A Parasitic Dinoflagellate of the Ctenophore *Mnemiopsis sp.*

by

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Abstract

In this study I have sought to characterize a previously unknown parasitic dinoflagellate, which is associated with the costal ctenophore *Mnemiopsis sp*. Here, I describe its general morphology, based on an identification system created by Charles Kofoid used specifically for dinoflagellates. The identification system, Kofoid plate tabulation, allows for identification of genera or possibly species. The plate tabulation is used to interpret the gross morphological characters, number of thecal plates, and their arrangement. The study will also present on an overview of its parasitic relationship with the host and its reproductive capacity. Lastly, the study finishs with the phylogenetic placement based on rDNA, ITS, and cyt b molecular analysis. I conclude that the dinoflagellate's phylogeny is placed tentatively into the genus *Pentapharsodinium* due to the inconsistencies within the monophyletic E/Pe clade. The life cycle of the dinoflagellate is characteristic of a parasite. However, the ability to successfully culture the dinoflagellate would suggest it is mixotrophic opportunistic parasite.

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Chapter 1: Introduction. The Ctenophore *Mnemiopsis leidyi* and its Symbiotic Protist Assemblage.

Ctenophore anatomy and placement

Comb jellies, or ctenophores (Phylum Ctenophora) are gelatinous marine planktonic organisms found throughout the oceans of the world. All are predators distinguished from Phylum Cnidaria by the lack of nematocysts, an oral-aboral body axis that positions the aboral organ and mouth at the most distant positions on the body, and

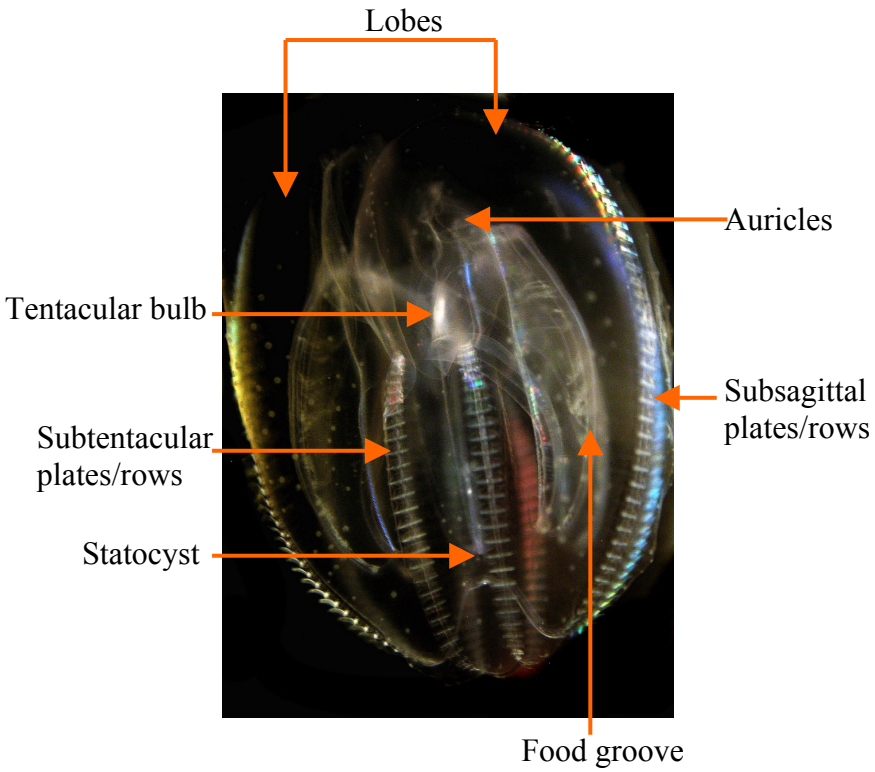

Figure 1. Ctenophore (*Mnemiopsis*) Gross Anatomy

eight rows of ciliary paddles called ctenes or comb plates, from which the group derives its name. The paddles are used for locomotion and food acquisition via fluid transport driven by ciliary beating (Colin, Costello et al. 2010). Ctenophores contain an extensive mesoglea that consists mostly of acellular components and water for structural support

(Harbison 1985). Ctenophores undergo embryological development via a distinctive, determinate series of cell divisions (Martindale and Henry 1999). All possess a single aborally positioned statocyst. Members of Class Tentaculata possess tentacles bearing specialized adhesive cells at some stage in their life cycle; all members of Class Nuda never bear tentacles at any stage.

Ctenophores are usually hermaphroditic, storing both gametes beneath their comb rows (Pang and Martindale 2008). Recent molecular phylogenies of ctenophores (Podar, Haddock et al. 2001; Dunn, Hejnol et al. 2008; Hejnol, Obst et al. 2009) agree with previous classical analyses (Hyman 1940) and place them into Phylum Ctenophora, which has approximately greater than 150 known species (Mills 2007) broken into two classes, Tentaculata and Nuda.

Mnemiopsis

The coastal ctenophore *Mnemiopsis leidyi* (Class Tentaculata; Order Lobata; (Fig. 1) is endemic to the Western Atlantic. It is a planktonic predator found from the Bay of Campeche along the North American Atlantic coast as far north as southern New England (Purcell, Shiganova et al. 2001).

Mnemiopsis routinely experiences transatlantic transport to the Old World via ship's ballast water (Harbison and Volovik 1993; Ruiz, Carlton et al. 1997; Ivanov, Kamakin et al. 2000; Bai, Zhang et al. 2005). *Mnemiopsis* is an exceptionally invasive organism, and in the 1980s invaded the Black Sea; in the 1990s, the Caspian; in 2006, the North and Baltic Seas, and most recently the Western Mediterranean. Like many invasive organisms, *Mnemiopsis* has severely impacted the ecology of numerous bays and estuaries in invaded regions. The lack of the naturally occurring New World predators

Beroë ovata and *Chrysaora quinquecirrha* (Burrell and Van Engel 1976; Finenko, Anninsky et al. 2001) inevitably results in swarms of overwhelming magnitude. Invasive *Mnemiopsis* populations are typically regulated by local seasonality and their inability to survive sustained water temperatures below 2 ºC. Invasions of *Mnemiopsis* into the Black Sea and Caspian Sea caused multibillion-dollar losses to local fisheries and exacted tremendous ecological damage (Ivanov, Kamakin et al. 2000; Purcell, Shiganova et al. 2001; Kideys, Roohi et al. 2005). Damage resulting from invasions into the North and Baltic Seas has yet to be assessed (Hansson 2006). Microsatellite analyses have recently revealed that *Mnemiopsis* invaded the Old World in two waves (Reusch, Bolte et al.)

Ctenophore parasites

Ctenophores are known to often carry a multitude of parasites, some which may use ctenophores as an intermediate host (Purcell and Arai 2001). Several species of parasitic nematodes (Køie 1993; Gayevskaya and Mordvinova 1994) have been observed to be associated with ctenophores. Trematodes (Yip 1984) are well established as a known ctenophore parasite and even a cnidarian (Bumann and Puls 1996). In the mid 1990s the Moss laboratory reported that *Mnemiopsis leidyi* of Mobile Bay and the Northern Gulf of Mexico harbored an assemblage of protistan symbionts (Moss, Estes et al. 2001). Four distinct organisms were observed: a trichodine (Fig. 2a) (Estes, Reynolds et al. 1997), two types of amoebae (Fig. 2b) (Smith, Versteeg, Rogerson, Gast and Moss, in preparation), and a large ectodermally attached dinoflagellate (Fig. 2c). The trichodine, *Trichodina ctenophorii,* preferentially attaches to the host at the aboral side of the auricular and locomotary comb plates. *T. ctenophorii* appears to have a commensal relationship with *Mnemiopsis.* A *Flabellula* – like gymnamoebae only appears on the

comb plates and forms a parasitic relationship with *Mnemiopsis*. A *Vexillifera* –like commensal gymnamoebae is found at low densities on the ctenophore ectoderm (Moss, Estes et al. 2001).

The dinoflagellate can be found attached to the ectoderm, embedded in the mesoglea, and freely swimming in the vascular canals. Here, I provide evidence that the dinoflagellate is a parasite. Moss and colleagues suggested that the dinoflagellate was indeed parasitic because it caused localized collapse of the mesoglea, particularly in regions near the aboral pole and where there were relatively large numbers of cells.

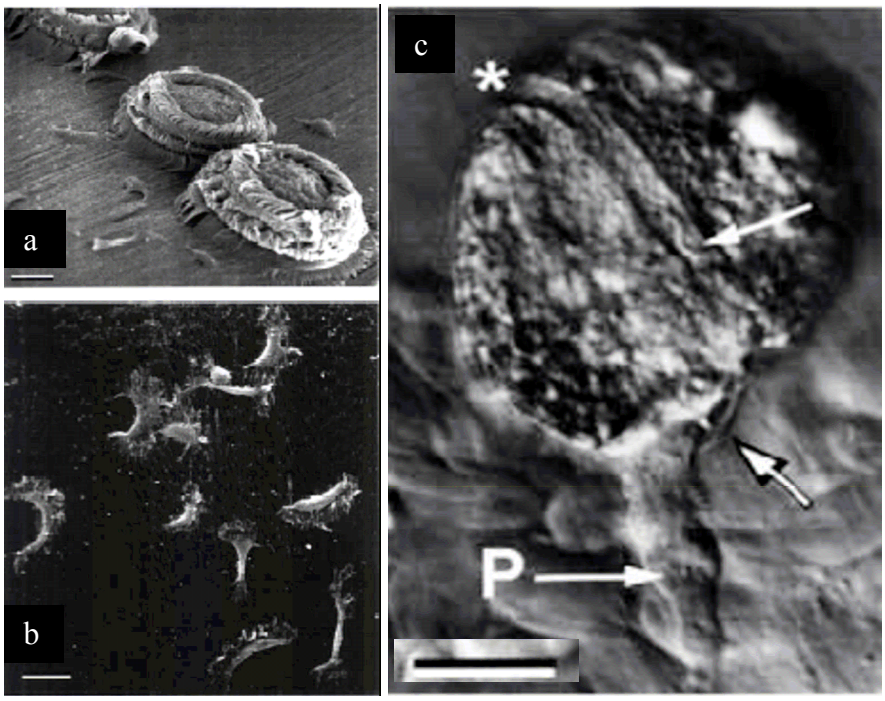

Figure 2. a) *Trichodina ctenophorii* attached *to Mnemiopsis* comb plate. Scale: 10µm b) Flabellulid gymnamoebae attached *to Mnemiopsis* comb plate. Scale: 5µm. c) Dinoflagellate attached to *Mnemiopsis*. Open arrow indicates longitudinal flagellum, asterisk indicates cingulum, and white arrow indicates transverse flagellum. Scale: 20µm (Moss, Estes et al. 2001)

Dinoflagellate cortex

The dinoflagellate cortex, also called the theca or amphiesma, is comprised of a plasmalemma, a thin peripheral cytoplasmic layer, a single layer of flattened cortical (amphiesmal) vesicles, and a layer of microtubules located within the cytoplasm beneath the cortical vesicles (Dodge and Crawford 1970). Another layer may also be present: termed the pellicular layer or pellicle, this fourth layer may be found under the

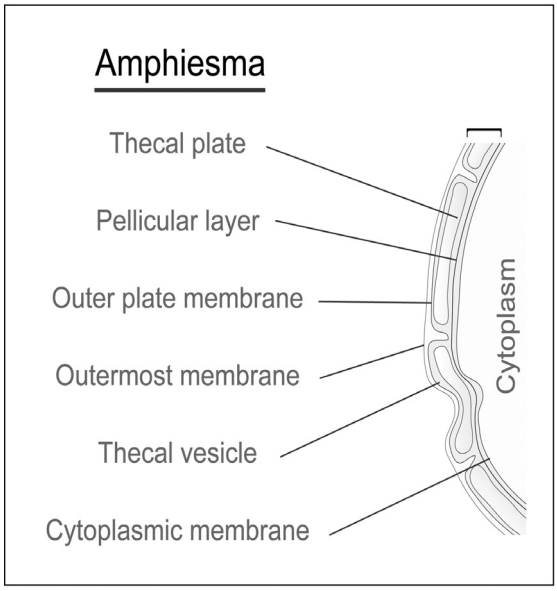

Figure 3. Dinoflagellate amphiesma depicting cortical layers (Kwok and Wong 2003). The outermost membrane is the plasma membrane.

dinoflagellate cell cortex. TEM studies performed by Dodge and Crawford (Dodge and Crawford 1970) the cortical vesicles may be filled with liquid, flocculent or granular material, a continuous sheet of dense material, or a thick rigid plate. Dense cellulosic plates observed within the cortical vesicles are the basis for armored dinoflagellates morphological plate tabulations (Fig. 3).

The structure, life styles, and life stages of dinoflagellates are very diverse. Dinoflagellate cell size can range from as small as 10µm to as large as 2.0mm. Dinoflagellate morphological taxonomy is based on the presence or absence of cellulosic plates, plate thickness, and plate outline. Morphology-based taxonomy is also based on very thin, transient, precursor thecal membranes thought to be precursors to formation of the thecal plates.

Dinoflagellate corticotypes

Dinoflagellates can be divided morphologically into five corticotypes based on the structure of the theca (Taylor 1980). The most common corticotypes are the

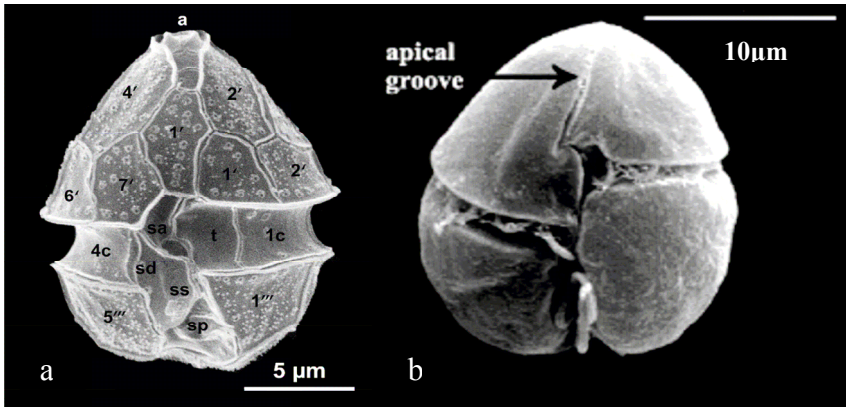

Figure 4. a)*Pentapharsodinium tyrrhenicum* is an example of an armored dinoflagellate containing cellulose within its thecal vesicles (Gottschling, Keupp et al. 2005). b) *Karenia brevis* is an example of an unarmored or naked dinoflagellate (Haywood, Steidinger et al. 2004).

gymnodinoids, peridinoids, gonyaulacoids, dinophysoids, and prorocentroids; however, a sixth corticotype, woloszynskoids, has been proposed (Netzel and Dürr 1984).

The prorocentroid corticotype (Fig. 5) is exemplified by dinoflagellates of the genus *Prorocentrum.* The prorocentroid amphiesma is made from two large plates, referred to as valves that usually possess trichocyst pores. Also a number of thecal plates

surround the flagellar pores (Fig. 6), periflagellar plates. The number of periflagellar plates range from eight to twelve, and is species-dependent.

The total plate count in dinophysoids is generally eighteen to nineteen plates (Balech 1980). The dinophysoid theca (Fig. 5) is very similar to that of prorocentroids, with regards to having two valves and numerous periflagellar plates. In addition to this dinophysoids have a four plate cingular girdle (Fig. 6) and a sulcal region (Fig. 6).

Gymnodinoids and woloszynskoids are generally referred to as the unarmored dinoflagellates (Fig. 4b). However, various gymodinoid and woloszynskoid species are known to possess very thin plate structures within their cortical vesicles (Dodge and Crawford 1969; Schnepf and Deichgräber 1972).

The gonyaulacoid and peridinioid corticotypes belong to the armored morphotypes (Fig. 4a and 5). The armored dinoflagellates possess five latitudinal plate series, apicals, precingulars, cingulars, postcingulars, and antapicals. Another non-latitudinal series, the sulcals, and an apical pore complex, or APC, is also present (Taylor 1987). Any additional plates are referred to as intercalary plates.

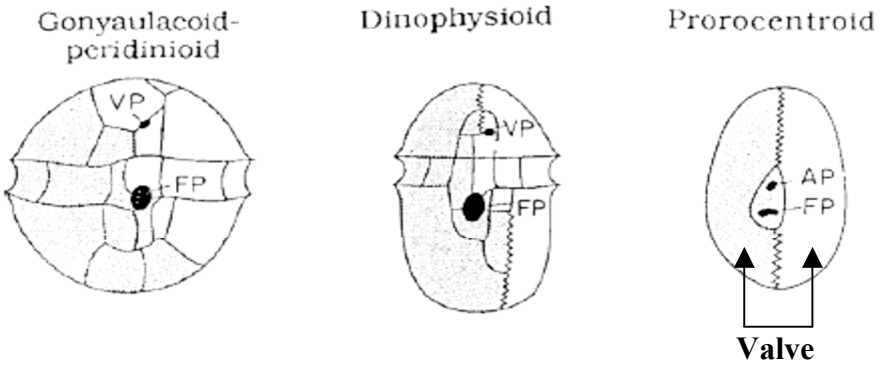

Figure 5. Dinoflagellate corticotypes (Lee, Hutner et al. 1985) FP, VP, and AP indicates the flagellar pore, ventral pore, and the accessory pore respectively.

Dinoflagellate morphological identification

Charles Kofoid developed a system of plate designation (Fig. 6) that is still used today for morphological identification (Kofoid 1907; Kofoid 1909). The gonyaulacoid and peridinioid cell is divided in reference to various landmarks, the sulcus, cingulum, apex, and antapex. The plates are designated based on their relative position to these landmarks (Kofoid 1907; Kofoid 1909). The plates are marked with a Kofoid label to

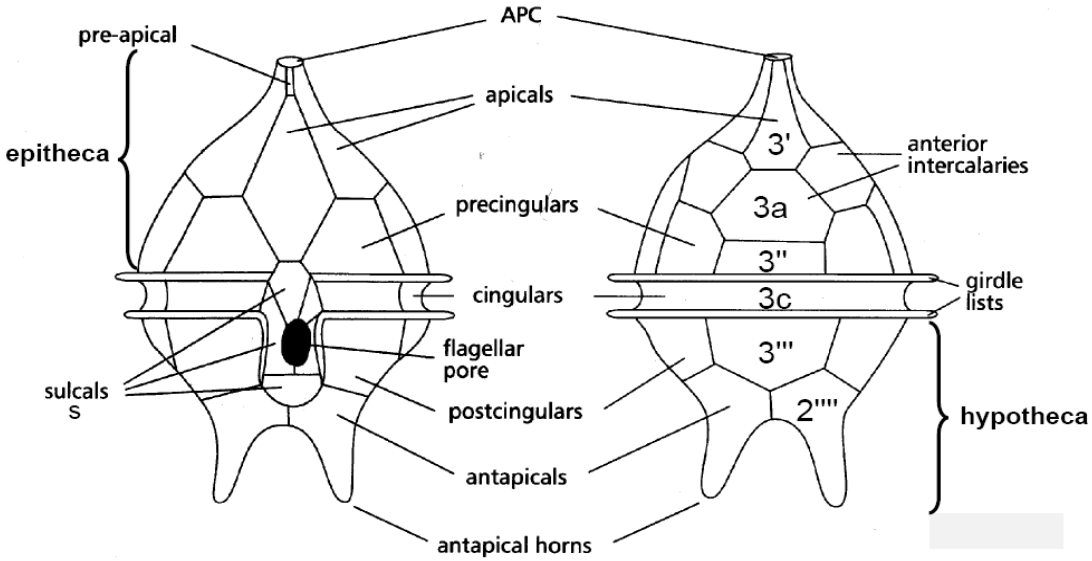

Figure 6. Peridinioid dinoflagellate diagram depicting Kofoidian plate designation and tabulation designations. The left figure depicts the ventral region and right depicts the dorsal region (O'Toole 2007).

designate their position. Armoured dinoflagellates are divided into three zones from the apical to the antapical end of the cell. The epitheca describes structures apical to the cingular girdle (or 'cingulum'). The cingulum is itself a zone, in which lies the transverse flagellum. Those regions of the cell posterior to the cingular girdle are referred to as the hypotheca.

The apical series that surround the APC are designated with a number starting with the ventral-most plate of that series being marked 1; the dorsal-most plate would be

marked 3, with one diacritical mark, e.g., 3' (Fig. 6). Thus, a number 3 precingular plate, which lies just above the cingulum, is designated by two diacritical marks: 3". Similarly, postcingular plates that lie just below the cingulum would be 3'".

Plates that make up the antapex region, termed antapical plates, bear four diacritical marks; e.g. 2"" (cf. Fig. 6). The intercalary plates that lie between both the apicals and the precingulars are designated "a;" thus 3a. Any intercalary plates that lie between the postcingulars and antapicals plates are designated "p" (not seen in Fig. 6). Cingulum plates are designated by "c". Sulcal plates are defined by their relative positions (left, right, anterior or posterior) and an additional "s".

