

## Taxonomy of *Pfiesteria* (Dinophyceae)

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### **Abstract:**

The dinoflagellate species originally described as *Pfiesteria shumwayae* Glasgow et Burkholder, recently transferred to a new genus, *Pseudopfiesteria* Litaker et al., is reclassified into the redefined genus *Pfiesteria* Steidinger et Burkholder, as *Pfiesteria shumwayae* within the order Peridiniales. This change is based upon consideration of a compilation of previous and new morphological analyses and molecular phylogenetic analyses. Morphological analysis with scanning and transmission electron microscopy supports previous findings except in the sulcal area. In the cells examined, the sulcus is partly concealed by the peduncle cover plate (p.c.), which originates at the right side of the sulcus along the left side of the 6c and 5''' plates. The fine structure of the p.c. appears similar to that of other thecal plates. The 1'' plate can also extend slightly over the sulcus. Transmission electron microscopy revealed that *Pfiesteria shumwayae* can have at least six sulcal plates; the number remains uncertain and may vary. The sulcal plates of this small, delicately thecate species have not been clearly discerned by scanning electron microscopy of membrane-stripped and/or suture-swollen cells. The Kofoidian thecal plate formula for the genus *Pfiesteria* is Po, cp, X, 4', la, 5-6'', 6c, p.c., ?s, 5''', 0p, 2'''. The monophyletic grouping of "pfiesteria-like" taxa within the order Peridiniales, as well as the grouping of *Pfiesteria piscicida* and *Pfiesteria shumwayae* within the same genus, is also supported by the preponderance of previous molecular evidence, and by the phylogenetic trees contributed in the present analysis. *Pfiesteria* appears to be closely related to as-yet informally described cryptoperidiniopsoids and calcareous dinoflagellates such as *Thoracosphaera*; thus, the family classification requires revision that is beyond the scope of this study.

**Keywords:** *Pfiesteria*; *Pfiesteria shumwayae*; Electron microscopy; Morphology; Plate tabulation; Ribosomal genes; Phylogenetic trees; Taxonomy; Toxicogenic

**Abbreviations:** a, anterior intercalary plate(s); APC, apical pore complex; bp, base pair; c, cingular plates; cp, closing plate, part of the APC; ITS, internal transcribed spacer; ITS1, internal transcribed spacer 1; ITS2, internal transcribed spacer 2; ML, maximum likelihood; p.c., peduncle cover plate; PAUP\*, Phylogenetic Analysis Using Parsimony Program; Po, pore plate; part of the APC; ', apical plate(s); '', precingular plates; ''', postcingular plates; ''', antapical plates; p, posterior intercalary plate(s); s, sulcal plates; SSU rDNA, small subunit ribosomal DNA; X, canal plate

### **1. Introduction**

The taxonomy of thecate (armored) dinoflagellates historically has been based upon their plate tabulations, most often following the system proposed by Kofoid (1909—reviewed in Fensome et al., 1993 and Carty, 2003), coupled with molecular sequence data where available (Fensome et al., 1999). Here we re-examine the taxonomy of *Pfiesteria* Steidinger et Burkholder, a toxicogenic genus (Burkholder et al., 2005) that has been

considered to include two thinly thecate dinoflagellate species (the type species, *Pfiesteria piscicida* Steidinger et Burkholder in Steidinger et al., 1996a, and *Pfiesteria shumwayae* Glasgow et Burkholder in Glasgow et al., 2001). The latter species recently was reclassified within a separate genus as *Pseudopfiesteria* Litaker et al. in Litaker et al. (2005).

The original description of the genus *Pfiesteria* and the type species *P. piscicida* (Steidinger et al., 1996a), the original description of the second species *Pfiesteria shumwayae* (Glasgow et al., 2001), and the recent reclassification of *Pseudopfiesteria shumwayae* (Glasgow et Burkholder) Litaker et al. (2005) were incomplete in description of certain morphological features that have since been recognized, such as the uncertainty and the potential for variation in the number of sulcal plates as recognized for various other dinoflagellate genera (reviewed in Steidinger and Tangen, 1997). The recent reclassification of *Pfiesteria shumwayae* secondarily was based upon a maximum likelihood phylogenetic analysis of rDNA sequence data, interpreted to suggest that *Pfiesteria shumwayae* and *P. piscicida* are no more closely related to one another than to other informally described “pfiesteria-like” taxa (Litaker et al., 2005), and in contrast to other published research (e.g. Litaker et al., 1999, Oldach et al., 2000 and Jeong et al., 2005). Since taxa of the genus *Pfiesteria* are toxigenic and of economic relevance (Marshall et al., 2000, Burkholder et al., 2005 and Gordon and Dyer, 2005), knowledge of their morphology and genetic identity is important. Therefore, we reinvestigated *Pfiesteria* taxonomy based upon (i) examination of historic information on the morphology and phylogenetics of *Pfiesteria*, followed by presentation of (ii) new data on the plate structure of *P. piscicida* and *Pfiesteria shumwayae* and (iii) new phylogenetic analyses of *Pfiesteria* and “pfiesteria-like” dinoflagellates.

