3). The chromosomes are fully condensed and have the banded appearance typical of dinoflagellate nuclei (Fig. 2). There is a prominent nucleolus (Fig. 3). Both oil droplets (Figs. 3, 4), and starch granules (Figs. 3, 4) are visible. Cytoplasmic processes penetrate fenestrations in the matrix from the center out to the periphery of the cyst (Fig. 4). A large group of chloroplasts lies adjacent to one side of the nucleus (Figs. 3, 4), and several individual chloroplasts are visible at the cell periphery and within cytoplasmic extensions (Fig. 4). The chloroplasts have triple thylakoid membranes (Fig. 7) and may occasionally give rise to a multiple-stalked pyrenoid that is not penetrated by the thylakoids (Figs. 5, 6). Sometimes the chloroplasts are extremely elongate and linked end to end (Figs. 3, 4). Granular secretions are often visible between thylakoids, and sometimes there are also dense inclusions. Accumulation bodies are distributed throughout the cyst, within the nucleus, and also in the peripheral cytoplasm (Figs. 3, 4). Intact mitochondria have not yet been observed.

Intracellular bacteria are found near the perim-
NOTE: Abbreviations used in figures: accumulation bodies (AB), bacteria (Ba), vegetative cyst (C), chloroplasts (Ch), polyhedral crystals (Cr), cyst wall (CW), cytoplasmic process (Cy), glycocalyx (G), fused gill filaments (GF), microbody (M), macrophages (Ma), mucous goblet cells (MG), mitochondria (Mi), nucleus (N), nucleolus (Nu), oil droplet (O), pyrenoid (P), superficial epithelial cells (SE), fenestrated skeletal matrix (SM), spores (Sp), starch granules (St), spore wall (SW).

FIGS. 1–3. Dinoflagellate vegetative cysts. FIG. 1. LM of 1 μm section through fused gill filaments of _Gasterosteus_ (L.) showing epithelial cells enclosing vegetative cyst. FIG. 2. TEM of dinokaryotic nucleus in vegetative cyst, showing condensed banded chromosomes (arrowheads). FIG. 3. TEM of vegetative cyst showing nucleus and chloroplasts enclosed by fenestrated matrix.
Fig. 4-7. Dinoflagellate vegetative cysts. Fig. 4. TEM of cyst showing nucleus and adjacent chloroplasts enclosed by fenestrated matrix. A long cytoplasmic process penetrates the matrix diagonally. Fig. 5. TEM of formalin-fixed vegetative cyst showing tripartite chloroplast with pyrenoid. Fig. 6. Similar to Figure 5. Fig. 7. TEM of chloroplast showing stacked triple thylakoid membranes.

eter of the cyst (Fig. 8), adjacent to the fenestrated matrix or embedded in it, and on rare occasions have been found within an indentation of the nucleus. Microbodies (Fig. 9), polyvesicular bodies, and polyhedral crystals (Fig. 3) have also been observed.

Asexual Reproduction

Binary fission. Vegetative cysts that were kept in distilled water for two months continued to divide by fission, becoming smaller with each generation.
At the outset the cysts measured 150 μm in diameter and after two months were reduced to 20 μm in diameter. During this period the intracellular bacteria multiplied and a spherical red body became evident in the cytoplasm of the smaller cysts. At each cell division the nucleus divided first, followed by the cytoplasm and then the fenestrated matrix, which formed between the two cells as a fenestrated partition (Figs. 10, 11). Sections of smaller cysts (30 μm) revealed a construction similar to full sized cysts, with no evidence of preparation for release as zoospores, such as flagella. Cysts that had died turned white and the contents degenerated, although the intracellular bacteria multiplied during the period of study.

Zoosporogenesis. Vegetative cysts were cultured on
agar plates (made with culture medium, see Timpano and Pfister 1985a). Five or more vegetative divisions occurred before the plates were flooded with culture medium with the expectation that zoosporogenesis would ensue and zoospores would be released (Timpano and Pfister 1985a).

**Palisporogenesis.** Some formalin-fixed vegetative cysts were found that had undergone palisporogenesis; numerous cell divisions had occurred internally and had given rise to dinospores contained within the parent membrane. When cysts were broken open the dinospores could be collected and sectioned. We frequently observed a rod-shaped bacterium attached to the external surface of a spore (Fig. 12). Often the bacterium had undergone fission. A few spores, found in preparations of homogenized cysts, had thick outer walls, large accumulation bodies and several mitochondria (Fig. 13). None of the dinospores observed showed any signs of developing flagella, such as basal bodies.