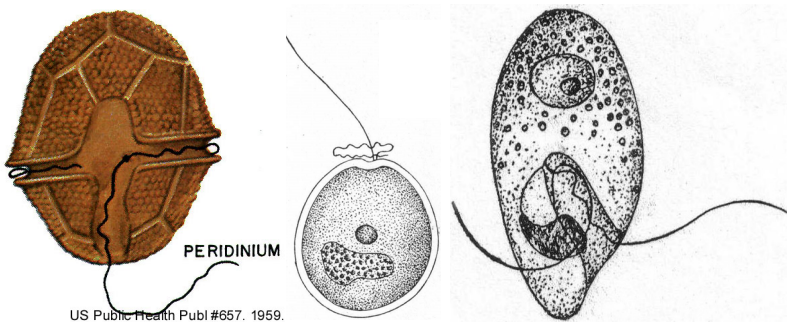

Figure 7. Dinflagellates representing the three types of flagellar arrangement a) *Peridinium* (dinokont) b) *Prorocentrum lima* (desmokont) (Calkins 2006) c) *Oxyrrhis marina* (opisthokont) (Calkins 2006)

Morphological identification is not based solely on plate tabulations. Although plate tabulations play an important defining characteristic in morphology-based identification, the flagellar arrangement of the mastigote, or swimming cell, is also used to help define dinoflagellate type. Three flagellar arrangements persist among dinoflagellates: dinokont, desmokont, and opisthokont (Fig. 7)(Taylor 1987). In the dinokont arrangement, the transverse flagellum has a ribbon-like appearance, and beats in such a way that it propels water at roughly 90 degrees to the orientation of the cingulum

(Leblond and Taylor 1976). The longitudinal flagellum beats posteriorly, propelling the cell forward. In contrast, desmokonts bear both flagella on the anterior end of the cell and are not associated with any grooves. The opisthokonts bear two flagella that arise from the cell posterior end.

Girdle displacement is also used in the morphological description of dinoflagellates. Rarely does the cingulum girdle meet up with itself in exact alignment (Fig. 6). The displacement is referred as either left-handed or descending and right-handed or ascending forming helices around the cell (Fig. 8). In some cases numerous turns of the cingulum encircle the cell (Taylor 1987).

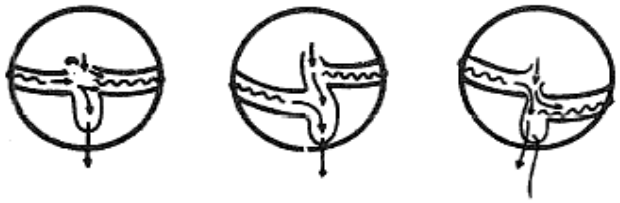

Figure 8. Types of cingular displacement (arrows indicate probable water flow) a) No displacement b) left-handed or descending c) right-handed or ascending (Leblond and Taylor 1976)

Infraphylum Dinoflagelleta is divided into two superclasses Syndinea and Dinokayota. The morphological separation that distinguishes these two superclasses is their nuclear structures. The Dinokaryota possess a dinokaryotic nucleus, a nucleus that contains permanently condensed chromosomes, while the Syndinea do not (Spector 1984a; Cachon and Cachon 1987). However the Dinokaryota do not possess histone associated DNA, (and so are lacking nucleosomes); but instead possess “histone-like” proteins (Spector 1984c). The Syndinea are differ from Dinokaryota by possessing histone proteins in their DNA (Lee, Hutner et al. 1985). A commonality this is shared

between these two groups is the presence of an unusual base, 5-hydroxymethyluracil, that is only found in dinoflagellate DNA (Spector 1984a).

Nutritional diversity

Dinoflagellates vary nutritionally from being strict autotrophs through mixotrophic to strictly heterotrophic organisms. Although strict autotrophs are very rare (Gaines and Elbrächter 1987), approximately half of known dinoflagellates possess some photosynthetic functionality. The overwhelming majority of photosynthetic dinoflagellates are mixotrophic, facultatively moving from autotrophic to heterotrophic depending on available resources. These photosynthetic species usually contain chlorophylls a, c_2, and rarely c_1, β-carotene. The preferred light-harvesting carotenoid used is peridinin, with a few exceptions using fucoxanthin, which is in turn usually derived from an endosymbiont.

Phagocytosis typically mediates the acquisition of other nutritive compounds such as vitamins or alternative carbon sources (Stoecker 1999). Phagotrophic ingestion can involve the consumption of an entire organism, or may involve piercing cells and removing cytoplasmic contents, a process known as myzocytosis (Schnepf and Deichgräber 1984). According to Jeong (Jeong 1999), phagotrophy is performed by either engulfment of a prey organism, break down and uptake by pallium formation (food web), or peduncle-mediated uptake of host materials. Engulfment usually involves ingestion at the flagellar grooves or the posterior end of the cell where the entire prey is consumed (Gaines and Elbrächter 1987). The use of a pallium or food web was first described by (Allman 1855) but it was not until (Odum 1971) that the term saprotrophy was used to describe the feeding mechanism. The dinoflagellate extrudes a delicate

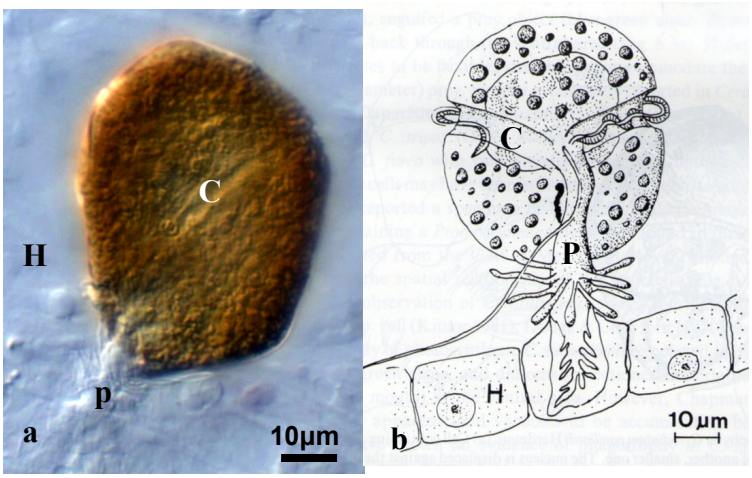

Figure 9. Ectodermal attachment of parasitic trophonts. a) Light micrograph of dinoflagellate attached to ctenophore host b) Ectoparasitic *Protoodinium chattonii* Hovasse dinoflagellate attachment via peduncle (Cachon and Cachon 1971a). H indicates the host tissue; p indicates the peduncle; and c indicates the cingulum.

cytoplasmic net from its thecal pores that digests and absorbs an ensnared prey. After the feeding event the net is retracting back into the cell allowing the cell to search for another food source. The last phagotrophic mechanism is through the use of a specialized organ referred to as a peduncle. The peduncle itself contains longitudinally-arranged microtubules, extensions of the internal microtubular basket (Lee 1977). In some dinoflagellates the peduncle (Fig. 9) is seen as cytoplasmic extension of the protoplasm originating within the epitheca and emerging from the cingular-sulcal interface near the flagellar pores (Spector 1984b). Parasitic dinoflagellates use peduncles to attach to the host, and as a means to collect nutrients from the host.

Dinoflagellate symbiosis

The nutritional requirements of dinoflagellates are elusive. While some are strict autotrophs, others are heterotrophic; still others are mixotrophic, i.e., capable of both heterotrophy and autotrophy. Many dinoflagellates form symbiotic relationships with

other organisms to achieve their nutritional goals. One example of a well-known genus that contains mutualist is *Symbiodinium.* A group of well-known, closely studied endosymbiotic dinoflagellate that actually represents a very wide range of phylogenetic variation (much more than represents a typical species) (Rowan and Powers 1992), *Symbiodinium* forms a symbiotic relationship with various corals and other marine organisms. The dinoflagellate is supported by obtaining nitrogen, phosphorus, carbon and other coral metabolites while providing fixed carbon, organic acids, and other various metabolites back to the host (Fitt, Rees et al. 1995; Hackett, Anderson et al. 2004). Some dinoflagellates themselves contain an endosymbiont and even plastids that are not dinoflagellate in origin. Such plastids are referred to as kleptochloroplasts (Sweeney 1971; Larsen 1988; Fields and Rhodes 1991); that can be photosynthetically active (Skovgaard 1998).

Parasitism is a common form of symbiosis for many dinoflagellates. It has even been shown in a laboratory environment that they can shift from mutualist to parasite (Sachs and Wilcox 2006) in the case of horizontal transmission of symbiont to host. It is generally accepted that parasitic relationships evolve from the need for specific, limiting metabolites required for survival of the parasite. Usually this concerns organic substrates not available by other means other than the physical removal from a host organism. Most parasitic relationships have very exacting requirements, and involve interactions that display a very limited range of species interactions. However, this is not always the norm with dinoflagellates. An example of multi-host parasitism is seen in *Amoebophrya ceratii*, a dinoflagellate that can parasitize a multitude of dinophyte species (Drebes 1984). Another example of a dinoflagellate having multiple host is an *Oodinium* sp.

known to parasitize a variety of ctenophores and a hydromedusa (Mills and McLean 1991). However, there are species-specific relationships as in the dinoflagellate *Myxodinium pipiens* and its host-parasite symbiosis with only *Halosphaera.*

Parasitic dinoflagellates

There are more than 2,000 formally described dinoflagellate species, of which approximately 140 are known to be parasitic (Drebes 1984). Dinoflagellates may parasitize organisms extracellularly and/or intracellularly. According to Jean Cachon, parasitic dinoflagellates were categorized into the polyphyletic groups Blastodinida and Duboscquodinida (Cachon 1964). The groups were formed on the basis of morphology, nuclear development, and their relationships with the host. Then Loeblich established two additional Orders of parasitic dinoflagellates based on biochemical data, the Syndiniales and another Order that encompassed members of the genus *Chytriodinium* and its relative (Loeblich 1982). Today numerous members belonging to the Class Blastodiniphyceae and the Syndiniophyceae, which now contains the Duboscquodinida, have been rearranged based on recent molecular data and moved into the Class Dinophyceae, in order to provide phylogenetic relevance to these groups (Coats 1999; Levy, Litaker et al. 2007; Gómez, Moreira et al. 2009; Coats, Kim et al. 2010).

The Blastodiniphyceae and some Dinophyceae are known ectoparasites found on or in other protists, or metazoans. The Blastodiniphyceae have a direct physical attachment to the host by a posterior stalk and display a slow morphological change from a free-living form into a parasitic form (Cachon and Cachon 1987). The ectoparasitic dinoflagellate may contain chlorophyll, as seen in *Protodinium, Piscinoodinium, and Crepidoodinium*, or may entirely lack photosynthesis at any stage of life. This strictly

heterotrophic condition is exemplified by *Myxodinium, Cachonella,* and *Amyloodinium* species (Coats 1999). If chloroplasts are present they are usually intensely modified and their pigmentation can disappear and reappear depending on stages of autotrophy or complete heterotrophy (Cachon and Cachon 1987).

Ultrastructure of the ectoparasitic dinoflagellate peduncle (Cachon and Cachon 1971a; Cachon and Cachon 1971b) shows that it can remain attached to the host surface or penetrate into the host cell in either event forming a network of rhizoids thought to function for uptake of host material. Cachon and Cachon observed the stalk of *Protoodinium* deeply embedded into its host cytoplasm and believed that it acted as a cytopharynx, a structure acting as a gullet to pass food material from the cytostome to the cell interior (Cachon and Cachon 1971a). The peduncle of *Amyloodinium* has been observed by (Lom and Lawler 1973) to transport small vesicles and organelles from the perinuclear cytoplasm into the host, which was interpreted by Lom as lytic substances used to digest host cellular material. In some dinoflagellates a stylet acts as a secondary structure that works in conjunction with the stalk. The stylet can provide support or aid with the removal of host material as noted in the (Lom and Lawler 1973) study on *Amyloodinium* or *Haplozoon* (Siebert Jr 1973). An unusual example of attachment is seen in *Chytriodinium* (Cachon and Cachon 1968) where instead of a peduncle the dinoflagellate uses its hyposome, ventral body, as a spear to penetrate through a crustacean egg, its host; and then later develops a set of organelles to hold itself in place once it has reached the host cytoplasm.

Ectoparasitic dinoflagellates are known to parasitize a variety of gelatinous metazoans. *Protoodinium hovassie* and *Cachonella paradoxa* are known parasites of

siphonophores, while *Protoodinium chattoni* is a known parasite of hydromedusae (Cachon and Cachon 1987). A species of *Oodinium* has been reported to parasitize several gelatinous animals in the Pacific Northwest, including arrow worms, ctenophores and hydromedusae (Mills and McLean 1991).

The Duboscquodinida are intracellular and even intranuclear parasites of protists (Cachon 1964; Drebes 1984), with the exception of *Sphaeripara,* a known metazoan parasite (Chatton 1920; Coats 1999). In general, Duboscquodinida lack theca, chloroplasts, and even mitochondria suggesting that they are indeed obligate intracellular parasites. Gaines and Elbrächter (Gaines and Elbrächter 1987) have stated that parasitic dinoflagellates have ". . . morphologically different feeding and reproductive stages and . . . produce . . . numerous progeny after only one feeding act." The parasitic criterion is very evident in the Duboscquodinida. Between their free-living reproductive phase, sporont stage, and their intracellular parasitic phase, trophont stage, every living stage of this group is specialized for the optimization of parasitism. The *Amoebophrya cerati* sporont is a free swimming biflagellate, pear-shaped cell possessing a helical girdle (Fritz and Nass 1992). *Amoebophrya* experiences an extreme morphological change during the trophont stage (Fig. 10). After infecting its host the cell increases in size, allowing the girdle to elongate and make additional rotations around the cell. The episome, the apical portion of the cell, sinks into the hyposome, the antapical portion of the cell. Concurrently, the hyposome is enlarged to fold up and over the episome, forming a cavity referred to as a mastigocoel (Cachon 1964). The trophont begins to undergo a growth and division phase during which proliferation of numerous nuclei and flagella are evident.

Finally the sporonts exit the host in a tightly coiled multinucleated structured referred to as a vermiform.

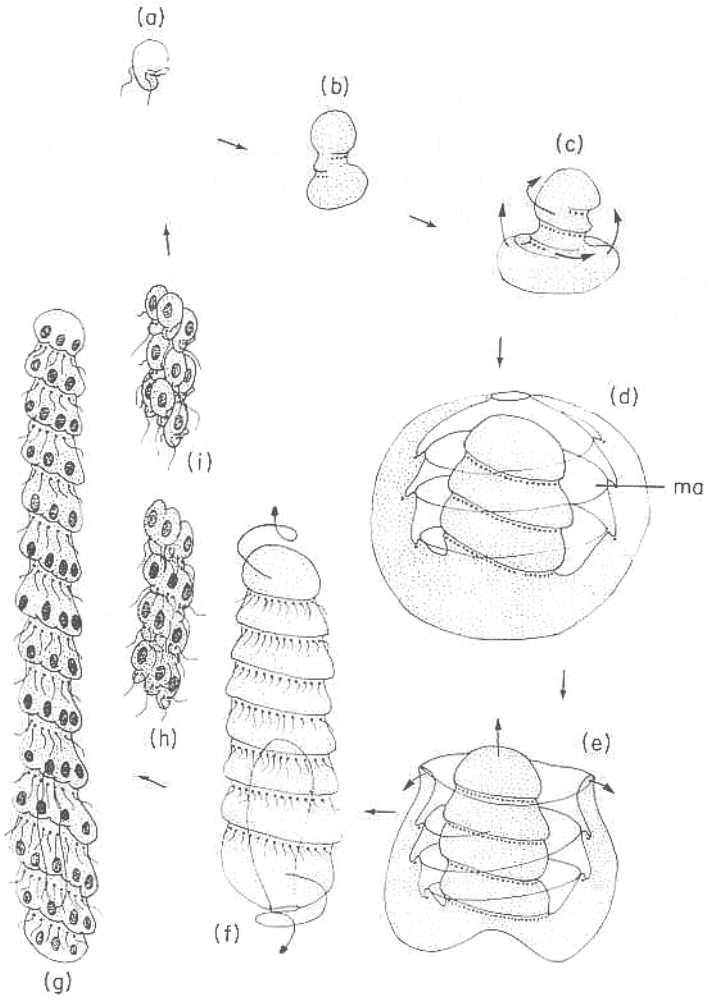

Figure 10. Diagram of the life cycle of *Amoebophrya*. a) dinospore; b,c,d) invagination of the growing intracellular trophont (Ma = mastigocoel); e,f) evagination of the trophont phagocytosis of the host and formation of a vermiform; g) lengthening of the vermiform; i,h) formation of the swarmers (Cachon and Cachon 1987).

Members of the Order Syndinida are an intranuclear group that parasitize protists and a wide variety of metazoa. Syndinida are dinoflagellates responsible for the decline of many invertebrate (Shields 1994; Appleton and Vickerman 1998; Stentiford and Shields 2005) and vertebrate (Gestal, Novoa et al. 2006) commercial fisheries. Upon the

onset of infection into the host hemal sinuses, vascular sinuses lacking a distinct lining and organs during the trophont stage, these parasites convert into a plasmodial form (Shields 1994). The plasmodia are then free to grow and produce a thin polysaccharidic cell coat until sporogenesis of micro and macrospores occur. The ingestion of host material is by performed by sapotrophy (Cachon 1964).

Parasitic Groups	Characteristics
Blastodiniphyceae	Ectoparasites of protists, metazoans, and algae. Exhibits a gradual modification of morphology from free-living to parasite.
Dinophyceae	Endo and ectoparasites of protists and metazoans. Exhibit a broad range of variation. Placement is based on molecular relevance. Possess permanently condensed chromosomes without histones (Spector 1984c).
Syndinida	Endoparasites of protists and metazoans. Parasites are colorless and from a thin polysaccharide cell coat. May or may not have a theca or cell wall. Chromosomes possess histones (Lee, Hutner et al. 1985)

Table 1: Zoological Nomenclature and characteristics of parasitic dinoflagellate groups

Parasitic dinoflagellate reproduction

The reproduction of parasitic dinoflagellates is based upon three mechanisms presented by (Cachon and Cachon 1987). In Syndinida, cells begin to divide within the plasmodial form. After completion, flagellated spores are produced and released. Another mode of reproduction, termed palintomy, occurs after the conclusion of a feeding event. The dinoflagellate, after reaching a substantial increase in size, will begin nuclear and cytoplasmic divisions producing sporonts, termed swarmers. Swarmers are produced when the dinoflagellate becomes multinucleated during a feeding event and can undergo multiple cell divisions either during the feeding event or after. The mechanism, termed

iterative sporogenesis or palisporogenesis allows for a single trophont to produce numerous generations of spores (Cachon and Cachon 1987). The production of numerous generations is accomplished when the divisions of new cells occur simultaneously with the parasitic feeding event. The new cells can grow in size and then further divide finally being released in a vermiform (Fig. 10).

A parasitic symbiont specifically associated with the ctenophore *Mnemiopsis*

Within the dinoflagellate genus *Pentapharsodinium* there have been no reported cases of parasitism. *P. tyrrhenicum* has been described by (Montresor, Zingone et al. 1993) as a marine benthic autotroph. *P. trachodium* and *P. dalei*, have not been found associated with a host and are always found in benthic samples, although neither have been formally described as either autotrophic of heterotrophic. In this study I describe a specific parasitic relationship between a dinoflagellate and its host *Mnemiopsis leidyi.* I provide a morphological and molecular identification and describe the life cycle of this parasitic dinoflagellate.

Chapter 2: Collection and Culture of a Dinoflagellate Parasitic on the Ctenophore *Mnemiopsis leidyi*.

Introduction

The ability to collect host and parasite has played a very important and limiting role within this study. Availability of the host, *Mnemiopsis,* was critically dependent on several factors, including weather and tide condition. Collection of the host was most successful during high tides.

Collection sites known for pristine water quality, such as Apalachicola Bay, produced few dinoflagellates associated with *Mnemiopsis*. However, sites that appeared hypereutrophic, for example Englehard, NC, Mobile Bay, AL, and Davidson Bay, Florida, provided *Mnemiopsis* with dense surface loads of the dinoflagellate. Greater numbers of parasitic loads were observed during the spring and summer months. *Mnemiopsis* and several other ctenophore species collected at multiple sites ranging from Port Aransas and Galveston Bay, Texas; Pascagoula, Mississippi; Mobile Bay at Dauphin Island, Alabama; Pensacola Bay, St. Andrews Bay, Apalachicola Bay, Dickerson Bay and St. Marks Bay, Florida. *Beroë ovata* were collected in the northern Gulf from Mississippi, and the Chesapeake and Delaware Bays, *Beroë cucumis* were collected from Pamlico Sound, North Carolina and Woods Hole and Sandwich Harbors, Massachusetts. *Pleurobrachia pileus* and *Euplokamis dunlapae* were collected from Cape Cod Bay at Sandwich Harbor and Cape Anne, Massachusetts. Close inspection of all these ctenophores never revealed any dinoflagellates.

Objectives and Rationale

Objective 1: Establish a viable cell culture of the parasitic dinoflagellate

The establishment of cell cultures allows for the independent study of the parasite without the host. Secondarily, collections of freshly caught ctenophores to obtain data on the parasite would not be required. The cultured dinoflagellate could then be used for DNA extraction without interference of host tissue and would also allow for a morphological description of the free-swimming cell, mastigote.

Objective 2: Koch's Postulate

The establishment of the cultures also allows for the ability to complete Koch's Postulates. Specifically, to fulfill Koch's postulates it would be necessary to: 1) find the microorganism in abundance in all organisms suffering from the disease 2) isolate the microorganism 3) reinfect the microorganism back into the host and show the same effect 4) reisolate the microbe and identify it as the original (Koch 1891).