## **2. Historic information on morphology and phylogenetics**

### **2.1. Plate tabulations of *Pfiesteria* species**

Steidinger et al. (1996a) and Glasgow et al. (2001) reported a plate formula for *Pfiesteria piscicida* and *Pfiesteria shumwayae*, respectively, that lacked a p.c. plate and included only four sulcal plates, and the findings of Seaborn et al. (2006) on the two *Pfiesteria* species supported those analyses. Clear micrographs in support of the described sulcal plates unfortunately were lacking in all three studies. Litaker et al. (2005) and Mason et al. (2003) reported that the suture swelling (Glasgow et al., 2001) or membrane stripping procedures (Steidinger et al., 1996b and Truby, 1997), each used alone to prepare cells for plate tabulation analyses of *Pfiesteria* spp., were insufficient to enable detection of the delicate sulcal plate sutures. They combined membrane stripping with cell swelling in attempts to discern the sulcal plates. Regardless of reported differences in the number of sulcal plates in *P. piscicida* and *P. shumwayae*, all five studies reported that the only difference in major plate number between the two species is the presence of an additional precingular plate in *P. shumwayae* which, in turn, affects the shape of the 1a plate.

Litaker et al. (2005) described in *P. shumwayae* a peduncle cover plate (PC, here p.c.), but were uncertain about its fine structure. In addition, photomicrographs provided by Mason et al. (2003) and Litaker et al. (2005) were ambiguous regarding the delineation of some sulcal plates that were described, and the photomicrographs in Litaker et al. (2005) did not clearly support drawings that were provided. In thinly thecate dinoflagellates, varying degrees of distortion, shrinkage, and sometimes other preparation artifacts may occur during fixation, as has been shown for many protists (Gifford and Caron, 2000 and Menden-Deuer et al., 2001) including *Pfiesteria* (Steidinger et al., 1996a). The problem of subjectivity in interpretation is also common in membrane-stripped preparations; by comparison, suture-swelling techniques may be less ambiguous (Glasgow et al., 2001), but suture swelling of small, thinly thecate dinoflagellates may miss the presence of small sutures such as those in the sulcal region (Mason et al., 2003). This analysis suggests that membrane stripping, alone or with suture swelling procedures, also may not provide sufficient resolution of the sulcal region. Overall, surface structure interpreted by Litaker et al. (2005) as sutures in the sulcal area cannot be clearly discerned as such, as opposed to artifacts of preparation.

## 2.2. Previous phylogenetic trees including *Pfiesteria* spp. and “pfiesteria-like” species

The available molecular sequence data used as the basis for constructing several phylogenetic trees that include *Pfiesteria piscicida*, *Pfiesteria shumwayae*, and various “pfiesteria-like” dinoflagellates were re-evaluated, and the approaches compared.

### 2.2.1. Litaker et al. (1999)

A phylogenetic analysis was conducted on a broad spectrum of taxa using the SSU rDNA region with maximum likelihood (ML) methods, along with some use of parsimony and distance methods. The outgroup was not specified, but apicomplexans broke out separately and were implicitly treated as a sister group to the dinoflagellates. Support was very weak for divergence order of major groups within the dinoflagellates. *P. piscicida* (AF077055, AF149793) and *P. shumwayae* (AF080098, then reported as a “pfiesteria-like” species) were weakly supported as sister taxa.

### 2.2.2. Oldach et al. (2000)

A minimum-evolution tree was constructed from ~2000 bp of the SSU rDNA from dinoflagellates. *P. piscicida* and *P. shumwayae* were weakly supported as sister taxa, with the only other “pfiesteria-like” sequence in the analysis (an organism submitted to GenBank as Cryptoperidiniopsis sp. brodyi AF080097 by Litaker et al., in July 1998; also see Litaker et al., 2000) in a basal position relative to the two *Pfiesteria* species.