**Resting cysts.** On three occasions we found a red cyst mixed in with the more common greenish-brown vegetative cysts in samples of the epithelial cell covering. Each cyst had undergone asexual reproduction internally, giving rise to numerous small nuclei located mainly toward the cell periphery (Fig. 14). These red cysts have extremely thick walls composed of five layers (Fig. 15). Layer 4 is the thickest and comprises a number of sub-layers, the outer sublayer being thinnest, suggesting compression and the addition of material from the inside. The chromosomes of these nuclei were condensed but lacked the characteristic banded pattern of the dinokaryon (Fig. 16). One nucleus was more centrally located and appeared slightly larger than the others; this was probably the second daughter nucleus. We presume that the multiple nuclei are precursors of dinospores, or gametes, which under certain conditions would be completed and then released into the surrounding water. However, no evidence was found for the production of flagella.

**Amoeboid stage.** The formation of an amoeboid stage from a living vegetative cyst was observed under the light microscope on two occasions. During this process the cyst began to rotate and a small cytoplasmic extrusion, possibly a microspore, was ejected. Following this the cyst wall was shed, and an amoeboid protoplast emerged that began moving across the culture dish. The shape of the amoeba changed from spherical to oval with the long axis in the direction of movement.

This and other known stages in the life cycle of the dinoflagellate, together with hypothetical intermediate stages, are summarized diagrammatically in Figure 18.

**DISCUSSION**

**Morphology and Life Cycle**

The morphology of the vegetative cyst and the various stages of the life cycle that we observed (Fig. 18) are similar to those of members of the Phyto- diniales (Dinococcales) such as *Cystodinium* (Timpano and Pfister 1985a, b). For example, the new species described here has the following features in common with *Cystodinium*: a coccoid vegetative cyst that is actively photosynthetic and forms a major part of the life cycle (this cyst is bounded by a continuous wall composed of microfibrils that is covered by an external glycoexy) and a life cycle which includes a resting cyst, an amoeboid stage, a sporogenous stage and an ability to divide asexually in the encysted state.

However, there are also similarities with some Blastodiniales. The parasitic genus *Blastodinium* retains its chloroplasts, and its nucleus undergoes structural changes during sporogenesis similar to those described here (Soyer 1971), although its external morphology is different and it usually lives in the guts of copepods.

Other features that are characteristic of dinoflagellates in general include a dinokaryotic nucleus with continuously condensed, banded chromosomes; a prominent nucleolus; accumulation bodies; starch granules; oil droplets; microbodies; polyhedral crystals; polyvesicular bodies and chloroplasts (Steidinger and Cox 1980, Dodge 1987, Taylor 1987). This dinoflagellate is unique in having a rigid internal fenestrated matrix filling the vegetative cyst. This matrix can be rapidly solubilized during formation of the amoeboid stage. Red droplets in the vegetative cyst and resting cyst are characteristic of many dinoflagellates (Dale et al. 1978, see also review by Spector 1984).

A typical parasitic trophont stage is either reduced or absent and yet the dinoflagellate still induces epithelial hyperplasia in its host. The reason for this may simply be that it is antigenic. However, it is also possible that the intracellular bacteria are releasing a toxin to which the host dinoflagellate is immune. Bacteria are well-known for producing potent toxins that were once thought to be produced by their hosts (Moore et al. 1982), and this may also be true of some toxic dinoflagellates that contain intracellular bacteria (De Silva 1981, Steidinger and Baden 1984). As we have described elsewhere, the stickleback host has clearly evolved mechanisms for reducing any pathogenic effects of the dinoflagellates (Reimchen and Buckland-Nicks 1989).

