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Classification and Identification of *Pfiesteria* and *Pfiesteria*-Like Species

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Dinoflagellates can be classified both botanically and zoologically; however, they are typically put in the botanical division Pyrrophyta. As a group they appear most related to the protistan ciliates and apicomplexans at the ultrastructure level. Within the Pyrrophyta are both unarmored and armored forms of the dominant, motile flagellated stage. Unarmored dinoflagellates do not have thecal or wall plates arranged in specific series, whereas armored species have plates that vary in thickness but are specific in number and arrangement. In armored dinoflagellates, the plate pattern and tabulation is a diagnostic character at the family, subfamily, and even genus levels. In most cases, the molecular characterization of dinoflagellates confirms the taxonomy on the basis of external morphology; this has been demonstrated for several groups. Together, both genetic and morphological criteria are becoming increasingly important for the characterization, separation, and identification of dinoflagellates species. *Pfiesteria* and *Pfiesteria*-like species are thinly armored forms with motile dinospore stages characterized by their distinct plate formulae. *Pfiesteria piscicida* is the best-known member of the genus; however, there is at least one other species. Other genetically and morphologically related genera, now grouped under the common names of "Lucy," "Shepherd's crook," and cryptoperidiniopsoid, are being studied and described in separate works. All these other heterotrophic dinoflagellate groups, many of which are thought to be benign, co-occur in estuarine waters where *Pfiesteria* has been found. **Key words:** *Pfiesteria*, cryptoperidiniopsoid, "Lucy," dinoflagellates, *Pfiesteria*-like, Kofoidian, plate patterns, morphology. — *Environ Health Perspect* 109(suppl 5):661–665 (2001). <http://ehpnet1.niehs.nih.gov/docs/2001/suppl-5/661-665steidinger/abstract.html>

Harmful algal bloom (HAB) species can produce a variety of biotoxins that can cause human illness, mass mortalities of aquatic organisms, and disease. Many of the biotoxins can be detected or assayed directly. In known toxin producers it is possible to determine when potentially harmful algal species are present in sufficiently high abundance levels to produce toxins, then to employ the more sensitive analytical techniques or bioassays to quantify the amount of toxin(s) present in the environment. Unfortunately, for some toxins there currently are no assays or appropriate analytical techniques because the toxins remain uncharacterized. In this situation the most efficient strategy for identifying a potential public health threat is to rapidly determine if high, potentially toxic concentrations of the organism are present so that appropriate response plans can be implemented. Quantifying the number of HAB species present in a sample can present difficulties because morphologically similar, but benign, species can be mistaken for the toxin-producing species. This is true for *Pfiesteria piscicida*, a small heterotrophic dinoflagellate that has been associated with fish kills and the production of a putative

biotoxin in North Carolina and Maryland (1–3). Originally, it was assumed that small heterotrophic dinoflagellates of a particular shape, and approximately 10 µm in length, were all *P. piscicida*. That morphologically similar co-occurring species could be misidentified with *P. piscicida* has been recognized (4). Because a number of related nontoxic *Pfiesteria*-like species co-occur with *P. piscicida*, assessment of the potential public health threat of this species is complicated. This article is part of the National Conference on "Pfiesteria: From Biology to Public Health" and covers what is currently known about the higher-level placement of the *Pfiesteria*-like organisms and the taxonomic relationship between *P. piscicida* and co-occurring morphologically similar species.

Higher-Order Taxonomic Placement of *P. piscicida* and Related Dinoflagellates

Pfiesteria and several *Pfiesteria*-like genera are dinoflagellates classified in the botanical division Pyrrophyta. They are morphologically and genetically related and may derive from a common ancestor. The only valid and available named species as of this conference (5) is

P. piscicida Steidinger and Burkholder (6), which has a zoospore stage characteristic of armored dinoflagellates. A second species, to honor Dr. Sandra Shumway, was named after the conference (7). Armored dinoflagellates have an outer wall of segments, or plates, arranged in specific, mostly horizontal series (Kofoidian series) (Figure 1). These series constitute a plate pattern and formula and are useful in separating genera (8). There is a good correlation between the phylogenetic relationships among dinoflagellates based on plate structure and those based on small subunit rRNA and rDNA sequences (9,10).