### 2.2.3. Jakobsen et al. (2002)

A minimum-evolution tree was constructed for dinoflagellates, using the entire SSU rDNA sequence. This analysis was very similar to that of Oldach et al. (2000), but also included an additional “pfiesteria-like” sequence (CCMP1873). A sister relationship was found for *P. piscicida* and *P. shumwayae*, but bootstrap support was <60%. The CCMP1873 sequence was strongly supported as basal to *Pfiesteria* spp. and an unnamed cryptoperidiniopsis designated as “cryptoperidiniopsis brodyi.”

### 2.2.4. Jeong et al. (2005)

Dinoflagellate SSU rDNA sequences were analyzed (including *P. piscicida* AY112746 and *P. shumwayae* AF080098), using Bayesian, maximum parsimony, and neighbor-joining methods. There was moderate support for a *P. piscicida*/*P. shumwayae* clade nested within a larger “pfiesteria-like” clade that consisted of the two *Pfiesteria* species, a cryptoperidiniopsis, and a “Lucy” sequence. The latter organism, variously named (“Lucy”, “lucy”, “lucie”; e.g. Steidinger et al., 2001), has been described as “pfiesteria-like” (e.g. Litaker et al., 2005 and Seaborn et al., 2006). A newly described species, *Stoeckeria algicida* Jeong, was weakly supported as basal to the “pfiesteria-like” clade plus the “shepherd's crook” sequence (CCMP1829) which, in turn, was strongly supported as basal to the “pfiesteria-like” clade. It should be noted that bootstrap values in Jeong et al.'s (2005) Figure 24 for the branch leading to the CCMP1833 [Lucy-3] and CCMP1928 [“Cryptoperidiniopsis sp. brodyi”] clade and the branch leading to the “pfiesteria-like” clade were transposed (H.J. Jeong, personal communication). These results indicate that the correct rooting for the unrooted tree of Litaker et al. (2005) was along the branch leading to “shepherd's crook.”

### 2.2.5. Litaker et al. (2005)

A ML analysis was performed on five taxa including *P. piscicida*, *P. shumwayae*, “lucy”, “shepherd's crook” and a cryptoperidiniopsis. No outgroup was included or specified. *P. piscicida* and *P. shumwayae* were weakly supported as sister taxa. Relative branch lengths alone were used to support classification of *P. shumwayae* as a separate genus from *Pfiesteria*, however, without consideration of topology or questions of root position. At a minimum, this tree would require an outgroup root in order to provide useful information on phylogenetic relationships of the taxa, which should be a primary criterion for classification (Felsenstein, 2004).

### 2.2.6. Rublee et al. (2005)

A neighbor-joining analysis was conducted on a 409 bp SSU rDNA segment. The primary focus was relationships within the “pfiesteria-like group,” which was sampled more extensively than in the other studies. *P. piscicida* and *P. shumwayae* were indicated as more closely related to each other than to

cryptoperidiniopsoids. The analysis did not show significant bootstrap support for *P. piscicida* and *P. shumwayae* as sister species, but it was complicated by a rather divergent sequence from a set of *P. shumwayae* samples from New Zealand.

### 2.2.7. Zhang et al. (2005)

Phylogenetic analysis was performed on a large (1469 bp) SSU rDNA segment and *cob* nucleotide and amino acid sequences, alone and in combination, using ML and Bayesian methods. Results with neighbor-joining and parsimony methods were also briefly mentioned, and were similar to the Bayesian and ML results. As in Litaker et al. (1999) and Seaborn et al. (2006), the branching order of the major groups was weakly supported and varied depending upon which genes were used. The basal and anomalous divergence of *Crypthecodinium cohnii* (Seligo) Chatton was possibly an artifact of outgroup rooting. In the combined tree, *P. piscicida* and *P. shumwayae* were moderately supported as sister taxa.

### 2.2.8. Seaborn et al. (2006)

Phylogenetic analysis was performed on full length SSU rDNA of a small set of taxa, using parsimony and ML methods. The outgroup consisted of 11 other dinoflagellate taxa. “Pfiesteria-like” dinoflagellates formed a single cluster similar to that described by Litaker et al. (1999), with a unique unidentified isolate (VDH034: “Dinophyceae sp. Bullet”) basal. *P. piscicida* and *P. shumwayae* were moderately supported as sister taxa, with other “pfiesteria-like” sequences basal to the two *Pfiesteria* species.