Materials and Methods

Host collection

M. leidyi were collected from locations along the Eastern US and Gulf Coast from shallow water, by wading at near-shore locations, and by boat from estuarine locations and open water sites. At shallow shore sites, ctenophores were collected whenever possible by surface dipping; when obtained *via* shipboard from the R/V Cape Henlopen or R/V Hugh Sharp (as guest of Dr. Eric Wommack and Dr. Wayne Coats during the MOVE 2007&2008 trips), ctenophores were often collected captured by a very slowly towed 325 μm mesh plankton net. Approximately 30 animals from each site were thoroughly examined for the presence of microorganisms with the frequency of 1-3

collections per month from 2005-2008. Ctenophores were held in 1-2 L plastic jars until arrival at the laboratory. The holding times ranged from a few minutes at the Marine Biological Laboratory and NERRS/Apalachicola, typically five to eight hours from the Northern Gulf coast locations, and as long as 48 hours from Eastern coastal (Rhode Island to South Carolina, or South Texas) locations. Animals were also obtained from Gulf Specimen Supply, captured from Dickerson Bay in Panacea, Florida, and the Marine Biological Laboratory. Upon arrival in the laboratory, the animals were observed at 20x–110x magnification by a dissection microscope to assess the protist assemblage. Shipboard-collected ctenophores were observed on site within 1-2 hours upon collection.

Site	GPS	Site	GPS
Bayview Ferry, NC		Station 744	N 37°43.98 W 76°10.94
Engelhard, NC	N 35°30.482 W 075°59.453	Station 804	N 38°04.39 W 76°12.76
Nags Head/Oregon Inlet		JS22(Del Bay)	N 39°12.74 W 75°17.06
Station 908	N 39°07.52 W 70°20.21	JS28(Del Bay)	N 38°48.97 W 74°58.13
Station 858	N 38°57.97 W 76°23.05	Lewes Harbor	N 38°47.119 W 75°09.405
Station 845	N 38°45.00 W 76°26.00	DISL	N 30°15.056 W 88°04.806
Station 834	N 38°34.62 W 76°26.23	DISL	N 30°15.158 W 88°04.719
Station 818	N 38°18.01 W 76°16.37	Apalachicola Bay	
Station 758	N 37°58.29 W 76°12.61	Galveston Bay	N 29°17’20.45” W94°52’28.06”
Off-shore site	N 36°22.97 W 74°26.10	FSU Marine Lab	N 29°54’50.74” W84°30’41.19”
Station 707	N 37°06.97 W 76°06.93	St. George Island, FL	
Station 724	N 37°23.94 W 76°04.75	Dickerson Bay Panacea, FL	

Table 2: Host collection sites and GPS coordinates. During each collection a minimum of thirty animals were collected.

Dinoflagellate collection

Ctenophores were surveyed for the presence of dinoflagellates after each collection. The density of dinoflagellates on *Mnemiopsis* varied. Dinoflagellates were removed only from hosts with cell densities ranging from 100 mm^{-2} to 150 mm^{-2} (Fig. 11). Dinoflagellates were collected from the ctenophore by two methods. Initially, we collected dinoflagellates by braking pipette, or by using a syringe-driven Gilson pipettor. Cells were collected directly by plucking from the surface or by penetration of the mesoglea and targeting specific cysts. This method, while very precise, proved impractical for repeatedly collecting a sufficient number of cells for molecular analysis. More importantly, cells collected in this manner were not viable for cultivation.

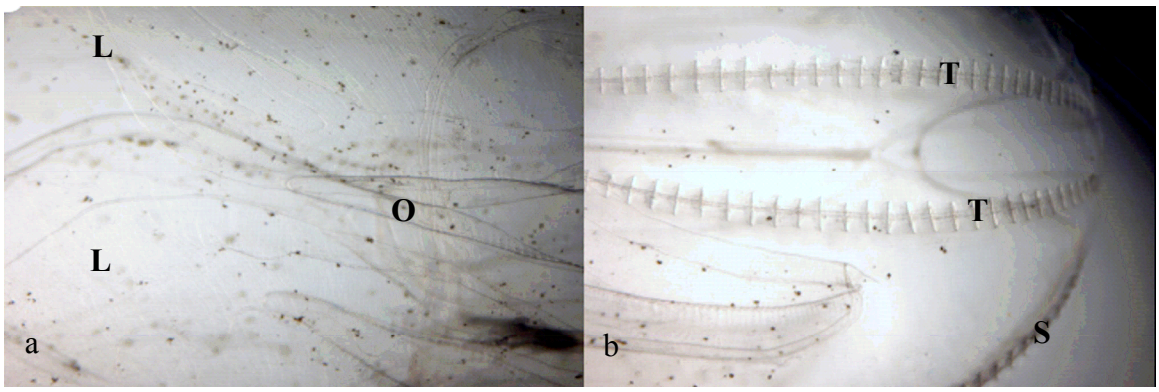

Figure 11. Light Micrograph of *Mnemiopsis* with high dinoflagellate surface density. a) (O) Oral and (L) lobe region. b) (T) Subtentacular comb rows and partial (S) subsagittal comb rows.

Presumably this method of collection damaged the cell and therefore impacted establishment of viable cultures.

Subsequently, I developed a more efficient method that enabled us to collect larger numbers of cells. Heavily infested tissues were removed by dissection and incubated at room temperature (approximately 23°C) in 0.1 % (w/v) RNAse and DNAse free protease (type XIV protease, *Streptomyces griseus*, Cat. No. 81748 Sigma Chemical

Co., St. Louis, MO, USA) dissolved in 0.45 μm filtered ambient seawater. After 1-4 hours the mesoglea collapsed, releasing encysted cells, which were collected by pipetting with a Pasteur pipette. Heavy infestations of ectodermally-attached dinoflagellates were pipetted directly from the remaining fragments of ctenophore ectoderm and remnant mesoglea. In all cases, cells to be analyzed by molecular techniques were washed in several dishes of sterile filtered artificial seawater then permeabilized, stabilized and fixed by being placed into acetone immediately upon collection.

Algal cultures

Two methods were used in the collection of algal cells for cell cultures. 1) Attached parasitic cells were removed from the host surface with a braking pipette. Cells were placed into several washes of site 0.45 μm – syringe filtered sterile seawater collected from the site of capture. 2) Alternatively ctenophores were placed in Petri dishes containing 0.45 μm filtered sterile seawater supplemented with full strength K medium (Keller, Selvin et al. 1987) minus silicate, to reduce the likelihood of diatom contamination. Ctenophores (and/or ctenophore tissue fragments) were incubated at room temperature overnight.

All algal cells were placed in 96 well culture plates containing fresh K medium. Cells were grown to a high cell density (Fig. 12) at 29 ± 1°C in sterile Corning 75 cm^2 canted neck flasks (cat. no. 430720, Corning, NY) in K medium (Keller, Selvin et al. 1987), in 30 ppt sterile filtered seawater base collected from St. Andrew's Bay, Florida. Cultures were illuminated at an irradiance level of 80 μmol photons$\cdot m^{-2} \cdot s^{-1}$on a 12:12 L:D cycle. Cell counts were performed using a hemacytometer (cat. no. 0267110, Fisher Scientific, Pittsburgh, PA).

The ctenophore parasite was compared with a known strain by culturing. *P. tyrrhenicum* strain SZN13, a known benthic autotroph, was provided by Monika Kirsch, Bremen University FRG. SZN13 was originally collected from the Bay of Naples, Italy (Montresor, Zingone et al. 1993).

Host reinfection

Ctenophores known to be free of dinoflagellates, were collected from the National Marine Fisheries jetty by surface dipping in Woods Hole, MA, or from Apalachicola Bay, Florida. After the arrival of *Mnemiopsis* in the lab, ctenophores were observed each day over a week for the presence of dinoflagellates; close inspection indicated that animals were entirely free of parasitic dinoflagellates.

Immediately before infestation, each ctenophore was given a final inspection. Two ctenophores were placed in dinoflagellate-free two liter holding tanks, in each of three groups; A, B or C. Group A (control) contained ctenophores not inoculated with cultured dinoflagellates. Group B consisted of ctenophores inoculated with a single culture of parasitic dinoflagellates isolated from Engelhard, North Carolina. Approximately 35 mL of culture containing approximately 9000 cells mL^{-1} were introduced into the holding tank and allowed to incubate at room temperature (~23 °C) for forty-eight hours. Group C consisted of ctenophores inoculated with a culture of *P. tyrrhenicum,* strain SZN13 at the same density as in Group B. Ctenophores were observed under a dissecting microscope (model SZ11, Olympus Corp., Center Valley, PA, USA) equipped with an oblique illumination base (model TLB3000, Diagnostic Instruments).

Results

Dinoflagellate cultures

Algal cultures were established and used in the morphological analysis, reinfection experiment, and molecular analysis of the dinoflagellate. When attempting to establish cultures fewer than 50% produced viable cell lines. Single cells grew well when initially inoculated into volumes of less than 100 µL; larger initial culture volumes did not produce viable cell lines. Healthy viable cells doubled over a 12 – 24 hour period. When cysts were observed, culture volume was doubled each week until they reached a total volume of 50 – 75 mL. Cell cultures plateaued at two months (Fig. 12) at approximately 9,000 – 10,000 cells per mL and then began to decline in numbers.

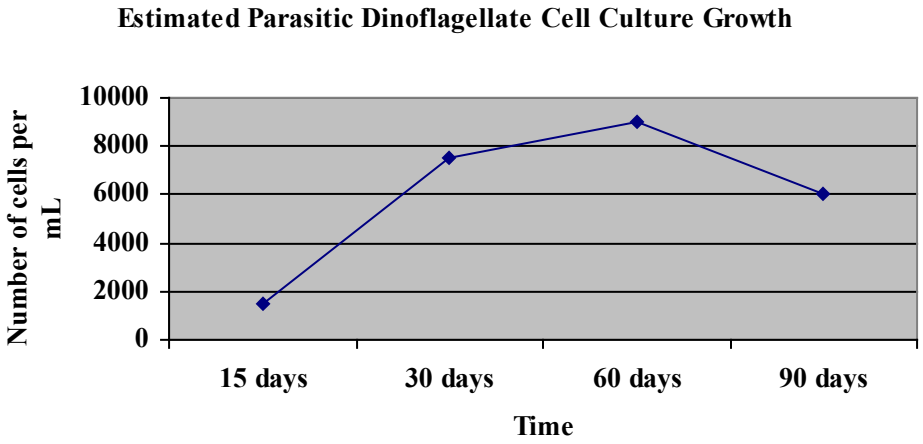

Figure 12. The chart represents the estimated growth of isolated parasitic dinoflagellates in culture. Days 15 – 30 are based on visual inspection estimating by orders of magnitude. Days 60 – 90 are based on accurate cell counts using a hemacytometer on established cultures.

Reinfection experiment

The first of Koch's postulates states that the microorganism must be found in abundance when a disease symptom is observed. Ctenophores collected in the field with dinoflagellates resulted in mortality in a time span of less then 12 hrs. to one week. Ctenophores collected that did not harbor dinoflagellates could be keep in holding tanks for several months. The reinfection of the microorganism back to the host after isolation is another criteria to complete Koch's postulates. Reinfection back to the host utilizing

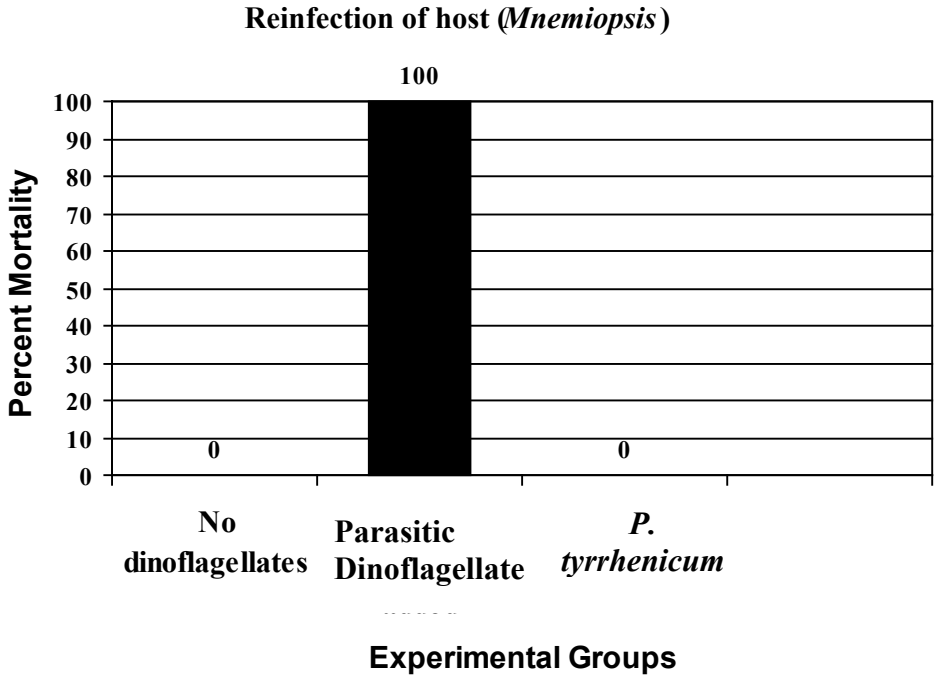

Figure 13. Chart representing mortality of host *Mnemiopsis* in response to reinfection with dinoflagellates. Due to a low number of replicates statistical analysis could not be applied.

the isolated dinoflagellate yielded approximately 20 – 30 trophont dinoflagellate cells attached to *Mnemiopsis.* The attached cells exhibited the typical change in morphological features and growth observed from trophonts on ctenophores captured in the field. However, attachment location was random and did not exhibit the same anatomical

preference as seen in low infestation rates of field captured ctenophores. Reinfestation of the host by the parasitic dinoflagellate caused mortality after two days (Fig. 13). Mortality was assessed by the complete disintegration of the ctenophore. Ctenophores that were dying would settle to the bottom of the tank, stop swimming, and begin to deteriorate. Attempts to infect *Mnemiopsis* with *P. tyrrhenicum* did not produce any attached dinoflagellates, (Fig 13) and were still alive after 48 hrs. Ctenophores that were not infected with either *P. tyrrhenicum* or the cultured isolate did not display any mortality within the 48 hrs. time frame.

The final component to Koch's postulates is to reisolate the microorganism and establish its identity as the original isolate. However, reisolation was not possible due to the host completely degrading and attempts to locate the dinoflagellate was nonproductive. Also, the low number of trials and sample size, N = 4 per experimental group, does not provide enough data to perform an analysis showing a statistically significant outcome.

Discussion and Conclusions

The collection of ctenophores from various sites was highly dependent upon weather conditions and tidal ranges. Collection trips were planned according to times that high yields were expected. Generally collections of *Mnemiopsis* yielded over 50 per site but a minimum of 30 was collected to provide a statistically viable collection size.

Dinoflagellate collection from hosts using direct physical removal yielded a lower efficiency in establishing viable cultures. It is likely that the forcible removal of the parasite caused damage to the cell leading to a high percentage of mortality. Dinoflagellates that were collected after self-detachment yielded more success in culture

establishment. The growth of cultures depended greatly on the source of the seawater used for culturing. Seawater that was collected from sites that produced dinoflagellates yielded the best results for achieving a high cell density (Fig. 12). Seawater used from sites that did not yield dinoflagellates or artificial seawater yielded no growth even when using K culture medium. Due to the low rate of growth produced by water other then water collected at sites of high dinoflagellate infestations only water collected from these sites were used in culturing.

The high degree of mortality and similarity of symptoms observed after infection of *Mnemiopsis* with a pure culture of the isolated parasitic dinoflagellate fulfills Koch's postulates to identify the dinoflagellate for as the causative agent of *Mnemiopsis* mortality. However, even though 100% *Mnemiopsis* mortality was observed, the number of cells that parasitized the ctenophores was low, less than 1% of the dinoflagellate cells introduced. I speculate that the transfer of the dinoflagellates into an artificial seawater medium caused mortality to the cells and or forced many cells to encyst. It is likely that the few cells that were able to reinfect the host were able to quickly locate the host and adapt to a parasitic form before a drop in viability or encystment. The inability for *P. tyrrhenicum* to form a symbiotic association with *Mnemiopsis* suggests that the cultured isolate has specific adaptations permitting establishment of a parasitic relationship with *Mnemiopsis*. Although, this experiment was assessed using a low number of trials and sample size the trend of high mortality over a short period of time relative to infected individuals verses noninfected individuals has been observed in all ctenophores captured.

The quick rate of mortality observed in the newly infected ctenophores could have been a compounded effect induced by stress. The typical time before mortality observed

from field infected ctenophores with low densities of the parasitic dinoflagellate is within seven days but generally greater than two. However, healthy ctenophores that have been placed in small tanks of artificial seawater will only last one to four weeks while healthy ctenophores held in large tanks > 5 L have been maintained for times exceeding two months. The other ctenophores involved in the experiments all died shortly after a weeks time period. It is thus my conclusion that the rate of mortality observed in the time frame of 48 hrs. was produced by a cumulative affect of parasite interaction and stress.

The parasitic cells that infected the ctenophores did not display the usual attachment pattern seen in low densities from field captured ctenophores. Infestations that involve a low density of surface attached dinoflagellates < 100 mm^2 are typically seen on the lobes and oral region (Fig. 11). A behavioral study of *Mnemiopsis* (unpublished data) shows the host frequently samples the flocculent benthic layer where it is thought the host first comes into contact with the dinoflagellate, triggering a stimulatory response leading to attachment of the parasitic trophont. However, the introduction of the isolate into an artificial environment prevents this from occurring. It is there for my conclusion that the attachment location of the experimental group is not a valid indicator of typical parasite-host attachment.

Chapter 3: Morphology of a *Pentapharsodinium* species parasitic on the ctenophore *Mnemiopsis leidyi.*

Introduction

Morphological characterization has been one of the most important classical methods of taxonomic classification of armored dinoflagellate species. All armored dinoflagellate species may be identified based on the cell cortex plate structure. The Kofoid system, developed by Charles Kofoid (Kofoid 1907; Kofoid 1909) provides the phycologist with a consistent means to morphologically distinguish among a multitude of dinoflagellate genera.

Over the years, several improvements have been developed, some resulting in entirely new classification systems (Taylor 1980; Evitt 1985). Barrows and Balech (Barrows 1918; Balech 1980) have noted polarity variation between the epitheca and hypotheca within armored dinoflagellates. They note that variations within the epitheca tend to be conserved and appear to be caused by intrinsic factors. In contrast, variations in the hypotheca appear to be caused by environmental factors.

In this study I conducted a thorough Kofodian plate tabulation based on cultured and host-associated cells. I generated a tentative phylogenetic placement based on this tabulation. During the study I also attempted to characterize the mode of cell adherence to the host and establish the nature of the symbiotic relationship between the ctenophore and the dinoflagellate.

Objectives and Rationale

Objective 1: Obtain an accurate plate tabulation based on the Kofodian system

The plate tabulation will allow for genus level placement of the dinoflagellate and reinforce phylogenetic placement based on molecular data.

Hypothesis 1: The dinoflagellate should be classified as a species of *Pentapharsodinium*.

Objective 2: Establish the type of symbiotic relationship the symbiont has with *Mnemiopsis*

The establishment of a host-symbiont relationship will allow for the study into the life cycle and behavior of the dinoflagellate.

Hypothesis 2: The dinoflagellate is a parasite strictly associated with the ctenophore *Mnemiopsi leidyi.*

Materials and Methods

Microscopy

Dissections were performed while observing the ctenophore or ctenophore tissue fragments with a dissecting microscope (model SZ11, Olympus Corp., Center Valley, PA, USA) equipped with an oblique illumination base (model TLB3000, Diagnostic Instruments) to provide improved contrast. Dinoflagellates were also examined with a compound microscope by differential interference, phase contrast and fluorescence (model BHS, Olympus Corporation, Tokyo, Japan). Images were collected with color (model Micropublisher 3.3, QImaging Corp., Vancouver, BC, Canada) or monochrome digital CCD cameras (model QICam, Qimaging Corp.). Image optimization and analysis was performed by using Image Pro Plus (Media Cybernetics) or Image J image analysis software. ThumbsPlus software was used for image archiving as well as post capture

digital image adjustment where necessary (Cerious Software, Charlotte, NC, USA). Image markup was performed with Macromedia Freehand or Photoshop (Adobe Systems Int'l. San Jose, CA, USA). Images of living cells were recorded to videotape with a monochrome Newvicon tube camera (model VE1000, Dage/MTI Corporation, Roeske City, MI, USA) or Sony HyperHAD CCD composite video camera (Sony Corporation, San Jose, CA, USA), and background-subtracted and digitally enhanced with a real-time image processor (model Argus 10, Hamamatsu, Japan). Images generated by the image processor were saved to S-VHS tape (model SE-180BQ Hitachi Maxell, Ltd. Osaka, Japan), digital 8 tape (digital HandyCam, model 240, Sony Corporation, San Jose, CA, USA), or, in the case of still shots, to an image capture card (Flashbus Spectrim, Integral Technologies, Inc., Indianapolis, IN, USA).

Fluorescence microscopy

Calcofluor White staining was used to reveal thecal plate boundaries according to the method of Fritz and Triemer (Fritz and Triemer 1985), with minor modifications to account for local salinity. Dinoflagellates were initially fixed in 2 % glutaraldehyde buffered with 0.1 M sodium phosphate made from 0.2 μm sterile filtered seawater collected at the dinoflagellate collection site and post-fixed in buffered 1 % osmium tetroxide. Calcofluor White M2R (cat. no. F3543, Sigma Chemical Corp) was added to a final concentration of 10-20 μg/mL and the cells viewed under UV fluorescence using a Hoechst Ploem cube (model 11000, Chroma Tech, Brattleboro, VT, USA; Olympus BHS microscope, Tokyo, Japan). The fluorescence images provided the basis for a Kofoidian plate tabulation, based on the number of thecal plates, their morphology and their relative arrangement.