The existence of intracellular bacteria in dinoflagellates is well-known (De Silva 1962, 1967, 1978, Gold and Pollingher 1971, Dodge 1973, Lee 1977), but there are no detailed studies of these associations or how the bacteria are transferred between generations. Most stages in the life cycle of a dinoflagellate would automatically receive a complement of the bacteria. For example, in the case of fission, each half would receive some bacteria and in the amoeboid stage the wall is lost but the bacteria are still contained within. However, sporogenesis poses a problem. Our study shows that during sporogene-
Figs. 12–17. Micrographs of various stages in the life cycle of the dinoflagellate. Fig. 12. LM of a 1 μm section of spores and attached bacteria ruptured from a formalin-fixed vegetative cyst that had undergone sporogenesis. Fig. 13. TEM of resistant spore from a ruptured vegetative cyst. Fig. 14. LM of a 1 μm section through a red resting cyst viewed with Nomarski optics. The cyst wall shows compression fractures (arrowheads) induced to allow penetration of fixatives and embedding media. Fig. 15. TEM through cyst wall showing five different layers. Note that layer 4 is composed of several similar sub-layers, which become progressively more compressed towards the external surface. Fig. 16. One of many nuclei from a red resting cyst showing condensed chromosomes without banding pattern (arrowheads). Fig. 17. SEM of young vegetative cyst trapped in stickleback gill filaments but not yet enclosed by epithelial cells. Possible site of archaeopyle is indicated (arrowhead).
Fig. 18. Diagrammatic representation of observed (solid arrows) and hypothetical (broken arrows) stages in the life cycle of this new species of parasitic dinoflagellate. The stickleback *Gasterosteus* is shown to be covered with cysts (dark dots), which predominate on the dorsal surface. A) A piece of fish skin is enlarged to show attached vegetative cysts. B) One vegetative cyst is drawn in cut-away to show nucleus and cytoplasm. C) A dividing vegetative cyst is shown in longitudinal section. D) Multiple asexual divisions result in a cluster of tiny cysts. We suspect that each cyst gives rise to a flagellated swarmer. E) A vegetative cyst can form an amoeboid stage. It first rotates and releases a small bit of cytoplasm (arrowhead), which is possibly a microspore. F) Then the cyst wall is shed and the amoeboid stage emerges. The amoeboid stage may undergo sporogenesis to produce flagellated swarmers as reported elsewhere (Pfiester and Popovsky 1979). G) The vegetative cyst can undergo sporogenesis which results in the production of numerous spores inside the cyst. H) Some spores have thick walls and are thought to be resistant. I) Other spores have thinner walls and almost always carry one or two bacteria attached to their external surface. Both types of spore possibly give rise to flagellated swarmers. J) A vegetative cyst can apparently become dormant and form a resting cyst (hypnozoygote) by increasing the wall thickness and altering internal structure. Later the resting cyst can undergo sporogenesis. K) Hypothetical flagellated swarmer. L) We suspect that a swarmer is responsible for infecting the host fish, but neither this nor a trophont stage has yet been observed. The stickleback is drawn actual size. For A–G, J scale bar = 20 µm. For K, H, I scale bar = 10 µm.

The bacteria become attached to the external surface of spores and are presumably transferred to the infective stage by this means. Since we did not find bacteria attached to the external surface of resistant spores, it is likely that some dinoflagellate cysts lack a complement of bacteria. This has been observed in some other dinoflagellate-bacteria associations (Steidinger and Baden 1984).
Classification

Previous descriptions of dinoflagellate parasites of freshwater fish are restricted to the genus *Piscinooodinium*, which belongs to the parasitic order Blastodiniales (Jacobs 1946, Lawler 1967, Lom and Schubert 1983), as do three other genera occurring in marine fish. The new species described here resembles *Piscinooodinium* because it parasitizes a fish host, contains chloroplasts (also in *Blastodinium*, although most Blastodiniales do not [Cachon and Cachon 1987]), has the ability to divide asexually in the vegetative cyst, and undergoes nuclear transformation in the resting cyst (hynnozygote). However, there are a number of significant differences. First, the major part of the life cycle is spent in the encysted, coccooid state in association with the fish host and not as a parasitic trophont. Second, the preferred site of infection is the dorsal part of the head; the lowest densities of cysts occur on the gills and mouth (Reimchen and Buckland-Nicks 1989), which is opposite to what one would expect for a *Piscinooodinium* (Jacobs 1946, Lom and Schubert 1983). Third, Oodiniidae generally cause substantial losses to fish infected by them and have been infamous as pathogens of aquarium fish (Jacobs 1946, Lom and Schubert 1983, Cachon and Cachon 1987). In the present case, although 99% of the fish had high infections of the gills and skin, there was no noticeable fishkill, and both young and adult fish of all size classes survived beyond the end of the parasitic season (Reimchen and Buckland-Nicks 1989). However, we have not kept fish in captivity to determine empirically the mortality rate among infected and non-infected fish. Furthermore, the reproductive activity of the fish does not appear to be affected as both gravid females and territorial males were found with heavy infections during the breeding season. Fourth, most freshwater dinoflagellates are alkalinophile (Pollinger 1987). The few genera that can tolerate low pH belong to other groups, and the majority of these species belong to the Phytodiniales. Of these, only a handful of species, such as *Peridinium* sp., can tolerate a pH as low as 4.0.