### 2.2.9. Summary

Litaker et al. (2005) asserted that their molecular phylogenetic analysis supported their morphologically based reassignment of *P. shumwayae* to a new genus. Yet, that analysis did not consider the importance of root position in the phylogenetic tree of *Pfiesteria* and “pfiesteria-like” sequences. The root position is critical in making phylogenetic inferences and interpretations about relationships among taxa. All seven other previous studies, using various phylogenetic methods (minimum evolution, neighbor-joining, maximum parsimony, maximum likelihood, and Bayesian analyses) showed the same consistent topology, and provided weak to moderate support for *P. piscicida* and *P. shumwayae* as sister taxa.

## 3. Materials and methods

### 3.1. Isolates and culture conditions for re-analysis of morphology of flagellate cells

The isolates used in the additional morphological and ultrastructural analyses contributed by this study are given in Table 1. Strains were collected following Burkholder et al. (2001a), or were obtained from the Culture Collection for Marine Phytoplankton [CCMP], Bigelow Laboratory for Ocean Science, Bigelow, Maine, USA. Each was cloned using techniques detailed in Parrow and Burkholder (2003) and cultured on a Chinook salmon (*Oncorhynchus tshawytscha* Walbaum in Artedi, 1792) cell line (American Type Culture Collection CRL-1681) as in Parrow et al. (2005), or on cryptomonad cells (*Rhodomonas* sp. CCMP757) as in Burkholder et al. (2001b).

Table 1  
Clonal isolates of *Pfiesteria piscicida* and *P. shumwayae* examined in the morphological and ultrastructural component of this study

Strain designation	Source location	Collection date
<i>Pfiesteria piscicida</i>		
CCMP2354	Marshall Creek, Chincoteague Bay, MD, USA	August 2000
CCMP2363	Marsh Side Pond, Hilton Head Island, SC, USA	April 2002
CCMP2423	Beard Creek, Neuse River Estuary NC, USA	May 2002
<i>Pfiesteria shumwayae</i>		
CCMP2357	Carolina Pines, Neuse River Estuary, NC, USA	July 1998
CCMP2089	Pamlico Estuary, NC, USA	November 1999
CCMP2359	Marshall Creek, Chesapeake Bay, Maryland, USA	August 2000

## 3.2. Morphological assessment and supporting analyses

### 3.2.1. Flagellate cells

The size range of flagellate cells from culture sub-samples of three clones each of *Pfiesteria piscicida* and *Pfiesteria shumwayae* (Table 1), 8 h after cryptomonads or fish cells were added (food-replete), were compared to cell size 5 days after feeding when the populations were prey-limited ( $N = 100$  cells each from prey-replete and prey-limited cultures; cells were preserved with 0.5% glutaraldehyde, final concentration – it should be noted that the preservative could have caused some cell shrinkage – e.g. see Choi and Stoecker, 1989). Cells were measured using an Olympus AX-70 microscope equipped with a water immersion  $60 \times 0.9$  NA objective, 0.8 NA condenser and a DEI-750 cooled chip CCD camera (Optronics Engineering, Goleta, CA).

Small, thinly thecate dinoflagellates typically do not stain well with calcofluor white M2R, and attempts to use this stain were unsuccessful in revealing fine details of the plate structure of *P. shumwayae*. For scanning electron microscopy, culture aliquots were treated with a 40% reduction in salinity for 30 min (i.e. 40% reduction in osmolality, monitored using a vapor pressure osmometer – Wescor, Inc., Logan, Utah, USA) and then combined with an equal volume of fixative cocktail (1%  $\text{OsO}_4$ , 2% glutaraldehyde, and 0.1 M sodium cacodylate final concentration) at 4 °C for 20 min. Fixed cells were filtered onto polycarbonate filters (3  $\mu\text{m}$  porosity), rinsed in 0.1 M sodium cacodylate, dehydrated through an ethanol series,  $\text{CO}_2$  critical-point-dried, sputter-coated with 25 nm Au/Pd, and viewed at 15 kV on a JEOL 5900LV scanning electron microscope. Plate tabulations for all but the sulcal plates were determined by scanning electron microscopy of suture-swollen cells as in Parrow et al. (in press). Plates in the sulcal area were examined by stripping the membranes from cells that had been pre-swollen by mild hypoosmotic treatment, following Mason et al. (2003).