Scanning Electron Microscopy and Transmission Electron Microscopy

On several samples a membrane stripping technique was utilized in order to visualize the thecal plates. Cells were placed into a 0.1% Triton X solution mixed in site 0.2 µm filtered site-collected seawater and allowed to incubate at room temperature for ten minutes. Fixation for electron microscopy was carried out using a 'simultaneous fixation' method (Tamm and Tamm 1981). All reagents were cooled to 0°C on ice for a minimum of 30 minutes prior to use; the sample was cooled on ice for a few minutes prior to the initial fixation. The primary fixation consisted of 1% paraformaldehyde, 2.5% glutaraldehyde, 1% osmium tetroxide buffered in 80mM sodium cacodylate and 0.2 µm filtered site water. Samples were fixed on ice for a minimum of 30 minutes to a maximum of one hour. Samples were subsequently washed 3X using ice-cold 80mM sodium cacodylate in 0.2 µm filtered site water and then allowed to incubate for 5-10 minutes. Post fixation osmication was carried out at 0°C using 1% osmium tetroxide in 0.2 µm filtered site water. Samples were incubated for a minimum of 15 minutes to a maximum of 30 minutes in OsO_4 seawater. Samples were then washed 3X in ultra pure water at room temperature. Samples prepped for TEM were stained *en bloc* overnight in saturated aqueous uranyl acetate. TEM and SEM samples were both then subjected to a graded ethanol dehydration series, 15%, 30%, 50%, 70%, 90%, 95% (2x), 100% (3x), and anhydrous (2x). SEM samples were treated with 3x exchange of hexamethyl-disilazane (CAS # 999-97-3, Electron Microscopy Sciences). The sample was left overnight in the final exchange of hexamethyldisilazane and then collected for viewing on Zeiss EVO 50 after the chemical had completely evaporated. TEM samples were infiltrated and embedded into Spurr's resin (Spurr 1969) then sectioned for viewing on

Zeiss EM 10C 10CR Transmission Electron Microscope. All reagents and supplies for the preparation were obtained from Electron Microscopy Sciences (Warrington, PA).

Results

Apical Plate Morphology

The free swimming mastigote was approximately 25-30 μm long and 20-25 μm wide, thereby presenting a pear shape cell body that exhibited dorso-ventral compression, a purely peridinioid characteristic (Fig. 6). It had a dinokont flagellar arrangement; i.e., both flagella were inserted on the ventral side of the cell. The thecal plates had a grainy, or 'pustulate' appearance (Williams, Sarjeant et al. 1978) and were covered with trichocyst pores placed in irregular patterns, with the exception of a concentric ring

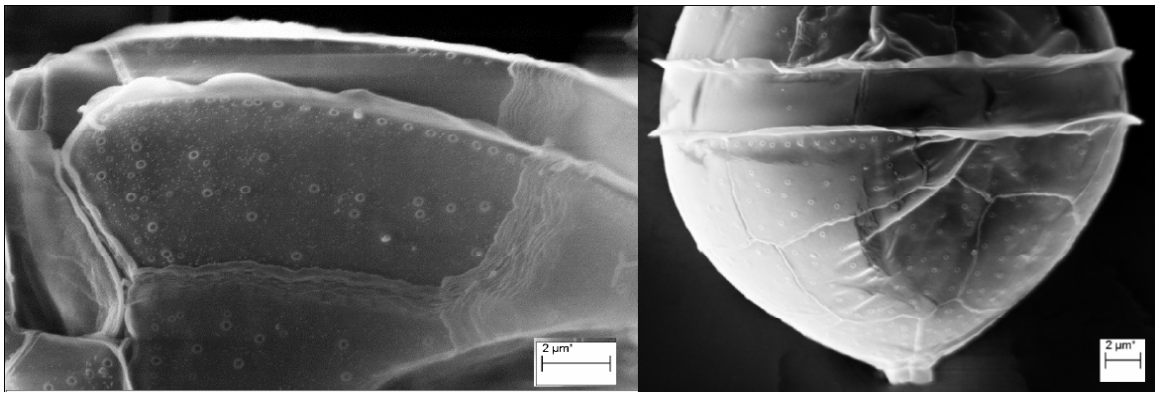

Figure 14. SEM of parasitic dinoflagellate showing surface ornamentations. The surface is covered in open pores, a texture referred to as pustulate. Note ring of pores immediately above and below the cingulum girdles.

above and below the cingulum girdles (Fig. 14). The plate tabulation formula matches that for *Pentapharsodinium* Po, X, 4′, 3a, 7′′ 4C + T, 4S, 5′′′, and 2′′′′ as proposed by Balech's description of *Peridinium tyrrhenicum n. sp*. (Balech 1990). The 1′ apical plate represents an ortho conformation, bordered by four apical plates (Fig. 15).

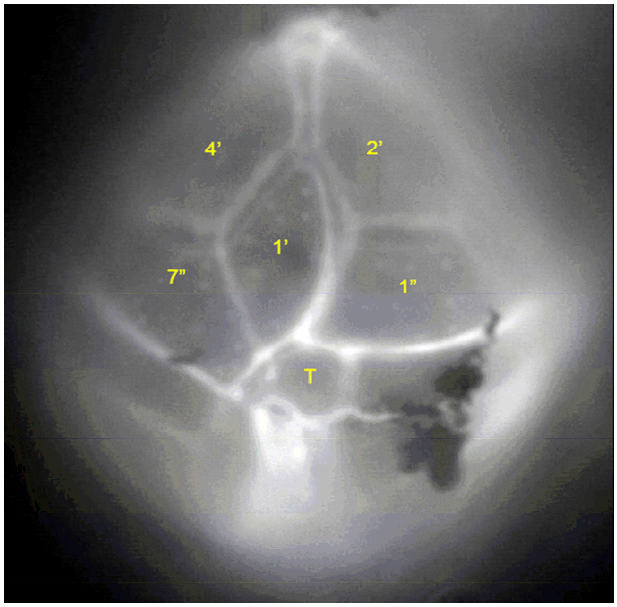

Figure 15. 1000X Light micrograph of parasitic dinoflagellate showing ortho conformation. The 1' plate is bordered by the four plates 7'', 4', 2', and 1'' giving the epitheca, anterior portion of the cell, an ortho conformation.

The 2a intercalary plate depicts a hexa or six sided conformation. On the basis of these plates it is assumed the epitheca is ortho-hexa. The epitheca appears conical without the presence of apical horns. Indelicato and Loeblich stress that the suture positions of

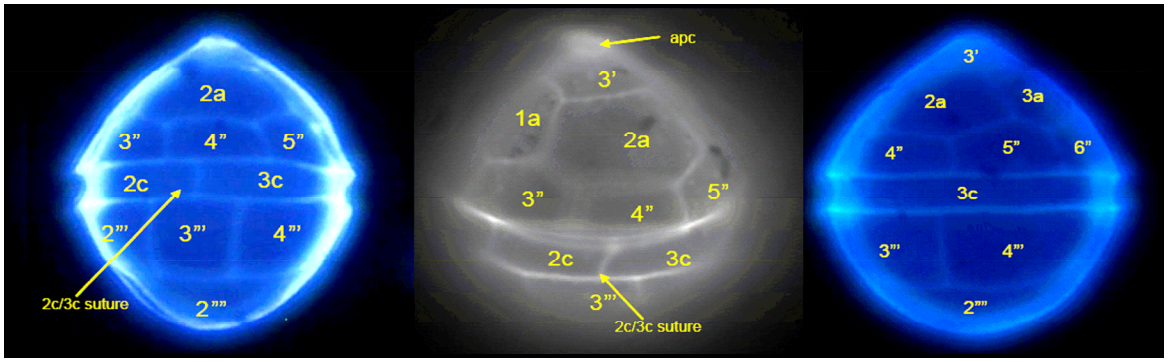

Figure 16. Calcofluor White staining under UV fluorescence. The parasitic dinoflagellate shows hexa conformation. The 2a intercalary plate is shown bordered by six plates 3'', 4'', 5'', 1a, 3', 3a giving the epitheca, anterior portion of the cell, a hexa conformation.

the cingular and hypothecal plates are a conserved feature within the peridinioid corticotype, which makes them useful tools in morphological identification (Indelicato and Loeblich 1986).

Cingulum Morphology

The cingulum is composed of 5 cingular plates (4C + T) and is displaced, descending from the proximal end. As part of the cingulum description, the transitional plate is designated as the T-plate, with the next attached cingulum plate being the 1C plate. The homologous cingular suture Y found in peridinioids (Indelicato and Loeblich 1986), lies between plates 1C/2C, apical to the 1/2 postcingular suture (Fig. 17a). In addition, the X suture lies between plates 3C/4C found apical to the 4/5 postcingular suture (Fig. 17b). This is the situation in genus *Pentapharsodinium*.

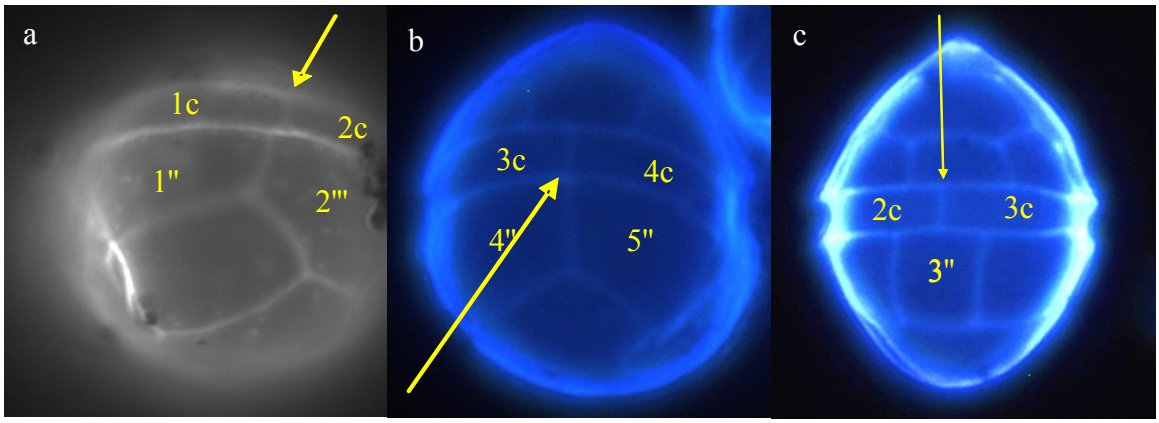

Figure 17. Calcofluor White staining under UV fluorescence depicting homologous cingular sutures. a) The arrow shows the Y suture. b) The arrow shows the X suture. c) The arrow shows the relative position of the 2C/3C suture to the 3 postcingular plate.

Another feature consistent with Indelicato and Loeblich's description of the *Pentapharsodinium* cingular sutures is the dorsal suture position that form the 2C/3C

border positioned at the center of the 3 postcingular plate (Fig. 17c) (Indelicato and Loeblich 1986).

Antapical and Sulcal Morphology

The antapical plate 1 is approximately one quarter the size of the antapical plate 2 (Fig. 18a), which in turn spans over the majority of the posterior region. The antapical

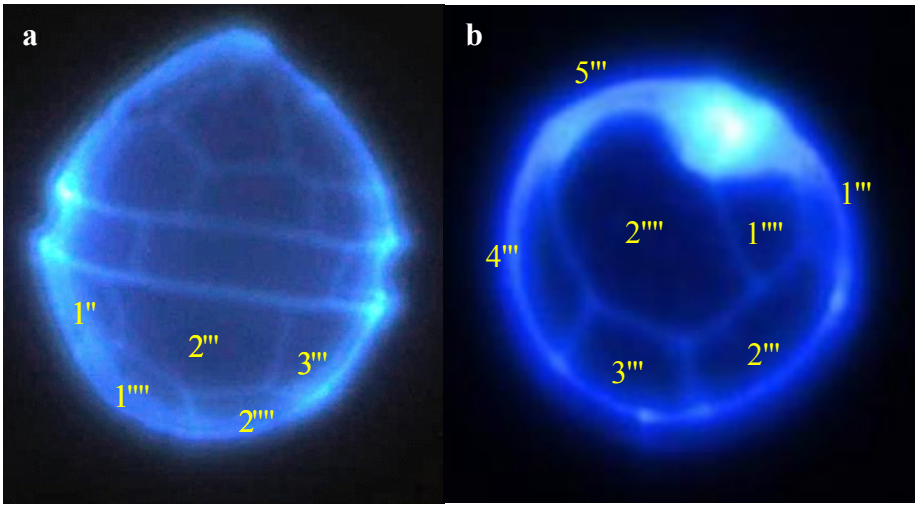

Figure 18. Calcofluor White staining under UV fluorescence depicting antapical plate morphology. a) Shows the asymmetry in the 2''' plate. b) Shows the relative size difference between the 1'''' and 2'''' plates and the border of the plates placed in the central region of the 2''' plate.

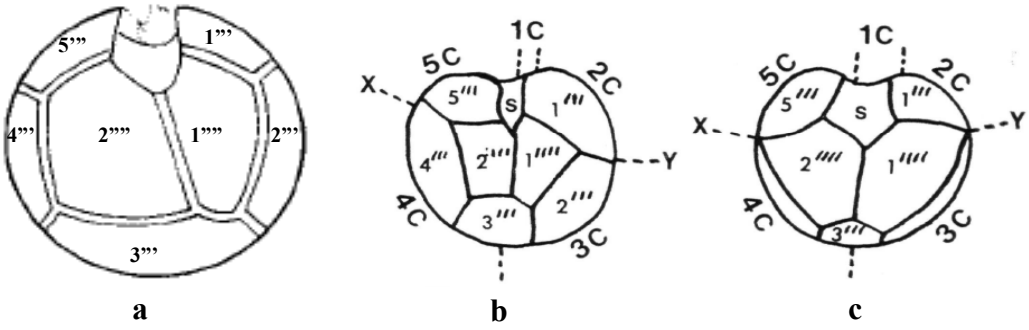

Figure 19. Diagram a) from (Balech 1990) depicting antapical plate morphology of *Pentapharsodinium tyrrhenicum*. b/c) (Indelicato and Loeblich 1986) depicting the antapical plate morphology and X and Y sutures of b) *Penatpharsodinium trachodium* and c)*Pentapharsodinium dalei*

plate 1 causes asymmetry of the postcingular plate 2 (Fig. 18b), creating a shorter left posterior margin than is seen on the right margin.

Four plates characterize the sulcal region, ventral portion of the cell where the flagella insert into the cell: sa, anterior sulcal plate; sp, posterior sulcal plate; ss, left sulcal plate; and sd, right sulcal plate (Fig. 20). The sa plate borders upon the longitudinal flagellar pore and also forms the right border of the transitional plate. The anterior portion of the sa plate forms the ventral border of the apical plate 1and the left border of the precingular plate 7. The posterior end of the sa plate forms the anterior border of the sd plate. The sa plate is somewhat quadrangular or pentangular and is more long than narrow. The sd plate is approximately 3x longer than it is wide. The anterior border is shared by the sa plate on the left and a portion of the precingular plate 7 on the right. The left border is shared approximately half way between the right border of the ss and the right border of the sp plates. The sd plate terminates on the posterior end forming a border with the anterior side portion of the sp plate. The right side of the sd plate forms the left borders of the postcingular 5 and cingular 4 plates. The ss plate forms an anterior border against the flagellar pore and the ventral midsection of the transitional plate. The left side forms the right border of the postcingular plate 1 and the left corner may or may not touch the antapical plate 1. The anterior border of the ss plate slightly protrudes into the sp plate forming its anterior border. The right forms a border with the mid to anterior left portion of the sd plate. The sp plate is approximately two times longer than it is wide. The ss plate concaves the anterior border. The left is bordered by the antapical plate 1. The posterior forms a border with mid-anterior portion of the antapical plate 2. The right

posterior border forms the right posterior border of the postcingular plate 5. The right anterior portion is bordered by the sd plate.

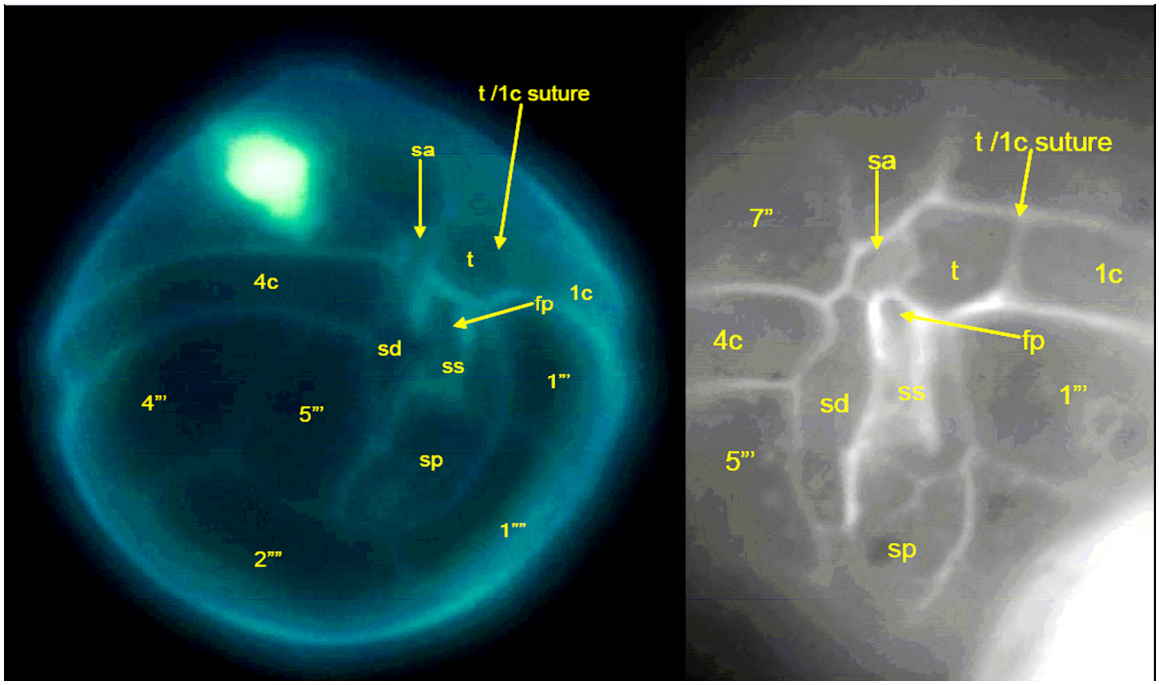

Figure 20. Calcofluor White staining under UV fluorescence depicting sulcal region. The sd represents the right sulcal plate; sa, anterior sulcal plate; sp, posterior sulcal plate; and ss, left sulcal plate.

<u>Life cycle and association with the ctenophore host</u>

Nonencysted parasitic dinoflagellates attached to *Mnemiopsis* range from approximately 19-26 µm long and 15-22 µm wide with a hyaline layer (Fig. 21b). Increased size variation has been observed in dinoflagellates attached over periods of time greater than three days. Such cells vary in size from 90-300 µm. Cysts present in *Mnemiopsis* vary from approximately 25 to 32µm in diameter and are always found embedded within the mesoglea (Fig. 21c). Concentrations of dinoflagellates on *Mnemiopsis* collected on the East Coast (U.S.) increase during the Spring and Summer months, usually reaching surface densities greater than 150 mm^2. In contrast, animals collected during the Winter and Fall months usually have very few to no dinoflagellates.

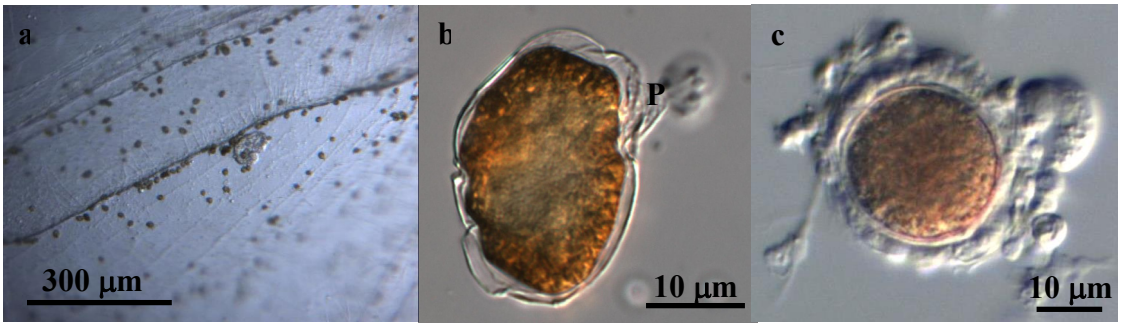

Figure 21. Dinoflagellate cells associated with host. a) Light micrographs depicting high dinoflagellate cell density on a ctenophore captured during the Spring. b) Dinoflagellate attached to host via peduncle (P). c) Encysted dinoflagellate cell embedded into host tissue.