Because dinoflagellates have characteristics that historically have been considered "botanical," e.g., presence of chloroplasts, as well as "zoological," e.g., flagella and heterotrophic mode of feeding, there are several classification schemes. Recently, Fensome et al. (11) proposed a single classification scheme that addressed fossil and extant groups and followed the International Botanical Code of Nomenclature. In the same article, the authors reviewed 39 classification schemes for living dinoflagellates dating back to the 1800s. Some of the classification characteristics included plate tabulation, life cycle, and the unique dinokaryon nucleus. In that work (11) and elsewhere, plate tabulation is considered the most important morphological characteristic to differentiate within the division Pyrrophyta at the family or subfamily rank. It is also used at the generic level in

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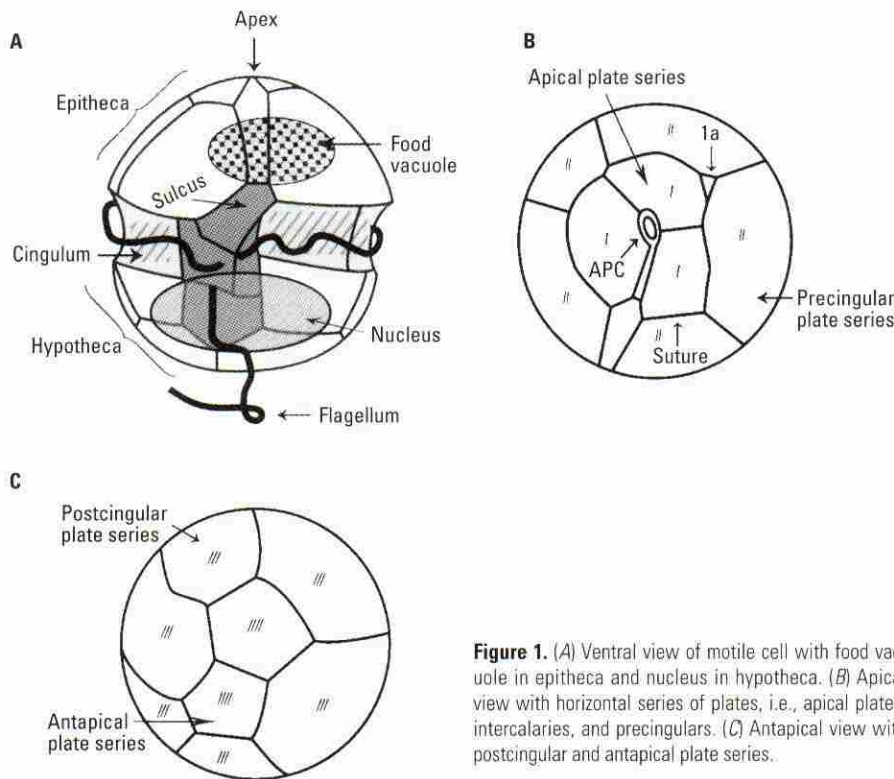


Figure 1. (A) Ventral view of motile cell with food vacuole in epithea and nucleus in hypotheca. (B) Apical view with horizontal series of plates, i.e., apical plates, intercalaries, and precingulars. (C) Antapical view with postcingular and antapical plate series.