For transmission electron microscopy, fixative stocks and buffers were prepared in sterile-filtered culture media, with the final pH adjusted to that of the culture. Cells were fixed in glutaraldehyde, osmium tetroxide, and 1.0 M sodium cacodylate buffer at a final concentration of 0.05%, for 10–20 min, and incubated in the dark at room temperature (Glasgow et al., 2001). Fixed cells were drop-filtered on 13 mm polycarbonate membrane filters (5  $\mu\text{m}$  pore size); post-fixed in 1% osmium tetroxide in cacodylate buffer for 30 min, and rinsed with three 0.1 M cacodylate buffer washes. The hydrated filters were embedded between glass slides in warm 2% agarose prepared in the same buffer, and then chilled to 4 °C. After complete gelling, the filters were gently removed leaving the agarose embedded cells behind, excess agarose was cut away with a razor blade, and the sample was cut into 1 mm<sup>3</sup> blocks. Blocks were stained and post-fixed in aqueous 2% uranyl acetate for 1 h at room temperature in a lightproof box. Cells were then rinsed two times in distilled water, dehydrated through a graded series of ethanol, and embedded in Spurr's resin. Cured blocks were trimmed and cut parallel to the plane of the filter to maximize the number of cell profiles viewed in the TEM. Ultrathin (750–800 Å) sections were stained with 4% aqueous uranyl acetate for 1 h in the dark, followed by Reynold's lead citrate for 4 min. Cells were viewed using a JEOL JEM100S transmission electron microscope at 80 kV.

### 3.2.2. Amoeboid cells

*Pfiesteria* originally was placed within the order Dinamoebales (Steidinger et al., 1996a), but amoeboid morphs have not been found in many strains (Burkholder and Glasgow, 2002). Some sequence data were obtained from a clonal amoebae culture (NCSU188A), a vahlkampfid-like amoeba (*Gymnamoebae* – Patterson, 1999) that was isolated from estuarine sediments of the Chicamacomico River, a tributary of eastern Chesapeake Bay in Maryland, USA. The organism (length 15–30  $\mu\text{m}$ ) was limax in form and had uroidal villi. Its locomotion was characterized by hemispherical, hyaloplasmic eruptions alternating to either side of the anterior end. It was cloned and fed cryptomonad microalgae for three months as routine procedure for screening estuarine amoebae isolates, to ensure that DNA from other *Pfiesteria* stages would not be present. The culture was observed under light microscopy in detail by coauthor JMB, and also by coauthor PAR. Only amoeboid forms were found. DNA from the culture was extracted and amplified by PCR. Purified DNA from the PCR reaction products was prepared with either *P. piscicida*-specific forward (108F: 5'-AGTTAGATTGTCTTTGGTGGTCAA-3') or reverse (311R: 5'-GATAGGTCAGAAAGTGATATGGTA-3') primers (Oldach et al., 2000), lyophilized, and sent to the Arizona State University DNA Laboratory (Dr. Scott Bingham) for sequencing. Amplicons were

sequenced on an ABI Prism Model 377 DNA sequencer using the BigDye™ version 2 Ready Reaction Dye Terminator Kit (Applied Biosystems, Foster City, CA).

### 3.3. Phylogenetic analyses

Phylogenetic relationships of *Pfiesteria piscicida* and *Pfiesteria shumwayae* versus closely related “pfiesteria-like” species were re-assessed considering the following small subunit ribosomal sequences from GenBank (www.ncbi.nlm.gov): “pfiesteria-like” dinoflagellate (“Lucy”) AY245689; cryptoperidiniopsoid species AY245690; *P. piscicida* AY245693; *P. shumwayae* AY245694; and dinoflagellate (“shepherd's crook”) AY590479. Since the interrelationships of these five taxa are not well understood, the dinoflagellate *Karlodinium* sp. (AY245692), which is not closely related to *Pfiesteria* and “pfiesteria-like” taxa (Zhang et al., 2005 and Seaborn et al., 2006), was used as an outgroup to root the tree. These sequences range from 3209 base pairs (bp) to 3434 bp and contain complete 5.8S, ITS1, and ITS2 sequences as well as partial 18S- and 28S-rDNA sequences. Sequences were aligned using ClustalX with default parameters. The alignment file (.aln) was converted to a Nexus file using MacClade 4.06, and visual examination indicated that no manual alignments were necessary. The Phylogenetic Analysis Using Parsimony Program (PAUP\* version 4.0) was applied for phylogenetic analysis (Swofford, 2002). A Maximum Likelihood (ML) analysis was run using the factor default parameters (Substitution model: Ti/tv ratio set to 2; base frequencies A = 0.25346, C = 0.21264, G = 0.26446, T = 0.26944; molecular clock not enforced; starting branch lengths obtained with the Rogers–Swofford method; trees rejected if approximate likelihood exceeded the target by more than 5%). One thousand replicates were used for bootstrap analysis, and all other parameters were left at default. A rooted bootstrap consensus tree was constructed. This approach was used because an unrooted tree (as in Litaker et al., 2005) only describes a degree of relatedness between taxa, while a rooted tree explains which species share a common ancestor and in which direction evolutionary change has taken place (Grauer and Li, 2000).