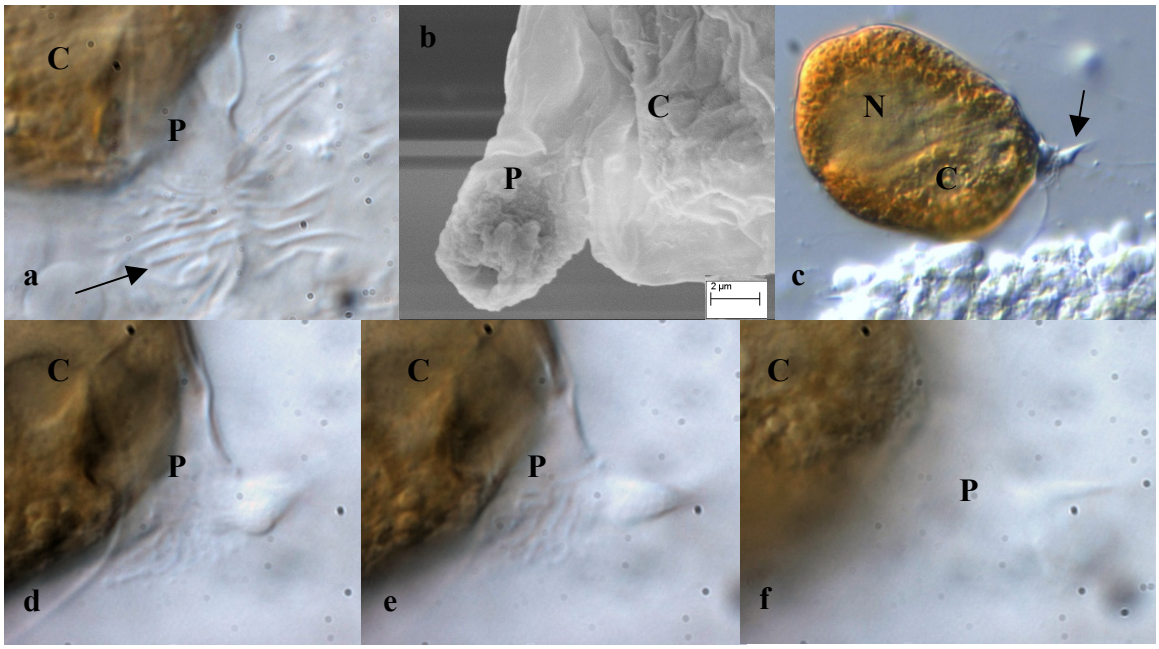

Figure 22. Light and SEM micrographs of dinoflagellate peduncle (P) and hypotheca (C) a) 1000X showing peduncle and numerous fimbre (arrow). b) SEM of unattached peduncle. c) Light micrograph of dinoflagellate skipping across host tissue. unattached peduncle (arrow) and nucleous (N) d-f) Light micrographs showing a focal series of unattached dinoflagellate peduncle.

The dinoflagellate attaches via a peduncle penetrating into the host ctenophore's epidermis (Fig. 22a). The dinoflagellate was usually found in greater abundance in the auricular grooves and the oral region, as previously described (Moss, Estes et al. 2001).

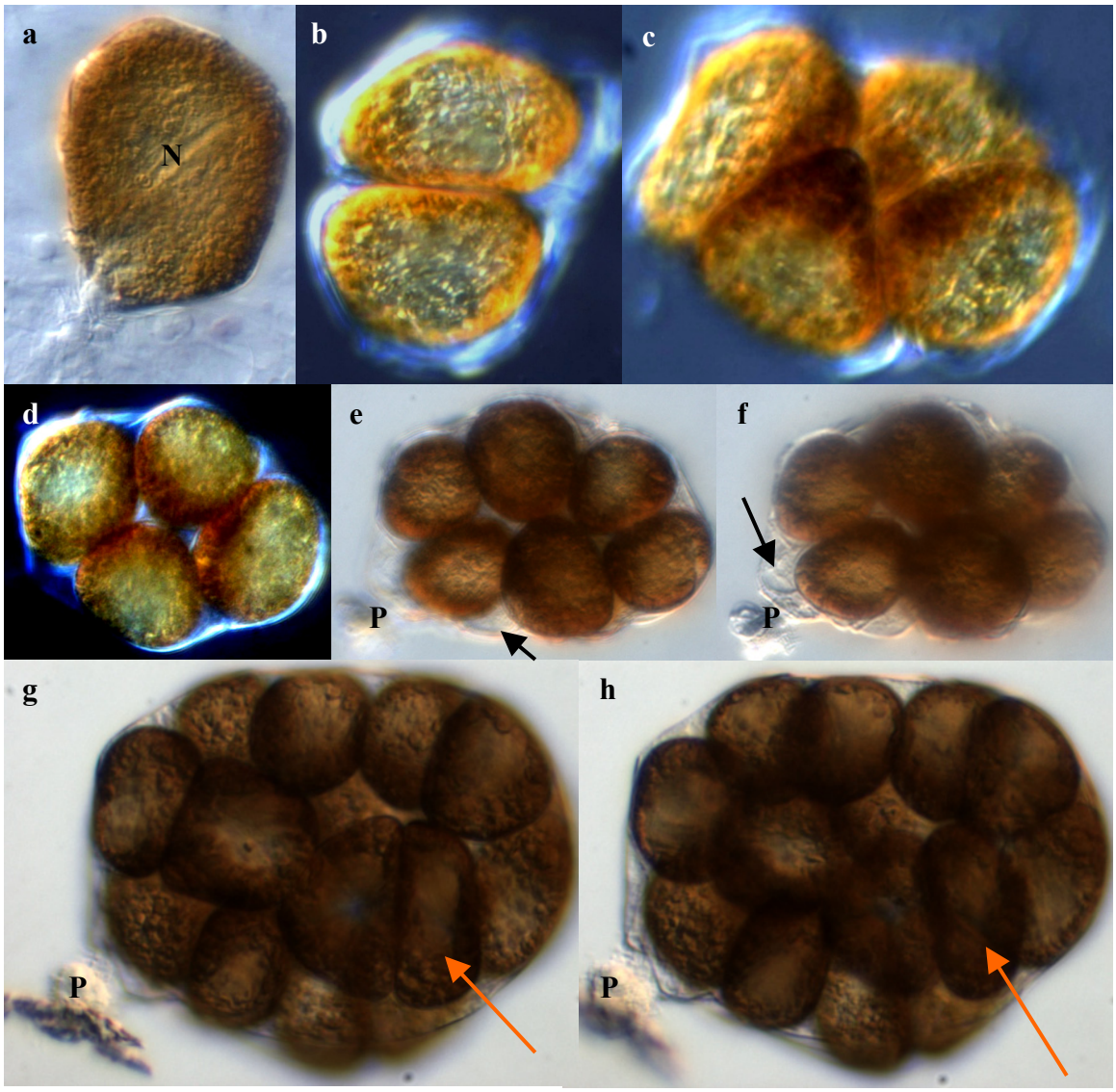

Figure 23. Light micrograph series showing parasitic reproduction, termed palintomy after disassociation with host. a) Shows single trophont as uninucleated (N). b) Detached cell undergoing equal holoblastic cleavage (2-cell stage). c-d) Detached cell undergoing a the 4-cell divison stage. e-f) Detached cell undergoing 8-cell stage the peduncle (P) can be seen and the outer cell membrane (arrow). g-h) Probable 16-cell stage multiple new dinospores are present (arrow).

Video-DIC micrography of detached dinoflagellates next to epidermal fragments revealed that it moved from place to place upon the host tissue until a location was found for attachment (Fig. 22c). Attachment occurred very quickly, in less than a minute. Cytoplasmic streaming, possibly of membrane-bounded vesicles containing lytic enzymes, was immediately and clearly observed undergoing orthograde transport within the peduncle. After a few minutes what is believed to be degraded host material was visualized to stream up the peduncle into the cell.

Parasitic Reproduction (the tomont)

The dinoflagellate associated with *Mnemiopsis* undergoes an unusual form of reproduction only seen in parasitic dinoflagellates: palintomy. The attached trophont enters a growth phase after attachment to the host. Cells that have been attached to the host for longer than three days have been observed to be as large as 100 – 300 μm in size. It is thought that after the feeding event is over the dinoflagellate enters into its reproductive phase, the tomont (fig 23 b-h). The cell begins to undergo division within the cell membrane and then ruptures, releasing a multitude of dinospores or sporonts or swarmers. Swarmers are produced by all parasitic dinoflagellates and are there for a good indicator of a parasitic lifestyle (Cachon and Cachon 1987). Swarmers possess two flagella, have a poorly developed girdle and sulcus, are morphologically variable compared to the Mastigote, and may be produced in macro or micro forms (Cachon and Cachon 1987). In the lab I have observed numerous tomonts rupture and produce dinospores that moved along the host tissue and appeared to possibly attach (Fig. 24) or swim away.

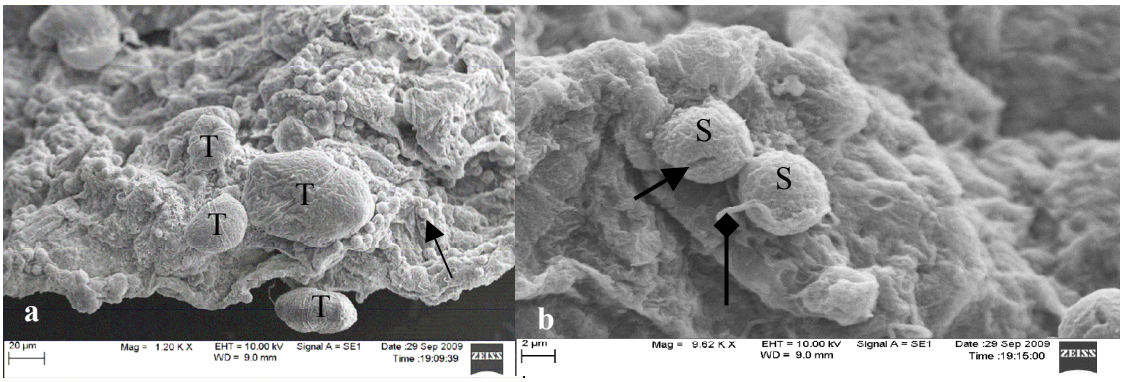

Figure 24. *In situ* SEM micrographs depicting putative dinoflagellate sporonts, swarmers and trophonts. a) Several different size trophonts ("T") appear, attached to host tissue. Thin arrow shows a sporont. b) Sporonts ("S") cingular girdle is indicated by a triangular arrow, while a boxed arrow shows a flagellum. Note tenfold difference in scales.

Discussion and Conclusions

Morphological analysis based on Kofoid's plate tabulation scheme places the dinoflagellate within the genus *Pentapharsodinium*. However, there are some discrepancies in the literature supporting the validity of the current plate tabulation designated for the *Pentapharsodinium* genus Po, X, 4′, 3a, 7″ 4C + T, 4S, 5‴, and 2⁗. A dinoflagellate belonging to the genus *Ensiculifera* based on molecular characterization of SSU, coupled with ITS analysis, has been noted to possess the same plate tabulation as the genus *Pentapharsodinum* (Hai-Feng and Yan 2007). The current plate tabulation used in the morphological identification of *Ensiculifera* is Po, X, 4′, 3a, 7″ 4C + T, 5S, 5‴, and 2⁗, (Matsuoka, Kobayashi et al. 1990) note the number of sulcal plates is 5S rather than 4S. Also, another morphologically distinct character of *Ensiculifera* is the presence of a long slender spine, about half the length of the epitheca, arising from the right anterior corner of the T plate (Fig. 25). The spine present in *Ensiculifera* is not without scrutiny as well. ITS analysis by the D'Onofrio group (D'Onofrio, Marino et al. 1999) could not separate *Ensiculifera* as an independent genus from *Pentapharsodinium* even

after coupled with a morphological anlaysis. D'Onofrio criticized the validity of the presence or absence of the spine as a valid taxonomic character at the genus level.

The epitheca of the parasitic dinoflagellate has an ortho-hexa conformation with no horns; both are indicative of the peridinioid group. The dinoflagllate adheres to the complete plate tabulation set for *Pentapharsodinium* Po, X, 4′, 3a, 7″ 4C + T, 4S, 5‴,

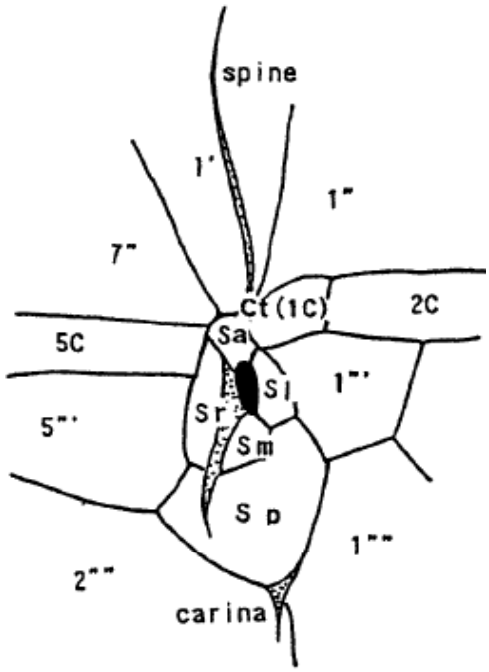

Figure 25. Diagram depicting the sulcal region o an *Ensiculifera* species (Matsuoka, Kobayashi et al. 1990). Note the large spine associated with the T plate and the presence of 5 sulcal plates.

and 2‴′. The Y and X sutures of peridinioids specifically fall within the description placed on the *Pentapharsodinium* genus. Another feature that is purely a *Pentapharsodinium* characteristic is the dorsal cingular suture position that forms the 2C/3C border positioned at the center of the postcingular plate 3. The antapical plate morphology is a defining characteristic for the genus *Pentapharsodinium.* The antapical plate size, shape, and general structure relative to the known *Pentapharsodinium* species

(Fig. 19) suggest that this dinoflagellate could possibly be an undescribed species. It is possible that the variation found in the antapical region is a result of the dinoflagellate adjusting to environmental changes as indicated by (Barrows 1918; Balech 1980) that caused it to adapt to a parasitic lifestyle. Another more likely possibility is that the antapical variation is a result of the dinoflagellates adaptation to parasitism.

Due to uncertainty in the literature involving *Pentapharsodinium* and *Ensiculifera*, in part due to their close genetic relationship and the high degree of morphological similarity, it is inconclusive whether if the dinoflagellate in this study belongs in the *Pentapharsodinium* genus. Given the current accepted plate tabulation of *Pentapharsodinium*, I tentatively place the new dinoflagellate into the *Pentapharsodinium* genus.

Evidence for parasitism

The peduncle appears to penetrate host epithelial cells, thereby facilitating myzocytosis. SEM (Fig. 22b) and video (not shown) all suggest that the peduncle is hollow, and possibly lined with microtubules and F-actin that transport lytic enzymes to penetrated host cells and recover digested host material back to the cell. Cell adherence occurs very rapidly, in less than one minute, and the transport of materials occurs almost immediately upon adherence. Cells that are observed attached to the host over a period of days grew very rapidly in size, some exceeding 200μM. Sporogenesis of this parasite is different from the description given by Cachon and Cachon for palintomy in *Protoodinium chattoni* Hovasse (Cachon and Cachon 1987). In *P. chattoni*, sporogenesis occurred after a feeding event was completed and the dinoflagellate detached from the

host. However, in this study, sporogenesis was observed while the cell was still attached to the host.

I propose that the host becomes infected by dinoflagellates because of its interaction with specific sediments that bear the dinoflagellate as a benthic form. A behavioral study of *Mnemiopsis* (Moss, Taylor, Odom, Stephenson and Welch, in preparation) revealed that the host frequently samples the flocculent benthic layer. Our operating hypothesis is that the ctenophore may recruit the dinoflagellate from currents generated as the ctenophore rests against the substrate. The heaviest initial infestations of mesogleal cysts occur in the stomodeal walls and in the tissues underlying the auricular grooves. Trophonts, sporonts and swarmers were observed in their greatest numbers after observation of mesogleal cysts, which were observed to migrate very slowly through the mesoglea after infestation, presumably via the feeding apparatus, to locations on the host surface.

Host specificity

Mnemiopsis was never systematically examined for transfer of the dinoflagellate to ctenophore predators such as the ctenophore *Beroë ovata* or the schyphomedusa *Chrysaora quinquecirrha*. However, even though each individual *Beroë* and *Chrysaora* certainly collect many hundreds of *Mnemiopsis* during their life span, I never observed the dinoflagellate associated with either species; nor was it ever evident on any fish known to ingest *Mnemiopsis*, such as *Petrilus burti* or *Menidia beryllina*.

Chapter 4: *Pentapharsodinium* Molecular Analysis

Introduction

The phylogenetic placement of an unknown organism can be ascertained through the relative comparison of phylogenetically conserved nucleic acid sequences in different organisms. Molecular phylogenetic analyses have enabled an additional, objective method for the classification of organisms, in addition to analyses of morphology. In 1991 a new taxon, the Alveolates, was established by virtue of molecular analyses. The Alveolates are comprised of ciliates, apicomplexans, protoalveolates and the dinoflagellates (Gajadhar, Marquardt et al. 1991; Wolters 1991). Infraphylum Dinoflagelleta is diverse , and is currently comprised of over 2,000 known species and 125 genera (Drebes, 1984).

In this study I determine the molecular phylogeny of a previously unknown parasitic dinoflagellate of the ctenophore *Mnemiopsis leidyi*. This study includes the analysis of three nuclear gene regions: 1) the (18S) ribosomal small subunit ; 2) the internal transcribed spacer region between the 18Sand 5.8S ribosomal DNA; i.e. ITS 1, and the 3) the ITS2 region, which lies between the 5.8S and the 28S regions. Finally, I present my results on 4) the extranuclear, mitochondrial gene cytochrome b. The sequences were used to construct phylogenetic trees, comparing the sequences with those of other organisms including known dinoflagellates.

Analysis of each of the selected gene regions has a particular role in the development of the phylogeny of organisms. The 18S rDNA gene sits within the eukaryotic ribosomal operon. The 18S rDNA encodes for 18S rRNA that is used as a scaffold for proteins to construct the 40S (small subunit) of the ribosome. Due to the

importance of the 18S rDNA gene insertions, deletions, or point mutations that would prevent the assemblage of the 40S subunit are selected against providing the gene with a conserved nucleotide sequence. Due to this level of nucleotide conservation, 18S rDNA is typically used to resolve to the genus level. Intronic sequences like the ITS are only restricted by structure and are under very little selection pressure. The ability to undergo genetic drift without causing detrimental affects to the cell allows the ITS region to be used in phylogenetics to resolve different populations within a species.

Cytochrome b is a mitochondrial gene used in the electron transport respiratory chain for the production of ATP. Mitochondrial genes display a higher rate of change with time, than is seen for nuclear genes (Brown, George et al. 1979). This allows the investigator to use mitochondrial genes to resolve differences at the population level (Conway, Fanello et al. 2000). Analyses that involve coupling sequencing of multiple genes of significantly different origin, like those for cytochrome b and 18S rDNA have provided robust phylogenetic trees within the alveolates (Rathore, Wahl et al. 2001).

Objectives and Hypotheses

Objective 1: Conduct a multi-locus phylogenetic analysis

Sequencing of targeted genetic regions will provide sufficient molecular sequence data to conduct a phylogenetic analysis for the parasitic dinoflagellate and related species.

Hypothesis 1: The dinoflagellate should be molecularly classified as a species of *Pentapharsodinium.*

Materials and Methods

DNA extraction

20-150 acetone-fixed parasitic dinoflagellates, derived directly from the host or from culture, or a similar number of cultured SZN13 cells, were centrifuged at 10,000g in a benchtop microcentrifuge (5415, Eppendorf, Federal Republic of Germany) for 10 minutes at 23 °C. Pelleted cells were extracted by the Cetyl Trimethyl Ammonium Bromide (CTAB) method modified after Gast et al., (Gast, Dennett et al. 2004) as modified from (Kuske, Banton et al. 1998).

Polymerase Chain Reaction (PCR) and sequencing

The 18S rDNA gene and the internal transcribed spacer (ITS) regions of the nuclear genome were amplified *via* PCR with primer pairs Dino18S5F1/Dino18S5R1, (Zhang, Bhattacharya et al. 2005) and the internal transcribed spacer region primer pairs ITS1/ITS4 (White, Bruns et al. 1990). The polymerase chain reaction (PCR) was carried out by incubating 50ng of template DNA with 10 μM primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM dNTPs, 1 U *Taq* polymerase and 2.5 mM $MgCl_2$ in a total volume of 25 μL. PCR was carried out as follows: an initial 60 s preheat at 94°C, followed by 34 cycles of 45 s denaturation at 94°C, 45 s annealing at 50 °C, 1 min elongation at 72°C, and a final period of elongation for 300 s at 72°C.

PCR amplification with the SS5/SS3Z primer pair (Rowan and Powers 1991) also used approximately 50 ng of template DNA, but instead began with 90 s denaturation period at 94°C, followed by 30 cycles of 60 s denaturation at 94°C, 60 s annealing at 56°C, 90 s elongation at 72°C, with a final elongation of 5 min at 72°C. The

amplification of mitochondrial primers Dinocob1F/Dinocob1R (Zhang, Bhattacharya et al. 2005) were performed under the same conditions as specified in that study.

The primer pairs 633DinoF/1051DinoR, D946F/D1582R, and D400F/D965R were developed during this study to obtain the internal nucleotide sequences across the 18S rDNA gene. After consensus sequences were obtained of the 18S rDNA flanking regions using the previously mentioned primers, new primers were built, using Amplify 3X (University of Wisconsin Ver. 3.1.4, Madison, WI, USA) to generate overlapping regions across the 18S rDNA gene in order to subsequently build contiguous sequences. PCR was carried out under the same conditions as stated for Dino18S5F1/Dino18S5R1.

The resulting amplicons were assayed by 1% agarose gel/TAE electrophoresis at 95 V for 35 minutes at room temperature in a Horizon 58 gel apparatus (Gibco/Bethesda Research Laboratories, Bethesda, MD, USA). Gels were stained with 0.1 % ethidium bromide. The gel was photographed with a high performance CCD camera (Cohu Inc., San Diego, CA, USA) equipped with a 4-48 mm zoom television lens equipped with an ethidium bromide 'rainbow' filter. Images were acquired by video capture (model LG3 capture board, Scion Corporation, Frederick, MD, USA), and image analysis performed by Gel-Pro software (Media Cybernetics, Bethesda, MD, USA). Successful amplicons were subjected to dye-termination sequencing at the Genetic Analysis Laboratory of the Auburn Research Instrumentation Facility on an ABI 33100 sequencer. The chromatograms were visualized, edited and assembled to produce consensus sequences using Sequencher (Gene Codes Corporation Ver. 4.8, Ann Arbor, MI, USA). The consensus sequences were then organized to form contiguous sequences.