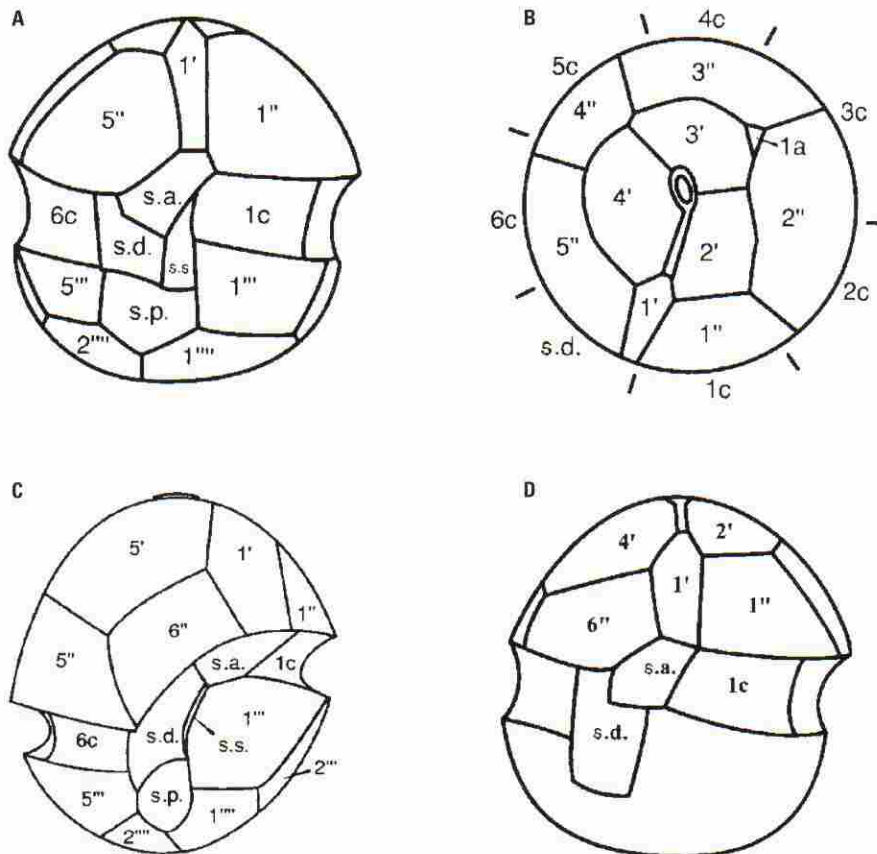


Figure 2. Schematic illustrations of *Pfiesteria* and *Pfiesteria*-like dinoflagellate. These four panels show how the epithecal plates fit together and how they are labeled. (A) Ventral view of *P. piscicida*. (B) Apical view of *P. piscicida*. (C) Ventral view of cryptoperidiniopsisoid. (D) Ventral view of the epithea of "Lucy."

most of the orders containing the majority of armored species, e.g., Gonyaulacales and Peridinales (12).

In general, dinoflagellates are thought to have affinities with ciliates and apicomplexans at the ultrastructure level because of similar organelle structures such as tubular mitochondrial cristae, type of spindle, rod-shaped trichocysts, and cortical vesicles, as well as at the genetic level (9,11,13). Dinoflagellates distinctly have a dinokaryon nucleus with continually condensed chromosomes throughout the entire life cycle, or in at least one stage of the life cycle, and characteristic flagella insertion.

A possible phylogenetic scenario or tree was presented by Fensome et al. (11), with support from the fossil record. This proposed phylogeny suggests that, structurally, unarmored dinoflagellates with unfilled cortical vesicles (hundreds) preceded armored forms in the evolutionary process; the armored forms then evolved from groups having many plates (vesicles filled with polysaccharide plate material) to those having only a few plates, as in the order Prorocentrales. In this scheme, the placement of *Pfiesteria* and related genera depends on several attributes that need further clarification. Originally, *Pfiesteria* was placed in the order Dinamoebales, which incorporates species with multiple life history stages, including dominant amoeboid forms. However, there are concerns that the dominant life history stage is a motile dinospore or coccoid stage with closer morphological affinities to the order Peridinales (14). Does *Pfiesteria* have dominant amoeba and/or coccoid stages? Even if the dominant state is an amoeba, there have been proposals to transfer *Pfiesteria* from the order Dinamoebales to the order Phytodiniales (11) or to place *Pfiesteria* specifically into the order Blastodiniales (9). Landsberg et al. (15) raised similar questions about life-cycle stages in the parasitic dinoflagellate *Amyloodinium*, and it was suggested that *A. ocellatum* belonged in the order Peridinales on the basis of the morphology of the dinospore stage. Recent phylogenetic work on the rRNA ITS and 5.8S regions supports the placement of *Pfiesteria* in the Peridinales or in a group between the Peridinales and the Blastodiniales (16). All these options need to be carefully considered and, for now, most are theoretical schemes based on limited morphological, genetic, and physiological life history data for these orders.