A maximum parsimony analysis additionally was performed on the data with PAUP\*, using default settings, 1000 bootstrap replicates, and rooting the bootstrap consensus tree with *Karlodinium* sp. as an outgroup. Finally, to investigate the possibility of intraspecific genetic variation, the 5.8S region of ten strains of *P. piscicida* and nine strains of *P. shumwayae* were compared to the 5.8S data of the “pfiesteria-like” taxa and various other dinoflagellate species, using maximum parsimony analysis with PAUP\*. One hundred bootstrap replicates were used and the tree was rooted using the dinoflagellate *Prorocentrum micans* Ehr. (GenBank AY499517) as an outgroup. We included 5.8S sequences from *P. piscicida* isolates CCMP2091, CCMP2363, CCMP2423, CCMP2354 and NCSU2177, and from *P. shumwayae* isolates CCMP2359, CCMP2089, CCMP2357, CCMP2360, and NCSU2172. For these isolates, DNA was purified using the DNeasy Plant kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A rRNA fragment was amplified by a high-fidelity Taq supermix (Invitrogen, Carlsbad, CA) using specific forward primers PPF: 5'-CGATTGAGTGATCCGGTGAATAA-3' for *P. piscicida* and PSF: 5'-GCACGCATCCAAGCATCTCAC-3' for *P. shumwayae*. The same reverse primer 5'-TTGCTGACCTGACTTCATGTC-3' was used for both species. The amplified fragment was cloned into pCR4-TOPO (Invitrogen, Calsbad, CA). After the plasmid was isolated using the Wizard Plus SV Miniprep kit (Promega, Madison, WI), the rRNA fragment was sequenced using the standard M13 primers and the ABI BigDye terminator cycle sequencing kit (v3.1). At least two clones from independent amplifications were sequenced to avoid any Taq-related errors. Reactions were run on an ABI3730 genetic analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequences were initially analyzed using Contig Express component of the Vector NTI suite of programs (Invitrogen, Carlsbad, CA).

## 4. Results

### 4.1. Morphology of *Pfiesteria shumwayae*

Due to the importance of accurate information about toxigenic organisms such as *Pfiesteria*, we here provide an extended description including new information on the plate tabulation.

*Pfiesteria shumwayae* Glasgow et Burkholder in Glasgow et al. (2001).

Nomenclatural synonym: *Pseudopfiesteria shumwayae* (Glasgow et Burkholder) Litaker et al. (2005).

Taxonomic synonym: *Pfiesteria* species B (Kempton, 1999; also in Glasgow, 2000, Glasgow and Burkholder, 2000 and Oldach et al., 2000).

Dinoflagellate with small, oval, cryptic peridinioid flagellate cells having a Kofoidian plate formula of Po, cp, X, 4', 1a, 6'', 6c, p.c.,?, 5<sup>m</sup>, 0p, 2<sup>m</sup>'. Thecate biflagellate vegetative cells range from ~9 to 25 µm in maximum cell dimension (Table 2). The convex epitheca is approximately equal in size to the hypotheca. The cells may enlarge by two- to three-fold during feeding (Table 2), as reported previously (Burkholder et al., 2001b and Parrow and Burkholder, 2003). For example, prey-replete and prey-limited *P. shumwayae* strain 1050c-b had cell dimensions (mean ± 1 S.D., *n* = 50) as 15.1 ± 2.6 µm × 12.3 ± 2.1 µm (estimated biovolume 1780 µm<sup>3</sup>) versus 11.0 ± 1.2 µm × 9.1 ± 2.1 µm (estimated biovolume, 720 µm<sup>3</sup>), respectively. Kleptochloroplastidy has been reported as an occasional phenomenon (Glasgow et al., 2001).