Primer	Sequence	Region	Specificity	Reference
Dino18S5F1	5’-AAG GGT TGT GTT TAT TAG NTA CAG AAC-3’	18S rDNA	194-220 *P. tyrrhenicum* ribosomal operon	Zhang et al., 2005
Dino18SR1	5’-GAG CCA GATR CWCA CCC AG-3’	18s rDNA	683 - 665 *P. tyrrhenicum* ribosomal operon	Zhang et al., 2005
SS5 (F)	5’-GGT TGA TCC TGC CAG TAG TCA TAT GCT TG-3’	18S rDNA	6-34 *P. tyrrhenicum* ribosomal operon	Rowan and Powers 1991
SS3Z (R)	5’-GCA CTG CGT CAG TCC GAA TAA TTC ACC GG-3’	18S rDNA	1686-1657 *P. tyrrhenicum* ribosomal operon	Rowan and Powers 1991
633DinoF	5'-GGA TTT CGT AGG ACG ACC GGT CCG C-3'	18S rDNA	633-657 Internal to contiguous sequence	This study
1051DinoR	5'-CCT CCA ATC TCT AGT CGG CAT GG-3'	18S rDNA	1051-1029 Internal to contiguous sequence	This study
D946F	5'-TTT GCC AAG GAT GTT TTC ATT GAT-3'	18S rDNA	946-969 Internal to contiguous sequence	This study
D1582R	5'-CTG ATG ACT CGC GCT TAC TAG GAA-3'	18S rDNA	1582-1559 Internal to contiguous sequence	This study
D400F	5'-AAC GGC TAC CAC ATC TAA GGA A-3'	18S rDNA	400-421 Internal to contiguous sequence	This study
D965R	5'-ATG AAA ACA TCC TTG GCA AA-3'	18S rDNA	965-946 Internal to contiguous sequence	This study

Table 3. List of 18S rDNA primers used in this study, their specificity and reference sources.

Primer	Sequence	Region	Specificity	Reference
ITS1 (F)	5'-TCC GTA GGT GAA CCT GCG G-3'	ITS 1&2	1770-1788 *P. tyrrhenicum* ribosomal operon	White et al.1990
ITS4 (R)	5'-TCC TCC GCT TAT TGA TAT GC-3'	ITS 1&2	2428 – 2409 *P. tyrrhenicum* ribosomal operon	White et al.1990

Table 4. List of ITS primers used in this study along with their specificity and references.

Primer	Sequence	Region	Specificity	Reference
Dinocob1F	5'-ATG AAA TCT CAT TTA CAW WCA TAT CCT TGT CC-3'	cyt b	61-92 *P. piscicida* cyt b operon	Zhang et al., 2005
Dinocob1R	5'-TCT CTT GAG GKA ATT GWK MAC CTA TCCA-3'	cyt b	877-850 *P. piscicida* cyt b operon	Zhang et al., 2005

Table 5. List of cytochrome b primers used in this study along with their specificity and references.

Sequence alignment and tree assembly

Dinoflagellate 18S, ITS, and cyt b sequences were obtained from GenBank to perform a phylogenetic analysis of the dinoflagellate in this study. Contiguous sequences and sequences obtained from GenBank were aligned using ClustalX ver. 2.0.10 set on Multiple Alignment Mode (Larkin, Blackshields et al. 2007). Aligned sequences were then entered into RAxML (Randomized Axelerated Maximum Likelihood) using a GTR (Generalized Time Reversible) set to 1,000 bootstrap replicates to produce a maximum likelihood phylogeny estimation based on nucleotide sequences (Stamatakis, Hovver et al. 2008). Output files were converted to phylogenetic trees using TreeView (Page 1996).

RAxML is a program for sequential and parallel Maximum Likelihood based inferences. Maximum Likelihood refers to the distribution that gives the observed data the greatest probability. ML is essentially an estimation that searches over all possible outcomes giving a specific model, in this case GTR, to produce the most likely scenario, a phylogenetic tree, based on the given data, the sequence alignments (Frongillo 2002).

The GTR model used in this study gives RAxML the parameter values used in its estimation to find the best phylogenetic tree with the given data, the sequence alignments. The GTR model was developed by Simon Tavaré (Tavaré 1986) when attempting to explain substitution rates in his study on the divergence time of rat and mouse; and is one of numerous Markov models of DNA sequence evolution. In these models a set of parameters are given based on the substitution rates of nucleotides. The models can either assume that nucleotide changes occur at equal frequencies – the JC and K2P models – or that the four nucleotides can change at different frequencies – the F84, HKY85, and GTR models. Specifically, in the GTR model, Tavaré takes into account the relative roles of substitution, insertion and deletion, duplication, and transposition as forces that change the structure of genes over time. The model assumes that nucleotide changes are "time reversible." In other words, if a nucleotide changes, it has the ability to revert back to the original nucleotide at the same rate. The model assumes that each substitution type between nucleotides occurs at different rates and that each nucleotide can occur at different frequencies (Hillis, Moritz et al. 1996).

Table 6a: List of dinoflagellate species and GenBank accession number for SSU analysis

Dinoflagellate species	Order	Family	BPs	Gen Bank No.
Ceratium fusus	Gonyaulacales	Ceratiaceae	1752	AF022153.1
Ceratocorys horricda	Gonyaulacales	Ceratocoryaceae	1744	AF022154.1
Alexandrium fraterculus	Gonyaulacales	Gonyaulacaceae	1801	AB088315.1
Alexandrium tamarense	Gonyaulacales	Gonyaulacaceae	1800	AB088333.1
Gymnodinium mikimotoi	Gymnodiniales	Gymnodiniaceae	1803	AF022195.1
Takayama cf. pulchellum	Gymnodiniales	Kareniaceae	1799	AY800130.1
Cachonina hallii	Peridiniales	Heterocapsaceae	1801	AF033865.1
Heterocapsa niei strain CCMP 447	Peridiniales	Heterocapsaceae	1753	AF274265.1
Heterocapsa triquetra	Peridiniales	Heterocapsaceae	1801	AF022198.1
Coolia monotis isolate CCMP1345	Peridiniales	Ostreopsidaceae	1778	EF492487.1
*Ensiculifera aff. loeblichii strain GeoB*220*	Peridiniales	Peridiniaceae	1797	HQ845328.1
Pentapharsodinium sp.	Peridiniales	Peridiniaceae	1569	(this study)
Pentapharsodinium sp. CCMP771	Peridiniales	Peridiniaceae	1755	AF274270.1
Pentapharsodinium tyrrhenicum	Peridiniales	Peridiniaceae	1803	AF022201.1
Peridinium cinctum	Peridiniales	Peridiniaceae	1751	AB185114.1
Peridinium wierzejskii	Peridiniales	Peridiniaceae	1755	AY443018.1
Scrippsiella nutricula	Peridiniales	Peridiniaceae	1802	U52357.1

Table 6b: List of dinoflagellate species and GenBank accession number for SSU analysis

Dinoflagellate species	Order	Family	BPs	Gen Bank No.
Scrippsiella sp. MBIC11168	Peridiniales	Peridiniaceae	1756	AB183677.1
Scrippsiella sweeneyae strain CCCM 280	Peridiniales	Peridiniaceae	1755	AF274276.1
Scrippsiella trochoidea strain CCCM 602	Peridiniales	Peridiniaceae	1753	AF244277.1
Pfiesteria-like	Peridiniales	Pfiesteriaceae	1787	AY033487.1
Pfiesteria-like sp. CCMP 1827	Peridiniales	Pfiesteriaceae	1752	AY456118.1
Pfiesteria-like sp. clone POC-8	Peridiniales	Pfiesteriaceae	1792	AY121856.1
Protoperidinium pellucidum	Peridiniales	Protoperidiniaceae	1751	AY443022.1
Prorocentrum micans isolate UTEX 1003	Prorocentrales	Prorocentraceae	1795	EF492511.1
Prorocentrum mixicanum strain CCMP 687	Prorocentrales	Prorocentraceae	1764	EU287485.1
Pyrocystis noctiluca	Pyrocystales	Pyrocystaceae	1792	AF022156.1
Symbiodinium sp. AP310	Suessiales	Symbiodiniaceae	1531	AY051087.1
Symbiodinium sp. K192	Suessiales	Symbiodiniaceae	1531	AY051093.1
Azadinium poporum isolate UTHD4	unclassified	unclassified	1740	HQ324899.1
Azadinium spinosum strain 3D9	unclassified	unclassified	1521	FJ217814.1
Dinophyceae sp. W5-1	unclassified	unclassified	1798	AY434687.1
Duboscquodinium collinii isolate VSM11	unclassified	unclassified	1801	HM483399.1
Thraustochytrium kinnei (outgroup)	Labyrinthulida	Thraustochytriidae	1705	DQ367053.1

Table 7a: List of dinoflagellate species and GenBank accession number for ITS analysis

Dinoflagellate species	Order	Family	BPs	Gen Bank No.
Dinophysis acuminata	Dinophysiales	Dinophysiaceae	578	AB192301.1
Dinophysis infundibulus	Dinophysiales	Dinophysiaceae	590	AB374987.1
Dinophysis rotundata	Dinophysiales	Dinophysiaceae	590	AB355141.1
Alexandrium fraterculus	Gonyaulacales	Gonyaulacaceae	584	AB436946.1
Alexandrium minutum	Gonyaulacales	Gonyaulacaceae	520	AJ312277.1
Alexandrium tamarense	Gonyaulacales	Gonyaulacaceae	608	AB233377.1
Alexandrium tamiyavanichi	Gonyaulacales	Gonyaulacaceae	605	AB436947.1
Karlodinium micrum	Gymnodiniales	Gymnodiniaceae	3264	EF036540.1
Heteocapsa pygmaea	Peridiniales	Heterocapsaceae	590	AB084094.1
Heterocapsa arctica	Peridiniales	Heterocapsaceae	597	AB084095.1
Heterocapsa sp M-2008	Peridiniales	Heterocapsaceae	568	AB445394.1
Heterocapsa sp NIES-473	Peridiniales	Heterocapsaceae	588	AB084100.1
Heterocapsa triquetra	Peridiniales	Heterocapsaceae	594	AB084101.1
Ensiclifera aff imariensis isolate D207	Peridiniales	Peridiniaceae	555	AY728076.1
Ensiculifera aff loeblichii GeoB 229	Peridiniales	Peridiniaceae	583	AY499513.1
Ensiculifera cf imariensis strain JB3	Peridiniales	Peridiniaceae	551	AF527814.1
Pentapharsodinium sp.	Peridiniales	Peridiniaceae	593	(this study)

Table 7b: List of dinoflagellate species and GenBank accession number for ITS analysis

Dinoflagellate species	Order	Family	BPs	Gen Bank No.
Pentapharsodinium dalei SZN19	Peridiniales	Peridiniaceae	554	AF527817.1
Pentapharsodinium tyrrhenicum	Peridiniales	Peridiniaceae	586	AY499512.1
Peridinium aciculiferum strain PASP6	Peridiniales	Peridiniaceae	558	EF417297.1
Peridinium cinctum SAG: 2017	Peridiniales	Peridiniaceae	570	EU445322.1
Peridinium willei	Peridiniales	Peridiniaceae	3602	AB232669.1
Pfiesteria piscicida clone CCMP 2091	Peridiniales	Pfiesteriaceae	677	DQ344043.1
Pfiesteria shumwayae clone CCMP 2359	Peridiniales	Pfiesteriaceae	772	DQ344035.1
Pfiesteria-like sp CCMP 1838	Peridiniales	Pfiesteriaceae	3320	AY590478.1
Prorocentrum belizeanum	Prorocentrales	Prorocentraceae	3208	DQ238042.1
Prorocentrum cassubicum strain VGO 835	Prorocentrales	Prorocentraceae	631	EU244475.1
Prorocentrum levis	Prorocentrales	Prorocentraceae	3323	DQ238043.1
Prorocentrum micans strain PM1V	Prorocentrales	Prorocentraceae	633	EU244467.1
Prorocentrum minimum strain AND3V	Prorocentrales	Prorocentraceae	619	EU244473.1
Sybiodinium sp. ex Amphisorus hemprichii	Suessiales	Symbiodiniaceae	745	EU786090.1
Symbiodinium sp. clade A	Suessiales	Symbiodiniaceae	232	FJ626951.1
Plasmodium vivax (outgroup)	Haemosporida		993	AF316893.1

Table 8: List of dinoflagellate species and GenBank accession number for Cty b analysis

Dinoflagellate species	Order	Family	BPs	Gen Bank No.
Dinophysis acuminata	Dinophysiales	Dinophysiaceae	925	EU130568.1
Ceratium longipes	Gonyaulacales	Ceratiaceae	932	EF036546.1
Ceratocorys horrida	Gonyaulacales	Ceratocoryaceae	932	EF036547.1
Alexandrium pseudogonyaulax	Gonyaulacales	Gonyaulacaceae	940	AB290130.1
Alexandrium tamarense	Gonyaulacales	Gonyaulacaceae	940	AB290126.1
Heterocapsa rotundata strain CCMP 1542	Peridiniales	Heterocapsaceae	948	EU126133.1
Heterocapsa triquetra strain CCMP 449	Peridiniales	Heterocapsaceae	936	EU126132.1
Pentapharsodinium sp.	Peridiniales	Peridiniaceae	751	(this study)
Peridinium centenniale CCAC0002	Peridiniales	Peridiniaceae	828	EF417340.1
Scrippsiella aff. hangoei K-0399	Peridiniales	Peridiniaceae	847	EF506569.1
Scrippsiella sweeneyae	Peridiniales	Peridiniaceae	926	EF036563.1
Pfiesteria piscicida	Peridiniales	Pfiesteriaceae	1112	AF357519.2
Pfiesteria shumwayae	Peridiniales	Pfiesteriaceae	926	AF502593.1
Pfiesteria-like sp CCMP 1827	Peridiniales	Pfiesteriaceae	926	AY456117.1
Prorocentrum micans strain CCMP 1589	Prorocentrales	Prorocentraceae	943	AY745238.1
Prorocentrum minimum strain JA0001	Prorocentrales	Prorocentraceae	949	DQ336067.1
Pyrocystis noctiluca	Pyrocystales	Pyrocystaceae	932	EF036562.1
Symbiodinium goreaui	Suessiales	Symbiodiniaceae	938	EU130574.1
Symbiodinium microadriaticum	Suessiales	Symbiodiniaceae	938	DQ082985.1
Plasmodium falciparum (outgroup)	Haemosporida		1131	AJ298787.1

Results

18S rDNA analysis

One thousand, five hundred sixty nine base pairs of the SSU rDNA were isolated from the parasitic dinoflagellate. Sequences were obtained from both cells removed directly from the host, as well as from cultured cells. Sequences obtained from either source were 100% identical. The sequence comparison by BLASTn with the GenBank nr/nt database returned a 99% similarity to *Pentapharsodinium tyrrhenicum*; in fact, sequence comparisons with *P. tyrrhenicum* revealed a difference of only three bases, all of which were ambiguities. The first difference, at nucleotide 212 of *P. tyrrhenicum*, resulted in an ambiguity code of W for *P. tyrrhenicum* and an ambiguity code of K for the parasitic dinoflagellate. The second difference was located at nucleotide 1062 of *P. tyrrhenicu,* resulting in an ambiguity code of W and a code of T for the parasitic dinoflagellate. Lastly, nucleotide 1344 of *P. tyrrhenicum* resulted in a code of C and an ambiguity code of M for the parasitic dinoflagellate.

Similarity in 18S rDNA sequences obtained from organisms of the same species is commonly accepted to be greater than 95% (Caron, Countway et al. 2009). The phylogenetic affiliation is within the Order Peridiniales (Fig. 26).

Here, the bootstrap values in the 18S rDNA provided strong support for the majority of the families presented including the Gonyaulacacea, Heterocapsaceae, Pfiesteriaceae, Prorocentraceae, and Symbiodiniaceae. Also the 18S rDNA analysis gives strong bootstrap values (i.e. >85%) to several unclassified dinoflagellates, *Azadinium poporum* and *Azadinium spinosum*, supporting their claim as a monophyletic group. The analysis also confirms the placement of *Duboscquodinium collinii* with the *Scrippsiella*

18S rDNA	CGGCAAAACTGCGAATGGCTCATTAAAACAGTTATAGTTTATTTGATGGT
	CATTCTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGCGCCC
	AAACCCGACTCCGTGGAAGGGTTGTGTTTATTAGKTACAGAACCAACCCA
	GGCTCTGCCTGGTCTTGTGGTGATTCATAATAACCAAACGAATCGCATGG
	CATCAGCTGGCGATGAATCATTCAAGTTTCTGACCTATCAGCTTCCGACG
	GTAGGGTATTGGCCTACCGTGGCAATGACGGGTAACGGAGAATTAGGGTT
	CGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCA
	GCAGGCGCGCAAATTACCCAATCCTGACACAGGGAGGTAGTGACAAGAAA
	TAACAATACAGGGCATCCATGTCTTGTAATTGGAATGAGTAGAATTTAAA
	TCCCTTTACGAGTATCGATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGG
	TAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCGGTTAAAAAGCTC
	GTAGTTGGATTTCTGCTGAGGACGACCGGTCCGCCCTCTGGGTGAGTATC
	TGGCTCGGCCTGGGCATCTTCTTGGAGAACGTAGCTGCACTTGACTGTGT
	GGTGCGGTATCCAAGACTTTTACTTTGAGGAAATTAGAGTGTTTCAAGCA
	GGCACACGCCTTGAATACATTAGCATGGAATAATAAGATAGGACCTCGGT
	TCTATTTTGTTGGTTTCTAGAGCTGAGGTAATGATTAATAGGGATAGTTG
	GGGGCATTCGTATTTAACTGTCAGAGGTGAAATTCTTGGATTTGTTAAAG
	ACGGACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTGATCAAGAAC
	GAAAGTTAGGGGATCGAAGACGATCAGATACCGTCCTAGTCTTAACCATA
	AACCATGCCGACTAGAGATTGGAGGTCGTTATCTTTACGACTCCTTCAGC
	ACCTTATGAGAAATCAAAGTCTTTGGGTTCCGGGGGGAGTATGGTCGCAA
	GGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCT
	GCGGCTTAATTTGACTCAACACGGGGAAACTTACCAGGTCCAGACATAGT
	AAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCA
	TGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGA
	ACGAGACCTTAACCTGMTAAATAGTTACACGTAACCTCGGTTACGTGGGC
	AACTTCTTAGAGGGACTTTGCGTGTCTAACGCAAGGAAGTTTGAGGCAAT
	AACAGGTCTGTGATGCCCTTAGATGTTCTGGGCTGCACGCGCGCTACACT
	GATGCGCTCAACGAGTTTATGACCTTGCCCGGAAGGGTTGGGTAATCTTT
	TTAAAACGCATCGTGATGGGGATAGATTATTGCAATTATTAATCTTCAAC
	GAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGTGCTGATTACGTCCCT
	GCCCTTTGTACACACCGCC

Table 9: Consensus sequence for 18S rDNA gene sequences from the parasitic dinoflagellate isolate.

group, inferred by Coats's recent redistribution of the dinoflagellate out of the Syndiniophyceae to the Dinophyseae (Coats, Kim et al. 2010). The Family Peridiniaceae, which includes *Pentapharsodinium, Ensiculifera*, and *Scrippsiella* is grouped relative to genus. The *Pfiesteria* group, Family Pfiesteriaceae, divides the Peridiniaceae, but is grouped as a single Family. Thus, *Pentapharsodinium* and *Ensiculifera* form a monophyletic group.

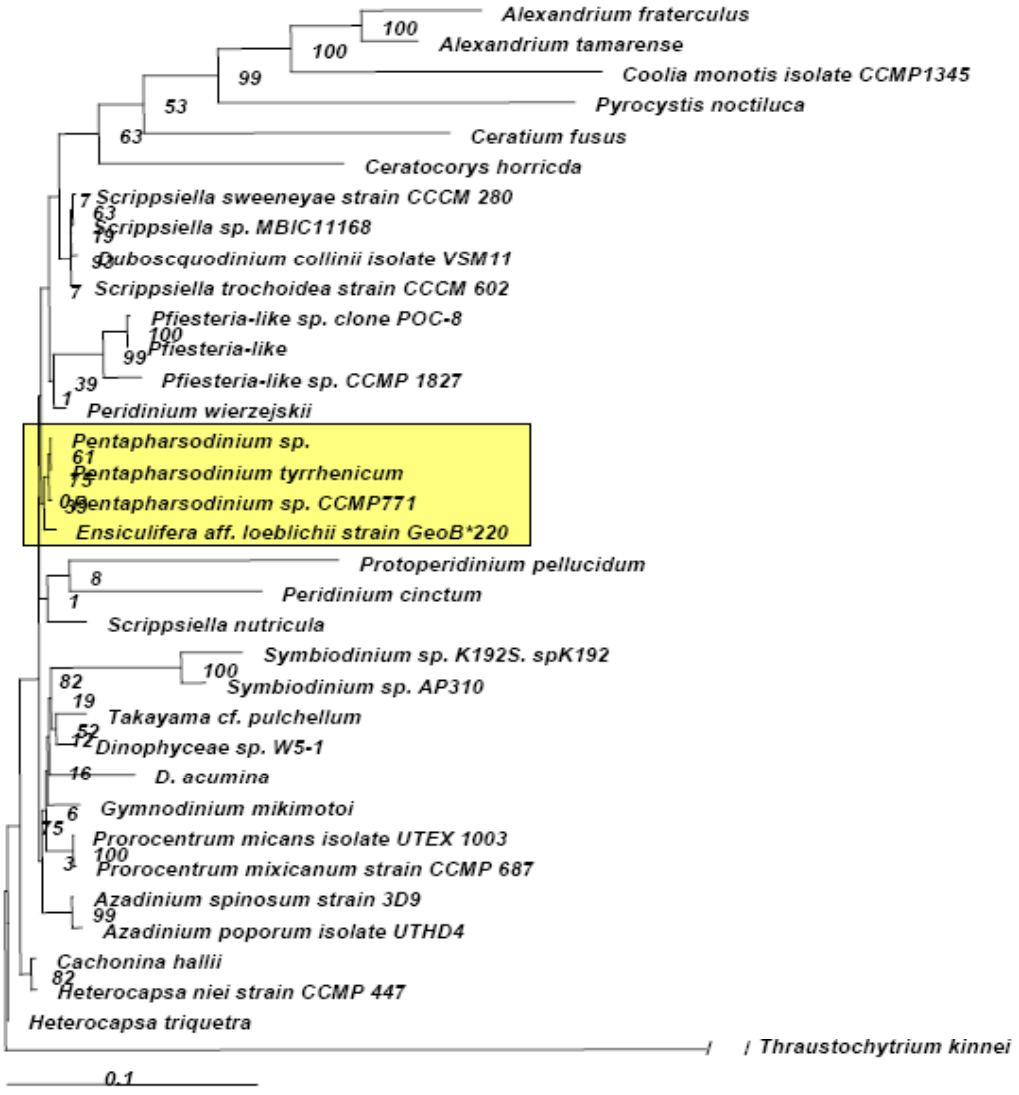

Figure 26. SSU rDNA Maximum likelihood 1,000 bootstrap replicates using the GTR model. The tree is a representation of phylogenetic relationships across various Orders of closely related dinoflagellates.