Methods and Materials Used to Isolate and Specifically Identify *Pfiesteria* and *Pfiesteria*-Like Organisms

Single cells of flagellated motile stages of *Pfiesteria* and *Pfiesteria*-like organisms were isolated from water samples collected in

Maryland, North Carolina, and Florida estuaries or from estuarine water incubated with microalgal prey. Isolated single cells were washed by transferring them between drops of diluted seawater using a micropipette. The cells were then introduced to individual wells in a 24-well plate containing filtered natural seawater diluted to the salinity of the original sample. These heterotrophs were fed cryptomonad algal prey (Provasoli-Guillard CCMP 1319; Bigelow Laboratory for Ocean Science, West Boothbay Harbor, ME, USA) and when growth occurred, contents of the well were transferred to flasks for maintenance (15 psu, 22°C, and 12:12 light:dark cycle) as a research culture in collection facilities at the Florida Marine Research Institute in St. Petersburg, Florida. Other cultures (17) were similarly isolated and maintained at other institutions. Clonal isolates were used to prepare specimens for scanning electron microscopy (SEM) so that the thecal plate patterns and tabulations could be determined reliably from one population. Over 63 clonal cultures were prepared for SEM, and they resulted in more than 1,000 micrographs of individual cells. In addition, more than 3,000 micrographs of individual cells were reviewed from field samples. The most frequently used fixation and preparation protocol was that of Truby (18), in which cells were either stripped or osmotically swollen to visualize sutures. Sutures (Figure 1B) are the visible lines between plates, like grouting between tiles. These same isolates were used for genetic studies (9,19–22) and for the development of whole cell and other molecular probes.

Results and Discussion

All the *Pfiesteria* and *Pfiesteria*-like dinospores are heterotrophic, typically with a dinokaryon nucleus in the hypotheca and food vacuoles in the epitheca (Figure 1A). The longitudinal flagellum has two parallel parts, and the peduncle, a feeding organelle, emerges from under the flexible right sulcal plate. The sulcus is not straight but offset. Food vacuoles can contain whole phytoplankton cells or whole chloroplasts. At least in *P. piscicida*, Lewitus et al. (23) documented the chloroplasts to be functional for a period of time. The genus *Pfiesteria* is characterized as having a plate formula/tabulation of apical pore complex (APC)

(pore plate, closing plate, and X plate), 4', 1a, 5'', 6c, 4s, 5''', 0p, 2'''' (Figures 2A, 2B, 3). An unnamed marine *Pfiesteria* (*Pfiesteria* sp. "marina," Figure 4) (24) has the same plate formula (Table 1). Both have a triangular 1a (first intercalary plate) (Figures 1B,2B) on the left shoulder, which is one of the characteristics of the genus. Another proposed *Pfiesteria* (7), *Pfiesteria shumwayae* (Figure 5), has a larger, almost rectangular 1a plate and a plate formula distinct from that of *Pfiesteria* (six precingular plates instead of five). This species, because of its difference in plate tabulation, should be reevaluated as to its genetic and morphologic affinity with *Pfiesteria*. Both species have been implicated as producing toxins (3,6,7), though a purified toxin has yet to be isolated and characterized for either species.