The known Blastodinida and Syndinida have life cycles with two periods. The first is a vegetative phase (trophont) and the second is a reproductive phase (sporous) with spores developing in situ (Cachon and Cachon 1987). During the present study we examined numerous infected fish with light microscopy, including some with SEM, and we never saw an obvious trophont stage. It seems likely that a flagellated swarmer would initially infect the host fish and then encyst, possibly without going through a trophont stage. Such a life cycle would represent the other end of the extreme suggested by Jacobs (1946) in which the parasitic phase has become reduced and the photosynthetic phase has become predominant.

The uncertainty as to whether this dinoflagellate fits into the present classification reinforces the point made by Timpano and Pfiester (1985b) that the sharp distinction currently made between the Phytodiniales and the Blastodiniales may not be a valid one. The fenestrated matrix and distinctive life cycle should be sufficient to recognize this dinoflagellate as a new genus, since it is not assignable to any of those described so far. However, we wish to resolve the whole life cycle before doing so.

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MORPHOLOGIC DETAILS OF SIX BENTHIC SPECIES OF PROROCENTRUM (PYRROPHYTA) FROM A MANGROVE ISLAND, TWIN CAYS, BELIZE, INCLUDING TWO NEW SPECIES

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ABSTRACT

Two new dinoflagellate species, Prorocentrum hoffmannianum and Prorocentrum ruetzleriianum, and four known species, Prorocentrum emarginatum Fukuyo 1981, Prorocentrum mexicanum Tajfall 1942, Prorocentrum concavum Fukuyo 1981, and Prorocentrum lima (Ehr.) Dodge 1975, from floating detritus and sediments in a subtropical mangrove island, Twin Cays, Belize, Central America are described from scanning electron micrographs. Differences in the following characters of surface micromorphology separated the species: ornamentation of thecal plates (shape, size, and number of valve pores and areolae) and the architecture of the periflagellar area and intercalary band.

Key index words: benthic dinoflagellates; mangrove habitat; Prorocentrales; Prorocentrum concavum; Prorocentrum hoffmannianum sp. nov.; Prorocentrum lima; Prorocentrum mexicanum; Prorocentrum ruetzleriianum sp. nov.; Pyrrophyta; scanning electron microscopy; taxonomy

Prorocentrum species are distributed worldwide in marine waters. Most well-studied species are planktonic. In contrast, benthic Prorocentrum species have received little attention, and only four species have been described so far. Some of these species are sources of ciguatera toxins in fish and shellfish in tropical areas (Nakijama 1965, Murakami et al. 1982, Tindall et al. 1984). Species of benthic Prorocentrum are associated with coral reefs (Fukuyo 1981, Bomber et al. 1985, Carlson and Tindall 1985), protected embayments (Carlson 1984), benthic debris (Steidinger 1983), and sand (Lebour 1925, Drebes 1974, Dodge 1985), or are confined to islands (Bannar 1976) or attached to floating detritus in the mangrove habitat (Faust 1990a).

The four benthic species described to date are Prorocentrum lima, P. emarginatum, P. mexicanum, and P. concavum. They occur most frequently in epiphytic associations (Baden 1983), attached to brown and red macroalgae (Fukuyo 1981) with mucous threads (Steidinger 1983), and exhibit substrate preferences (Bomber et al. 1985). I describe here two additional benthic Prorocentrum species that co- exist with the above four species. These broader based associations were discovered in floating detritus and sediment in the Twin Cays mangrove island where they constitute a large portion of the dinoflagellate biomass.

There are only a few reports on the taxonomy and classification of benthic Prorocentrum species (Dodge 1975, Fukuyo 1981, Carlson 1984). These studies used low magnifications which could not reveal the minute features of the surface structure of the cells. Surface morphology is critical for classification of dinoflagellates (Pavillard 1916, Lebour 1925, Schiller 1933, Nie 1947, Dodge 1965, 1975, 1982, Abe 1967, Loeblich 1969, 1976, Taylor 1980, Faust 1990b). Here I present details of the surface morphology of the valves and the architecture of the flagellar pore area and intercalary band of six benthic species of Prorocentrum from sediments and floating detritus from a mangrove habitat of Twin Cays, Belize. Scanning electron microscopy revealed details of Prorocentrum surface morphology not apparent in previous studies that is useful for identi-

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