Internal transcribed spacer region analysis.

Five hundred ninety three base pairs of ITS sequence were obtained from the parasitic dinoflagellate. Sequences were obtained from both cells that were removed from host and cells in culture. Sequences obtained from either source were identical.

ITS	TCATTCGCACGCATCCAAATGAACCACTGTGAATCATTGGCGTGAGGTTC
	TGCATGGGGGACGGAGATTGCATCAATTCCCCCATGCAGAAGCTCGAGGG
	CGGCAGGGCAGGATGGGTGTTTGTCACCTCCTTTCTGTTCTTGTCGTCAT
	GTACCTTGCATGCTGATCTTTACATCCTCATGAACTCTGGAGTGCTTGCC
	CACTCCTTTTTCTTTCTTACAACTTTCAGCGACGGATGTCTCGGCTCGAA
	CAACGATGAAGGGCGCAGCGAAGTGTGATAAGCATTGTGAATTGCAGAAT
	TCCGTGAACCAATAGGGACTTGAACGTACACTGCGCTTTCGGGATATCCC
	TGAAAGCATGCCTGCTTCAGTGTCTATTCCATCTTCTGCCAGTGACGTCT
	TCCACCTCGTGTGGTCCAGTCGCTTGTGCGTGCTTGTGCGTTAAGGAGCT
	GTGCTGCCCCTGACGCATTCAGTGCATGGGGAGTTTCCGTGACTTGCAAC
	TTACCATACATTGCTGATGTTATTTGTTGCTGTGCCACTGGAAAGAGCCC
	TTGTGTGGAGTATGTCTCATACTTCTCTAAGACATGAAGTTAG

Table 10: Consensus sequence for ITS sequences from the parasitic dinoflagellate isolate.

In contrast, the sequences from specimens obtained from the Northern Gulf of Mexico, North Carolina and the Gulf of Naples, Italy were only 94% identical. Sequence comparisons with *P. tyrrhenicum* showed a total of three gaps, eighteen transitions, and nine transversions. The bootstrap values in the ITS analysis provided strong support for all of the major Families presented including the Dinophysiaceae, Gonyaulacacea, Heterocapsaceae, Pfiesteriaceae, Prorocentraceae, Symbiodiniaceae and Peridiniaceae. The only notable exception is with the genus *Peridinium*. Its placement with *Pfiesteriaceae* and *Prorocentraceae* is likely to be artifactual. As seen in the 18S rDNA analysis the two genera *Pentapharsodinium* and *Ensiculifera* both belong to the Family Peridiniaceae, and form a monophyletic group.

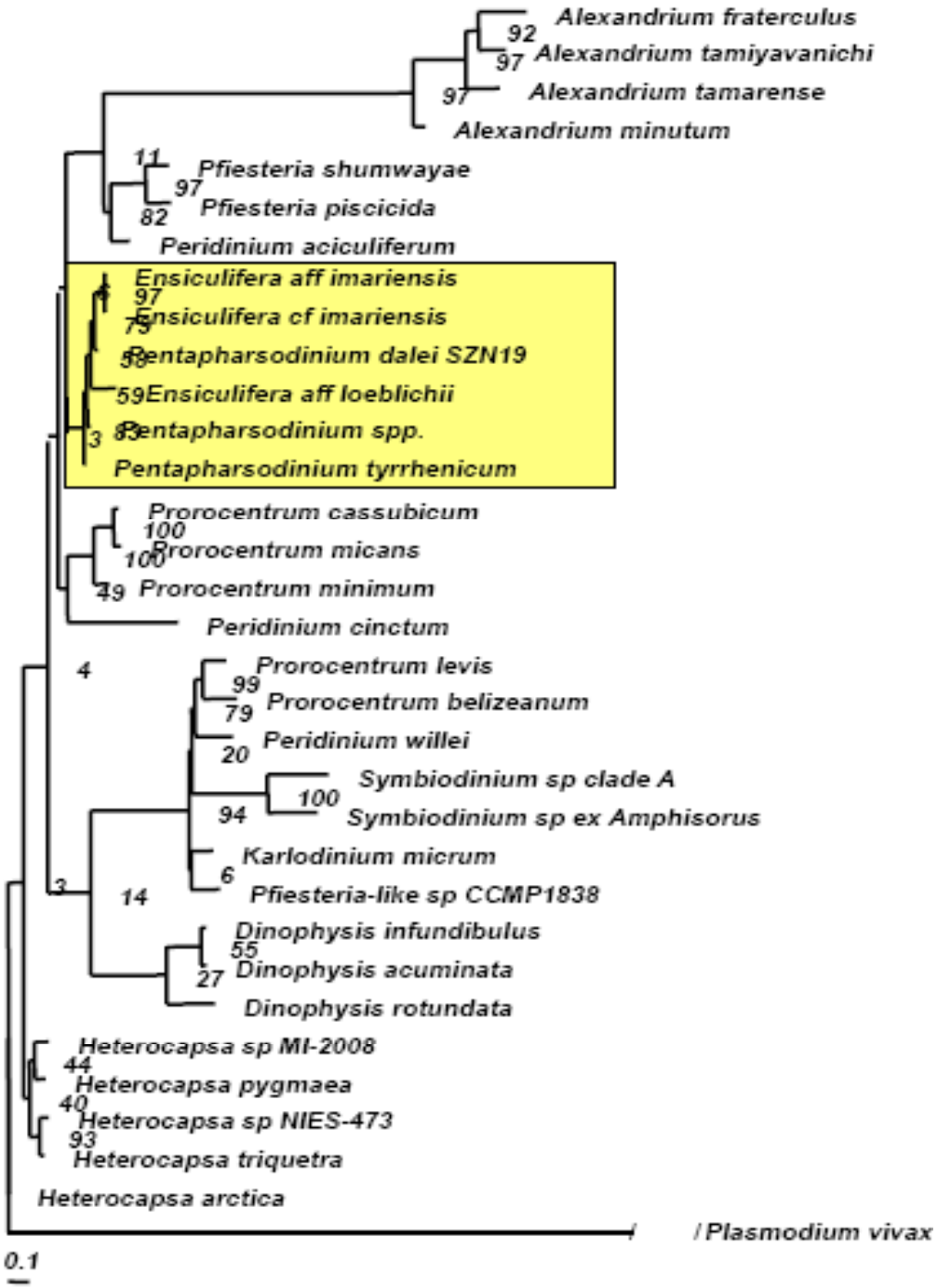

Figure 27 ITS Maximum likelihood search with 1,000 replicates using GTR model. The tree is a representation of phylogenetic relationships across various Orders of closely related dinoflagellates.

Molecular analysis cytochrome b (cyt b).

Seven hundred fifty one base pairs of cyt b sequence were obtained from the parasitic dinoflagellate. Sequences were obtained from both cells that were removed from host and cells in culture. The sequences obtained from both host and cultures were

Cyt b	TGGAATTACTATTATATTACAAATTATTACTGGAATCTTATTATCTTTAC
	ATTATACTTCAGATATTAATAGTGCTTACTTCTCTATATTCTTTATTATA
	AGAGAAATATTCTTTGGATGGTCTTTACGTTATTTACATTCTTCAGGTGC
	ATCATTTGTATTCTTATTTGTATTCTTTCATATTGGAAGAGGTATATTTT
	ATGGTTCATATTTCTATAATCCAAATACTTGGTTTTCTGGTATTATTCTT
	TTATTATTTTTAATGGCTATAGCATTTATGGGTTATGTCTTACCTTTTGG
	ACAAATGAGTTTCTGGGGAGCTACAGTAATTACAAATTTATTATCACCTT
	TTCCATGTGTAATAGAATGGGTTTCTGGAGGATATTATGTTTACAATCCA
	ACTTTAAAGAGATTTTTTATATTCCATTTCTTATTACCATTTCTATTATG
	TGGATTTACTATTCTTCATATTTTTTATCTTCATTTACTATCTTCTAATA
	ATCCTTTAAGGAATTCTACTAATAATAAAATCCCATTTTTCCCTTATATA
	TTTCAAAAAGATGTATTTGGTTTCATTATAATCCTTACTATATATCTTCT
	TCAAACTAATTTTGGTATATCTTCTTTATCACATCCAGATAATGCATTAG
	AAGTTTGTTCCTTACTTACTCCTTTACATATAGTACCTGAATGGTATTTC
	CTATGCCAATATGCTATGTTAAAAGCTGTACCCAACAAAAATTCAGGATT
	C

Table 11: Consensus sequence for cyt b gene sequences from the parasitic dinoflagellate isolate.

identical. The sequence comparison by BLASTn against the GenBank nr/nt database returned relatively low homology with several dinoflagellate genus belonging to the Order Peridiniales in the 86% - 88% range. The bootstrap values in the cyt b analysis provided support for the Families Gonyaulacaceae, Heterocapsaceae, Pfiesteriaceae, Prorocentraceae, Peridiniaceae, and Symbiodiniaceae. The phylogeny of the parasitic dinoflagellate is not easily resolved due to the lack of taxa available for analysis. However, the parasitic dinoflagellate does fall within Order Peridiniales.

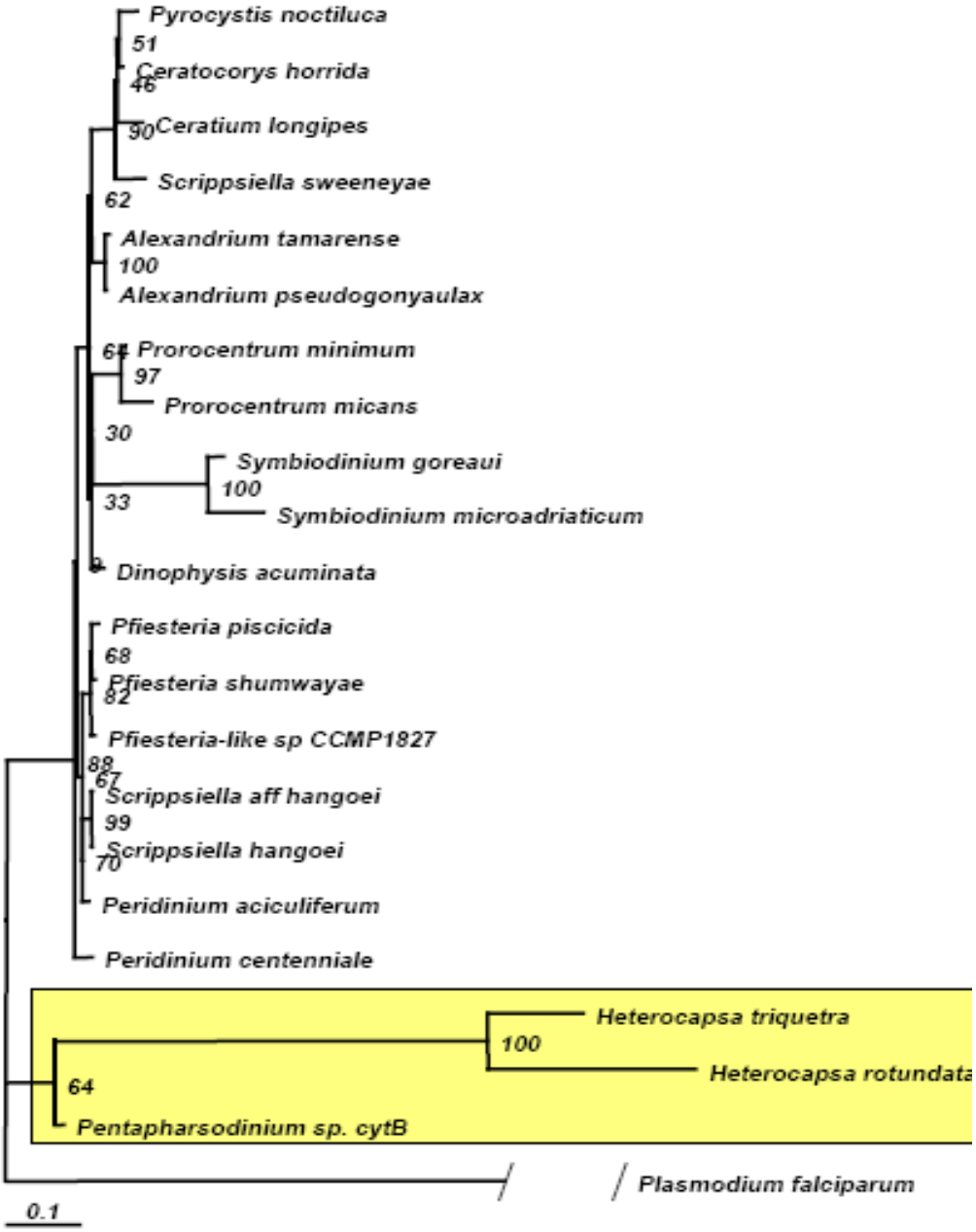

Figure 28 Cyt b analysis based on maximum likelihood, with 1,000 replicates using GTR model. The tree is a representation of phylogenetic relationships across various Orders of closely related dinoflagellates.

Discussion and Conclusions

It is instructive to recall that analyses of the cell morphology indicates that the parasitic dinoflagellate could be described as a *Pentapharsodinium* species and it is morphologically indistinguishable from *P. tyrrhenicum*, except for the antapical plates, which would be the ones most likely to be modified for attachment by the trophont. The molecular analyses appear to weaken that conclusion.

The two genera *Pentapharsodinium* and *Ensiculifera* sit in a monophyletic group (fig 26 and 27). In another study the Gottschling group concluded, based on phylogenetic analysis using ITS, 5.8S rRNA, and domains D1 and D2 of the LSU, that the two genera *Pentapharsodinium* and *Ensiculifera* form a monophyletic clade, termed E/Pe (Fig. 29) (Gottschling, Renner et al. 2008). The results of a study using ITS and morphological analyses of multiple calcareous dinoflagellates were also interpreted to provide strong evidence of monophyly and even showed the mixing of genera among *Pentapharso-dinium* and *Ensiculifera* (Fig. 30) (D'Onofrio, Marino et al. 1999).

In this study, 18S rDNA places the parasitic dinoflagellate within the E/Pe clade. The parasitic dinoflagellate falls within that clade with other *Pentapharsodinium* species and so is tentatively recognized as a *Pentapharsodinium* species. The strong homology between *P. tyrrhenicum* and the parasitic dinoflagellate, based on 18S rDNA, could suggest that the parasitic dinoflagellate is a variant of *P. tyrrhenicum*. Other *Pentapharsodinium* species have not been officially reported in North American waters. The observation that several *Ensiculifera* have been found in the Gulf of Mexico could suggest that the parasitic dinoflagellate is a species belonging to the E/Pe clade. It may be undergoing rapid evolution. In the study mentioned earlier by the Gottschling group

(Gottschling, Renner et al. 2008) evidence is presented suggesting that *Pentapharsodinium tyrrhenicum* evolved from a lineage that included the *Ensiculifera* genus sometime during the Cretaceous (Fig. 31).

ITS analyses showed the parasitic dinoflagellate appears to fit within the E/Pe clade (Fig. 27). ITS is used in phylogenetics to resolve genetic drift between populations. In this case, it reveals the mixing of genera on the E/Pe clade. Internal transcribed spacers 1 and 2 are introns known for particularly high rates of mutation (Blouin 2002). The variability within the ITS regions arises from the lack of evolutionary constraint. This occurs because they do not encode for a protein that can be acted upon by natural selection, and therefore can change without consequence for fitness. The mixing of genera could suggest that this molecularly and morphologically closely related group may need to be recharacterized and that members belonging to either taxa may be incorrectly categorized. Molecular analysis based on ITS in other studies in the E/Pe clade (D'Onofrio, Marino et al. 1999) provide evidence of genera mixing, and suggest that *E. imariensis* could be a species of *Pentapharsodinium* (Fig. 30). In another study involving ITS analysis (Hai-Feng and Yan 2007) the investigators placed a previously undescribed dinoflagellate into *Ensiculifera*, although the cell possessed the morphological plate tabulation of *Pentapharsodinium*. Due to inconsistencies within the E/Pe clade I feel that the resolvability of the ITS analysis can only show accurately that the dinoflagellate is a member of the Family Peridiniaceae.

Cytochrome b is a mitochondrial gene used as part of the electron transport chain involved in cellular respiration and the production of ATP. The mutation rates of mitochondrial genes are known to be more elevated than many nuclear genes (Brown,

George et al. 1979). The cause of a higher mutation rate is though to be attributed to the editing function of the mtDNA replication and the lack of enzymatic capability to remove or repair thymine dimers (Lansman and Clayton 1975). Therefore, changes in mitochondrial genes give a fine-tuned molecular clock, allowing for taxonomic assignment even beyond the species level.

In this study, analyses of cytochrome b gene sequences were unable to resolve the parasitic dinoflagellate to the level of Family. The problem arose from the lack of taxa needed to do a thorough phylogenetic analysis based on the cyt b gene. My analyses were able to resolve the parasitic dinoflagellate only to the level of Order Peridiniales (Fig. 28).

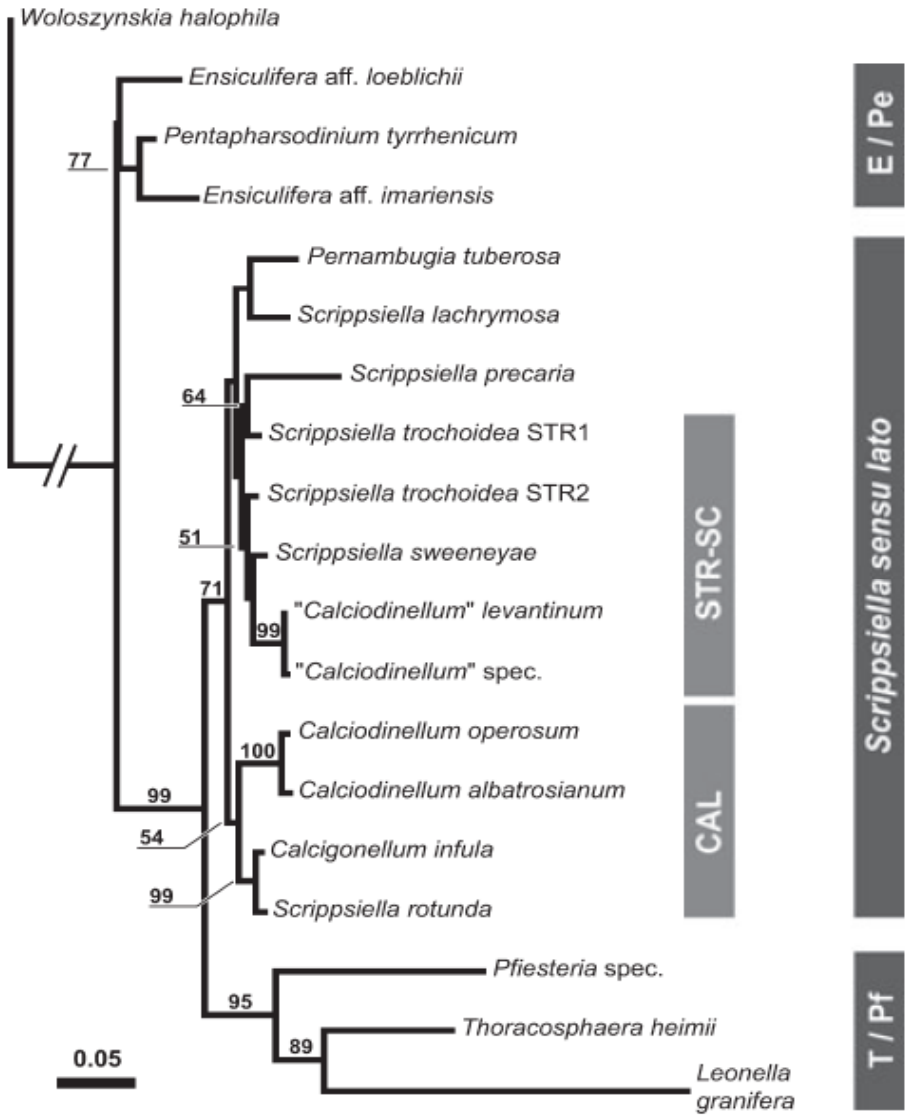

Figure 29. Maximum Likelihood (ML) phylogeny for calcareous dinoflagellates based on sequences of the LSU rRNA domains D1and D2, the 5.8S rRNA, and helices I and II of ITS1. Numbers above branches indicate ML bootstrap support from 1,000 replicates GTR model (Gottschling, Renner et al. 2008).