Pfiesteria-like organisms, by our definition, include numerous species that are superficially similar when viewed at the light microscopic level but distinct at the ultrastructural level. These are small (10–20 μm) cells lightly armored with defined plate formulae/tabulations and occupying ecological niches similar to *Pfiesteria*. Many of these species are not harmful but may be closely related genetically to those that are. The following summarizes the current knowledge regarding the taxonomic relationships of these similar groups and genera.

Cryptoperidiniopsoids (inferring that they are cryptically related to *Peridiniopsis*) are morphologically similar heterotrophs but have a plate formula of APC, 5', 0a, 6'', 6c, 4s, 5''', 0p, 2'''' (Figures 2C, 6; Table 1). The

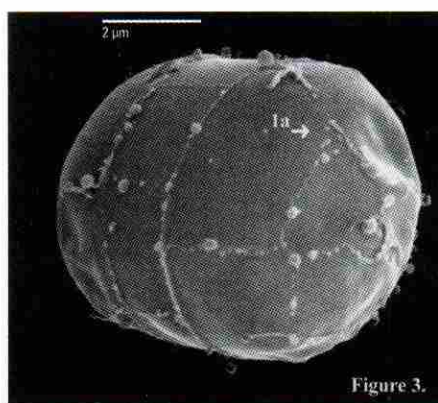


Figure 3. *P. piscicida*. SEM micrograph shows triangular 1a plate adjacent to 3'. Cell < 15 μm.

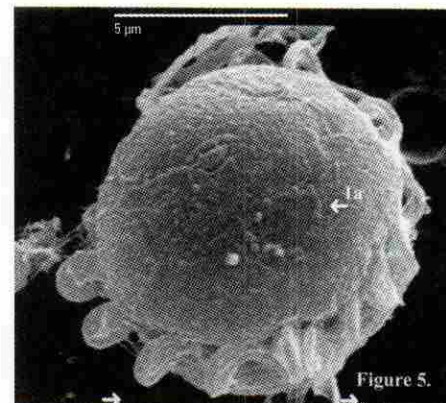


Figure 5. *Pfiesteria shumwayae*. SEM micrograph showing oblong plate in between 2' and 3'. Cells < 15 μm.

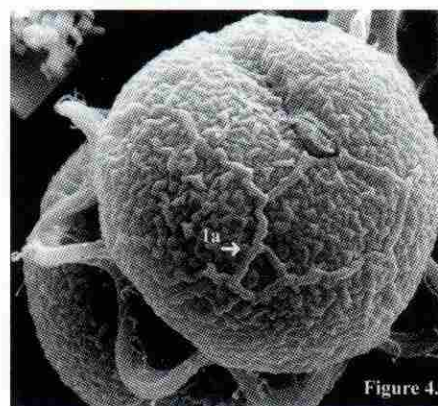


Figure 4. *Pfiesteria* sp. "marina". SEM micrograph shows triangular 1a plate adjacent to 3'. Cell < 15 μm.

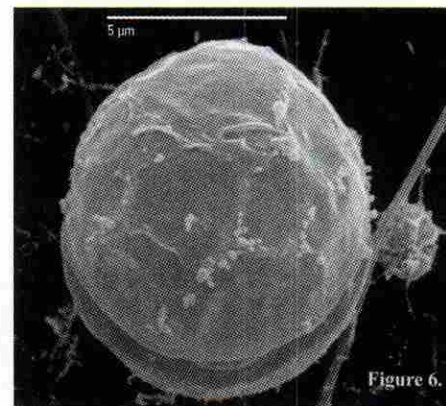


Figure 6. Cryptoperidiniopsoid species. SEM micrograph shows absence of anterior intercalary plates. Cell < 15 μm.

Table 1. Kofoidian plate tabulations for *Pfiesteria* and *Pfiesteria*-like species.