Table 2  
Comparison of some morphological differences among biflagellate vegetative cells of *Pfiesteria piscicida* and *Pfiesteria shumwayae*

Feature	<i>Pfiesteria piscicida</i>	<i>Pfiesteria shumwayae</i>
<b>A. Plates</b>		
Epitheca tabulation	Po, cp, X, 4', 1a, 5''	Po, cp, X, 4', 1a, 6''
1a plate(s)	Three-sided (triangular)	Four-sided (rectangular)
1a placement	Touches 2'', 3'', 3'	Touches 2'', 3'', 2', 3'
<b>B. Other features</b>		
Size range	9–17 µm	9–25 µm
Mean dimensions ± 1 S.D. (l × w, flagellate cells)	11.5 ± 1.9 µm × 9.1 ± 1.9 µm	13.8 ± 3.6 µm × 11.1 ± 3.2 µm

Size data were based upon three clones of each species used in this study. Clones examined in this comparison were the three listed for each species in Table 1 (*N* = 100 preserved cells per clone, from both prey-replete and prey-depleted conditions). Considering the small number of clones examined for each species, the size data should be expected to change depending upon the number of clones examined and the culture conditions. Plate dimensions (as in Litaker et al., 2005) were not included because plate overlap would confound measurements from scanning electron microscopy.

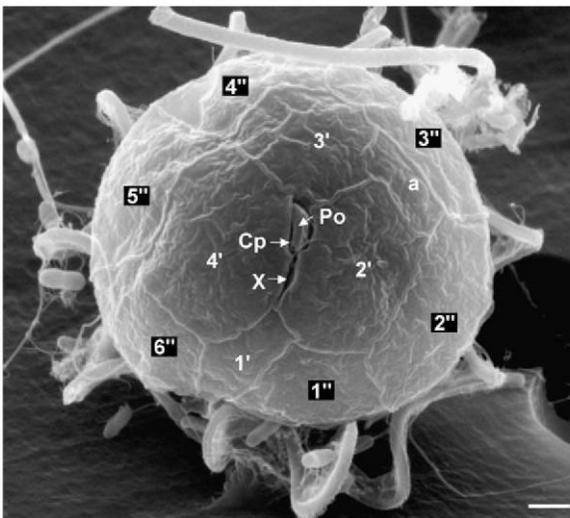


Fig. 1. Scanning electron micrograph (SEM) of the apical area in a flagellate cell of *Pfiesteria shumwayae*, showing the characteristic apical pore complex (Cp, closing plate; Po, pore plate, X, canal plate), "a" plate, four precingular plates with the 1' extending to the cingulum, and six precingular plates. Scale bar, 1 µm.

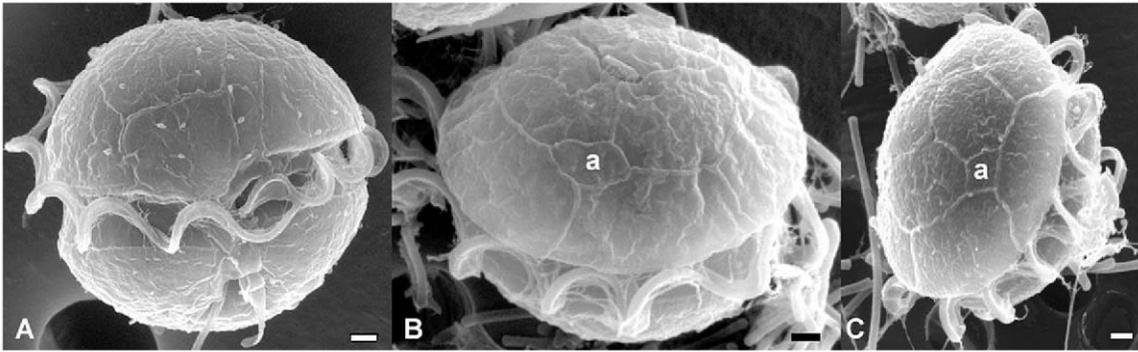


Fig. 2. SEMs of the epitheca of flagellate, suture-swollen *Pfisteria shumwayae* cells: (A) ventral view showing displacement of the cingulum; (B and C) differences in the shape of “a” plate, from irregular and roughly rectangular (B) to diamond-shaped (C). SEM in (C) reprinted with permission from *Phycologia* (Glasgow et al., 2001). Scale bars, 1  $\mu\text{m}$ .