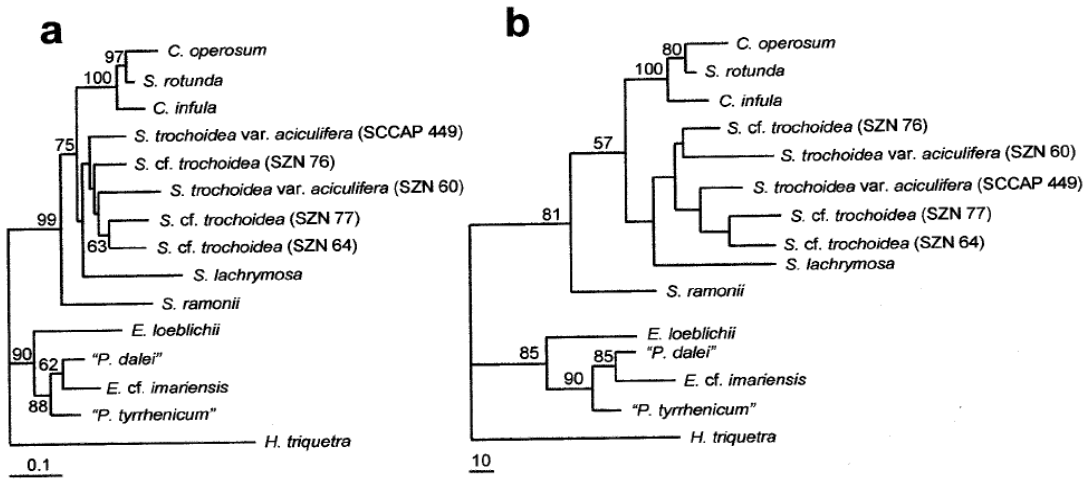

Figure 30 The phylogenetic trees obtained using ITS1 and ITS2 sequences. Numbers at internal branches indicate percentage of bootstrap (500 replicates); values < 50% have not been included. (a) Neighbor-joining analysis; scale bar = 10% divergence. (b) Parsimony analysis; scale bar = 10 steps (D'Onofrio, Marino et al. 1999).

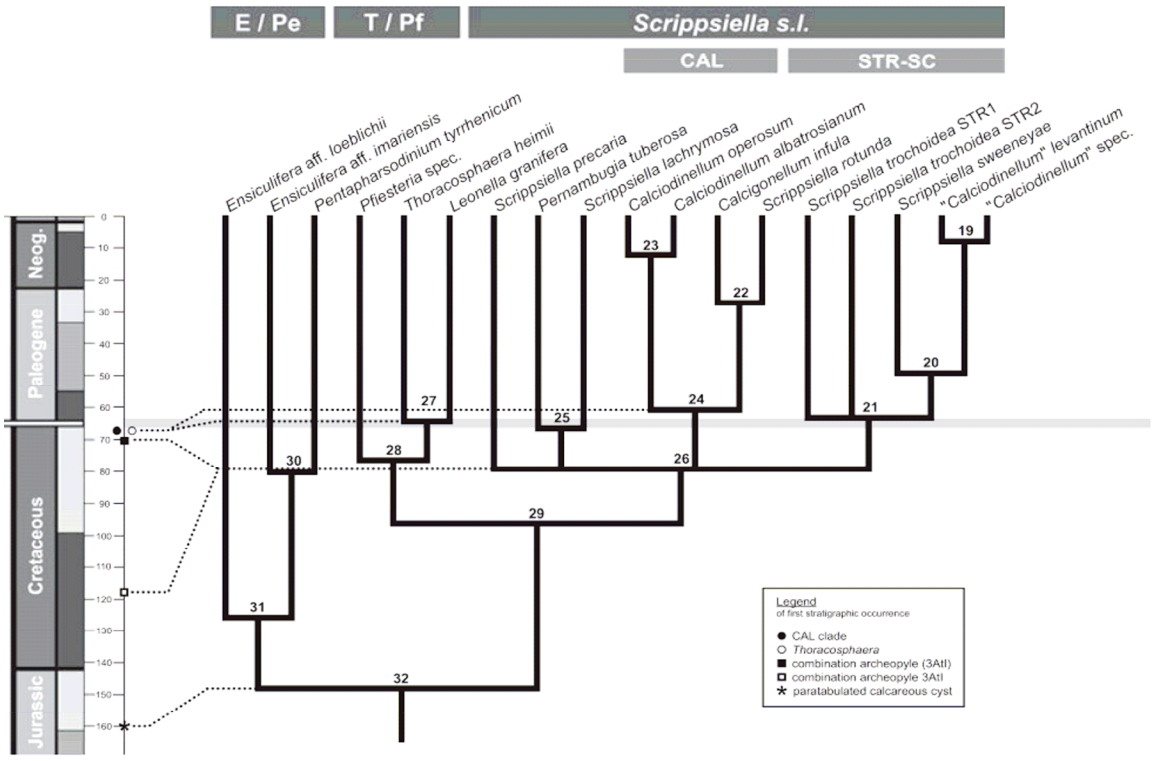

Figure 31. Chronogram depicting the separation of the E/Pe clade into separate genera during the Cretaceous period (D'Onofrio, Marino et al. 1999).

Overall conclusions

The molecular analyses presented here provide strong multiple levels of support that the parasitic dinoflagellate is a member of Order Peridiniales, Family Peridinaceae, and could provisionally be identified as a member of genus *Pentapharsodinium*. The molecular analyses, particularly at the 18S level, suggest near-identity with *P. tyrrhenicum*, but there is insufficient resolving power with the ITS and cyt b analyses to provide finer resolution than at the Family level.

Morphological analyses strongly suggest very close affinities to *P. tyrrhenicum*; yet the antapical plates strongly suggest that this cell is a distinctly different organism from the benthic autotroph described in 1993 by Montressor et al. It is instructive to consider that the antapical plates, which are the only ones that show morphological differences, would of course be the plates that would be most likely to be modified in an organism that exhibits cell attachment and nutrient capture via a ventroposterially emergent peduncle.

Analyses of the behavior and life cycle indicate that this organism is a mixotrophic dinoflagellate capable of parasitizing a ctenophore in a very specific host/parasite relationship. To date, since its first discovery in the early 1990s by Moss and colleagues, it has not been observed on any other host, despite the fact that the host ctenophore is prey to several species of fish (including *Menidia beryllina, Petrilus burti*, and *Mugil cephalus*) or the common sea nettle (*Chrysaora quinquecirrha*), a schyphozoan which is a major predator on *Mnemiopsis* (Moss, pers. communication). In addition, it has never been seen on other ctenphores The dinoflagellate is not an obligate parasite as evidenced by my ability to culture it *in vitro*.

Acknowledgements

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Appendix: 1 Molecular Analysis of *Trichodina ctenophorii* and an unknown amoeba associated to the comb plates of *Mnemiopsis*

During the course of this study some molecular and ultrastructural (SEM) work was performed on the other protists found associated with *Mnemiopsis.* DNA extractions and PCR amplifications were as described in chapter 4. The universal eukaryotic primers 18ScomF1 (forward) 5'-GCTTGTCTCAAAGATTAAGCCATGC-3' and 18ScomR1 (reverse) 5'-CACCTACGGAAACCTTGTTACGAC-3' that amplify the flanking regions of the 18S rDNA gene (Zhang, Bhattacharya et al. 2005) were used to amplify the 18S rDNA region of the organisms.

Trichodina ctenophorii were removed from the host by either direct pipetting or treating with a 100mM KCl solution. Cells were washed several times in sterilized artificial seawater to remove any ctenophore tissue and collected into acetone. After DNA extraction, PCR was performed, producing amplicons that were sequenced at the Genetic Analysis Laboratory of the Auburn Research Instrumentation Facility on an ABI 33100 sequencer. The chromatograms were visualized, edited and assembled to produce consensus sequences using Sequencher (Gene Codes Corporation Ver. 4.8, Ann Arbor, MI, USA). Contiguous sequences and sequences obtained from GenBank were aligned using ClustalX ver. 2.0.10 (Larkin, Blackshields et al. 2007) set on Multiple Alignment Mode. ClustalX was then used to construct a neighbor-joining phylogenetic tree.

18SCOMF1	TGGGCTTAATCTTTGAGACAAGCAGTTGCGTGGACTCATAGTAACTGATCG
	GATCGCTTCGGCGATGAGTCATTCAAGTTTCTGCCCTATCAGCTTTGATGG
	TAGTGTATTGGACTACCATGGCAGTCACGGGTAACGGAGAATTAGGGTTCG
	GTTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCA
	GGCGCGTAAATTACCCAATCCTGATTCAGGGAGGTAGTGACAAGAAATAAC
	AACCTGGGGCTTTGCTTTCGGGATTGCAATGATCGTAATCTAAAGCAATTA
	GAAAGAAACCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCA
	GCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTCAA
	CTTCTGCCCCGGGGCCGAGAGGCGACTCGGAGGTCCCGGGGCATCCGTTCC
	GCACCACGTCTACGCGTGAGGGCGGACAGTTTACCTTGAGAAAATTAGAGT
	GTTCACGCAGGCGTAGCCAGTATACATTAGCATGGTATATGGTAAGAGGAC
	TCCAAGCCGTTGTTGGT

Table 12: Contiguous sequence of *Trichodina ctenophorii* using primer 18SCOMF1

18SCOMR1	GGGACGTAATCAGCGCAAGCTGATGACTTGCGCTTACTAGGAATTCCTCGT
	TCACGACCCATAATTGCAAGGGTCGATCCCAATCACGGCACACCCTGACAG
	GTTACCCGGCTCCCTTCGGATCAGGAAACTCGCTGTGTGTGCCATTGTAGC
	GCGCGTGCGGCCCAGGACATCTAAGGGCATCACAGACCTGTTATTGCCTCA
	AACTTCCGTGCGATAGGCTCGCACAGTCCCTCTAAGAAGCACCTTCCGTTG
	AGACGGGGTGCTAGTTAGCAGGTTAAGGTCTCGTTCGTTAAAGGAATTAAC
	CTGACAAATCACTCCACCAACTAAGAACGGCCATGCACCACCACCCGTAGA
	ATCAAGAAAGAGCTCTCAATCTGTCCATCACACCCACGTTTTGACCTGGTA
	AGTTTCCCCGTGTTGAGTCAAATTAAGCCGCAGGCTCCACTCCTGGTGGTG
	CCCTTCCGTCAATTCCTTTAAGTTTCAGCCTTGCGACCATACTCCCCCCAG
	AACCCAAAGACTTTGATTTCTCGTACGGACCCAGCCAGGGACAATCCCTGA
	CTGAATCCGAGTCGGTATGGTTTATGGTTTAGGACTAGGACGGTATCTGAT
	CGTCTGTGATCCCCTAACTTTCGTTCTTGATCAATGAAAACATCCTT

Table 13: Contiguous sequences of *Trichodina ctenophorii* using primer 18SCOMR1

Trichodina species	BPs	Gen Bank No.
Trichodina ctenophorii	1237	(this study)
Trichodina sinipercae	1704	EF599288.1
Trichodina hypsilepis	1693	EF524274.1
Trichodina heterodentata	1698	AY788099.1
Trichodina reticulate	1702	AY741784.1
Trichodina sp. LAH-2003a	764	AY363960.1
Trichodina nobilis	1698	AY102172.1
Cryptomonas paramecium	1984	AJ420676.2

Table 14: List of Trichodina species and GenBank accession numbers

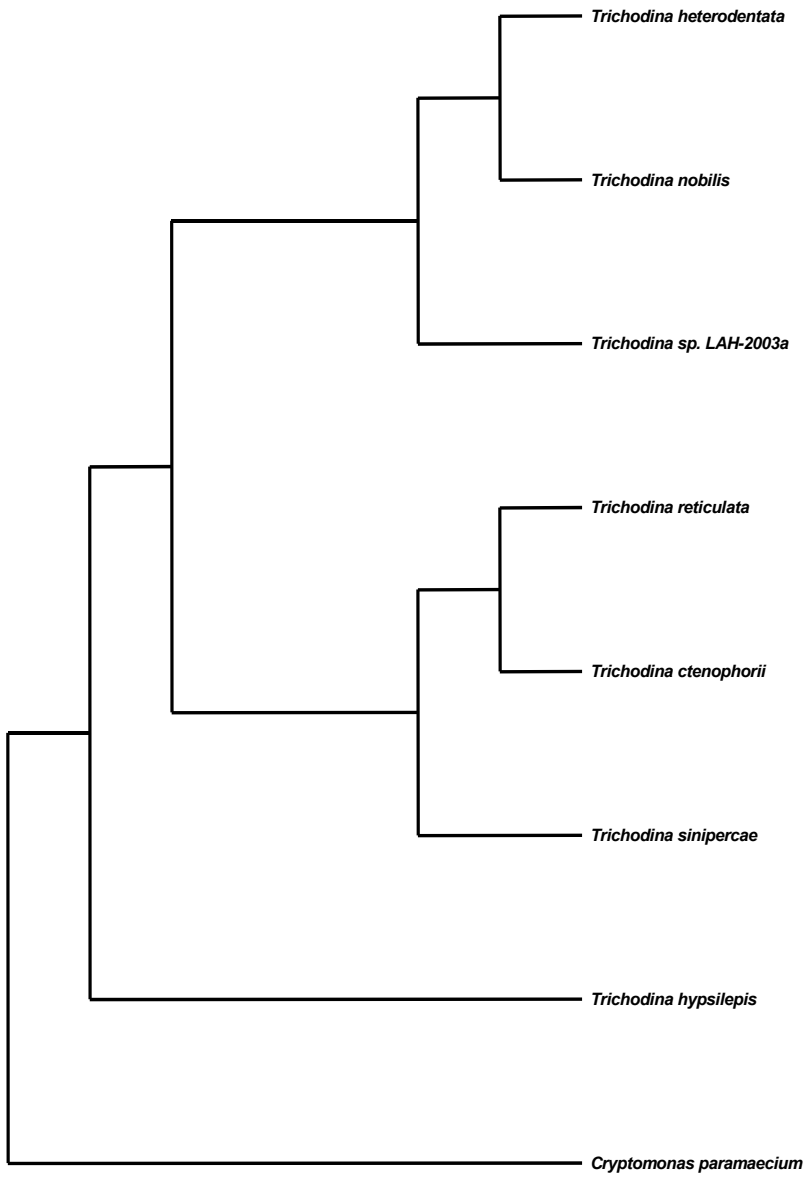

Figure 32 Neighbor Joining Tree of *Trichodina* showing that *Trichodina Ctenophorii* sits within the *Trichodina* group.

The resulting data placed *Trichodina ctenophorii* within the genus *Trichodina* under the family Trichodinidae. The phylogeny of *T. ctenophorii* may not be completely accurate due to the lack of a complete gene sequence. However, based on the presented data it is clear that *T. ctenophorii* belongs to the genus *Trichodina*.

The *Flabellula* – like gymnamoebae associated with the comb plates of *Mnemiopsis* may possibly be an unknown marine stramenopile. The universal eukaryotic

primers mentioned previously were utilized in the molecular analysis (Table 13). Amoebae were collected from cultures grown in MY100 medium at room temperature. Cells were centrifuged at 10,000g and the medium decanted off. The pelleted cells were used for DNA extraction as described in chapter 4.

Primer	Sequence	Region	Specificity	Reference
23FPL	5′-GCGGATCCGCGGCCGCTGCAGAYCTGGTYGATYCTGCC-3′	18S rDNA	Not specified in publication	(Barns, Fundyga et al. 1994)
518R	5'-ATTACCGCGGCTGCTGG-3'	18S rDNA	V3 region of 16S rDNA	(Muyzer, de Waal et al. 1993)
SimF	5'-AYCTGGTTGATYYTGCCAG-3'	18S rDNA	Universal primers that encompass the V1, V2, V3, V4, V7, and V8 regions	(Sims, Aitken et al. 2002)
SimR	5'-TGATCCATCTGCAGGTTCACCT-3'	18S rDNA	Universal primers that encompass the V1, V2, V3, V4, V7, and V8 regions	(Sims, Aitken et al. 2002)
18ScomF	5’-GCTTGTCTCAAAGATTAAGCCATGC-3’	18S rDNA	Flanking regions of the 18S rDNA	(Zhang, Bhattacharya et al. 2005)
18ScomR	5’-CACCTACGGAAACCTTGTTACGAC-3	18S rDNA	Flanking regions of the 18S rDNA	(Zhang, Bhattacharya et al. 2005)

Table 15: List of PCR primers for *Flabellula* – like gymnamoebae analysis

18ScomF1	TGTCAGTTAAGCGACTTTTTACTGTGAACTGTGAACGGSTCATTACATCG
	GTTCTAGTCTCTTTGGTAGTTCATCGTGTGTGTCATCTTCCCTTTCGGGG
	AGAGCACGCAAGGTTTARTTGGATAACTGTCATAATTTGAGAGCTAATAC
	ATGCCTAAAAGTCCTCGGTTGCTGCTTTTTGCAGGGATGGGGATGCGTTT
	ATTARATTGAGACCGGAGGCGCGCAAGCGTCGTTTTGTAAGGTGACTCAC
	AATAACCACTCGGATCGCTCTTCGTGAGCGATGTACCATTCSAGTTTCCG
	TCCTATCATGCTTGGAAGGKAAGGTATCGGCTTACCTTGGCGTTAACGGG
	CAACGGARAATTAGGGTTCGGTTCCGGARAGGGGGCCTGAGACATGGCCA
	CCACATCCAAGGAAGGCAGCAGGCGCGTAAATTACCCAATCCTAACTCAG
	GGAGGTAGTGACAATAACTAACGATGGTGCGCGCATGTTCCGTTTATCGG
	AAGATCGTACACCAATCGTCATGAGAACAATCTAAACACCTTATCGAGGA
	ACCATTGGAGGGCCAGTCTGGTGCCAG

Table 16: Contiguous sequences of *Flabellula* – like gymnamoebae using primer 18SCOMF1.

23FPL	TCATACGCTTGTCTCAAGATTAAAGCCATGCATGTCAAGTTAAAGCGACT
	TTTTAACTGTGAAACTGTGAACGGCTCATTACATCGGTTCTAGTCTCTTT
	GGGAGTTCATCGTGTGTGTCATCTTCCCTTTCGGGGAGAGCACGCAAGGT
	TTAGTTGGATAACTGTCATAATTTGAGAGCTAATACATGCCTAAAAGTCC
	TCGGTTGCTGCTTTTTGCAGGGATGGGGATGCGTTTATTAGATTGAGACC
	GGAGGCGCGCAAGCGTCGTTTTGTAAGGAGACTCACAATAACCACTCGGA
	TCGCTCTTCGTGAGCGATGTACCATTCGAGTTTCCGTCCTATCATGCTTG
	GAAGGTAAGGTATCGGCTTACCTTGGCGTTAACGGGCAACGGAGAATTAG
	GGTTCGGTTCCGGAGAGGGGGCCTGAGACATGGCCACCACATCCAAGGAA
	GGCAGCAGGCGCGTAAATTACCCAATCCTAACTCAGGGAGGTAGTGACAA
	TAACTAACGATGGTGCGCGCATGTTCCGTTTATCGGAAGATCGTACACCA
	ATCGTCATGAGAACAATCTAAACACCTTATCGAGGAACCATTGGA

Table 17: Contiguous sequences of *Flabellula* – like gymnamoebae using primer 23FPL.

518R	TGGCCTCCATGGTTCCTCGATAAGGTGTTTAGATTGTTCTCATGACG ATT
	GGTGTACGATCTTCCGATAAACGGAACATGCGCGCACCATCGTTAG TTAT
	TGTCACTACCTCCCTGAGTTAGGATTGGGTAATTTACGCGCCTGCTG CCT
	TCCTTGGATGTGGTGGCCATGTCTCAGGCCCCCTCTCCGGAACCGAA CCC
	TAATTCTCCGTTGCCCGTTAACGCCAAGGTAAGCCGATACCTTACCT TCC
	AAGCATGATAGGACGGAAACTCGAATGGTACATCGCTCACGAAGA GCGAT
	CCGAGTGGTTATTGTGAGTCACCTTACAAAACGACGCTTGCGCGCCT CCG
	GTCTCAATCTAATAAACGCATCCCCATCCCTGCAAAAAGCAGCAAC CGAG
	GACTTYTAGGCATGTATTAGCTCTCAAATTATGACAGTTATCCAACT AAA
	CCTTGCGTGCTCTCCCCGAAAGGGAAGATGACACACACGATGAACT ACCA
	AAGAGACTAGAACCGATGTAATGAGCCGTTCACAGTTTCACAGTTA AAAA
	GTCGCTTTAACTTGACATGCATGGCTTTAATCTTTGAGACAAGCGTA TGA

Table 18: Contiguous sequences of *Flabellula* – like gymnamoebae using primer 518R.

The contiguous sequences were BLASTned against the NCBI database. The sequences that resembled the most homology were marine stramenopiles. However, the homology was only 87%, suggesting that the sequence data may be unreliable or possibly that the organism is undescribed. The literature does set precedence for marine stramenopiles parasitizing marine invertebrates (Raghukumar 2002). However, given the morphology of the organism it is highly unlikely that it is a marine stramenopile. It is therefore my conclusion that the sequence data is from a contaminating organism held in culture.

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