	APC	Apicals	Anterior intercalaries	Precingulars	Cingulars	Sulcels	Postcingulars	Posterior intercalaries	Antapicals
<i>Pfiesteria</i>	APC	4'	1a	5''	6c	4s	5'''	0p	2''''
Cryptoperidiniopsoid	APC	5'	0a	6''	6c	4s	5'''	0p	2''''
"Lucy"	APC	4'	2a	5''	6c	4s	5'''	0p	2''''
<i>Peridiniopsis</i>	APC	3–5'	0/1a	6/7''	6c	3–5s	5'''	0p	2''''

Abbreviations and symbols: a, anterior intercalary; c, cingular; ', apical; p, posterior intercalary; s, sulcal; '', precingular; ''', postcingular; ''', antapical. Figure 2 illustrates the ventral view and plate designations for *Pfiesteria*, "Lucy," and cryptoperidiniopsoid.

genus *Peridiniopsis* has a plate formula APC, 3–5', 0–1a, 6–7'', 6c, 3–5s, 5''', 0p, 2'''''. When a range in epithecal (top half of the cell) in plate tabulation is evident, in the epithecal series (top half of the cell), the presence of more than one genus is indicated (although there are several exceptions to this general rule, most notably the genus *Pyrophacus*). An example of a similar situation occurred with the genus *Gonyaulax*. In the 1930s, the plate formula was APC, 3–6', 0–3a, 6'', 6c, 6''', 1p, 1'''''. It is now recognized that there are at least four genera: *Gonyaulax sensu stricto*, *Alexandrium*, *Amylax*, and *Lingulodinium* (Table 2). Each genus has a specific number of apical and anterior intercalary plates (8). In the order Peridinales, genera are even differentiated on the basis of the number of cingular plates, e.g., 3 versus 4 versus 5 versus 6 (Table 3) (12). Cryptoperidiniopsoids representing several species are one of the most common groups of dinoflagellates found in samples with *Pfiesteria*. There is no evidence that the cryptoperidiniopsoid species produce ichthyotoxins, but they do have bioactive compounds (25). Hence, misidentifying the cryptoperidiniopsoids as *P. piscicida* or *P. shumwayae* may overestimate any potential public health threat.

Another group of new species, with a plate formula of APC, 4', 2a, 6'', 6c, 4s, 5''', 0p, 2'''' (Figure 2D), is being referred to as "Lucy" (Figure 7), after a common name given to a Florida isolate. These species also have a posterior dinokaryon nucleus in the dinospore, are heterotrophic, possess a peduncle that is extendable from under the right sulcal plate, and have a sulcus that is offset to the right. "Lucy" cells have two diamond-shaped anterior intercalaries (1a and 2a) at the left and right side of the 3' plate (Figure 2D). "Lucy" is distinct from *Pfiesteria* and the cryptoperidiniopsoids, both morphologically and genetically, yet these groups are

closely related. Preliminary evidence indicates that "Lucy" produces bioactive ichthyotoxic compounds (25).

Another new heterotroph species in the order Peridinales, with a common name, "Shepherd's crook," because of its unusual crook-shaped canal or X plate, co-occurs geographically with species in the family Pfiesteriaceae, but it does not have the appropriate plate formula or offset sulcus to be included in that family (Figure 8). The species needs further workup to properly assign it to a genus. Its potential to produce toxins is unknown, but the species is thought to be benign.

Pfiesteria and its relatives are widely distributed, and like other dinoflagellates, they can have benthic stages, which could account for their recurrence in specific areas. Burkholder and Glasgow (26) and colleagues (27) have found there are benign stages of *P. piscicida* and that when exposed to fish, the organism may become toxic and produce an ichthyotoxic compound. However, neither the morphological (4,6) nor the biochemical identification of species, using molecular probes (19,20,28),

differentiates toxic from nontoxic or non-inducible *P. piscicida* forms. Hence, from a public health standpoint, the presence of *P. piscicida* alone can potentially overestimate any toxic threat from this species. The same situation applies to *P. shumwayae*, as both species are part of the same toxic *Pfiesteria* complex (26).

In addition to *Pfiesteria* and its related species, many other toxic or potentially toxic strains of HAB species occur in the coastal regions of the United States. These species include armored and unarmored dinoflagellates, rhabdophytes, diatoms, and blue-green algae or cyanophytes such as *Karenia* spp., *Chattonella* spp., *Pseudonitzschia* spp., and *Microcystis* spp. Many of these species produce well-characterized, highly toxic compounds that adversely affect both ecosystems and human health. The economic and ecological impacts from these blooms can be substantial, and the public health risks are still being assessed. It is important that we not lose sight of these harmful algae and their impacts in our pursuit to address harmful algal issues regarding *Pfiesteria*.

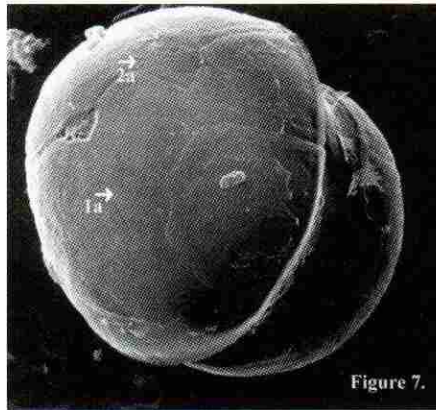


Figure 7. "Lucy." SEM micrograph showing diamond-shaped 1a and 2a plates separated by 3'. Cell < 15 μ m.

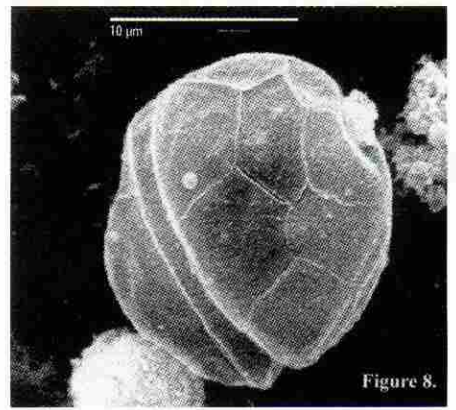


Figure 8. "Shepherd's crook." SEM micrograph of adjoining 1a and 2a plates. Cell > 15 μ m.

Table 2. Kofoidian plate tabulations for Gonyaulacoid.

	APC	Apicals	Anterior intercalaries	Precingulars	Cingulars	Sulcels	Postcingulars	Posterior intercalaries	Antapicals
Kofoid's <i>Gonyaulax</i>	APC	3–6'	0–3a	6''	6c	s	6'''	1p	1''''
<i>Gonyaulax</i>	APC	3'	2a	6''	6c	7s	6'''	0p	2''''
<i>Alexandrium</i>	APC	4'	0a	6''	6c	9–10s	5'''	0p	2''''
<i>Amylax</i>	APC	3'	3a	6''	6c	7–8s	6'''	0p	2''''
<i>Lingulodinium</i>	APC	3'	3a	6''	6–8c	7s	5'''	0p	2''''

Table 3. Kofoidian plate tabulations for Peridinioid.

	APC	Apicals	Anterior intercalaries	Precingulars	Cingulars	Sulcels	Postcingulars	Posterior intercalaries	Antapicals
<i>Glochidinium</i>	APC	4'	0a	6''	3c	4s	5'''	0p	2''''
<i>Protoperdinium</i>	APC	4'	2/3a	7''	4c	6s	5'''	0p	2''''
<i>Peridinium</i>	APC	4'	2/3a	7''	5/6c	5/6s	5'''	0p	2''''
<i>Scrippsiella</i>	APC	4'	3a	7''	6c	4/5s	5'''	0p	2''''
<i>Amyloodinium</i>	APC	4'	1a	7''	6–8c	7s	5'''	0p	2''''

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