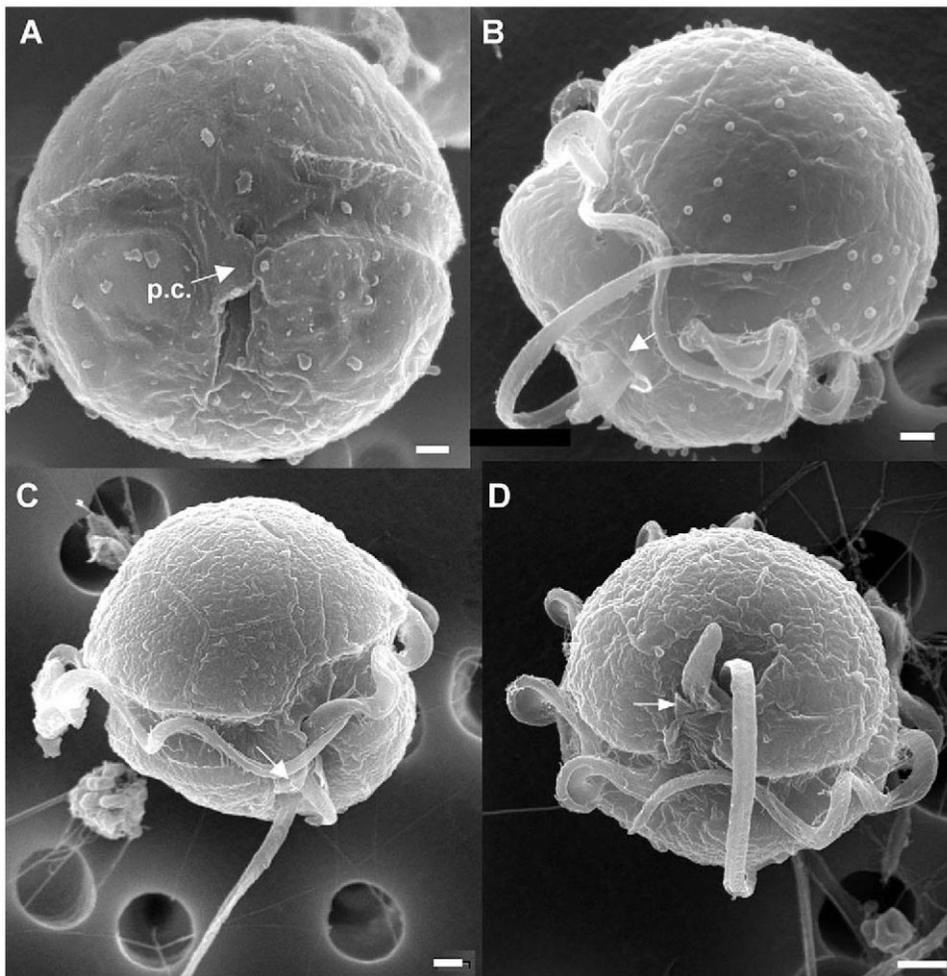


Fig. 3. SEMs of flagellate cells of *Pfisteria shumwayae*, showing the peduncle cover plate (p.c.) (A–C, ventral views); (D) antipical view, showing the peduncle to the right of the p.c. (arrow). Scale bars, 1  $\mu\text{m}$ .

The small apical pore complex (APC) has a round or oval pore plate (Po) that abuts the 2', 3', and 4' plates, and a closing plate (c.p.; Fig. 1). The APC is at a slight angle to the canal plate (X). The APC is located between the 2', 3', and 4' plates, and is connected to 1' by the X plate. The four-sided (diamond- or rectangular-shaped) 1a plate can exhibit polymorphism (Fig. 2), and differs from the three-sided (triangular) 1a plate of *P. piscicida*. The cingulum of asexual flagellate stages is median, slightly displaced about 0.5 $\times$  at the sulcus (Fig. 2A) as in *P. piscicida* (Steidinger et al., 1996a). The cingulum consists of six plates that are not rimmed by lists. The

hypotheca, typically slightly smaller than the epitheca, is symmetrical and consists of 5 *m* and 2 *m'* plates. There are no posterior intercalary plates. The sulcus is excavated and descends to about three-fourths of the length of the hypotheca. The sulcus is narrow and offset to the right of the anterior sulcal plate (s.a.), and is partly concealed by an overlying peduncle cover plate (p.c.) that originates at the right side of the sulcus along the left side of the 6c and 5 *m* plates. The p.c. plate, previously described as a presumed plate of uncertain structure (Litaker et al., 2005), appears to be very thinly thecate with fine structure similar to that of other plates (Fig. 3 and Fig. 4). The sulcus is median in the hypotheca or slightly displaced to the right, and extends slightly into the epitheca at the rhomboidal-shaped 1' plate (see Glasgow et al., 2001). Sulcal plates (at least six in number) are sometimes overlain or overlapping with other plates (TEMs; Fig. 5 and Fig. 6), and are poorly defined or ambiguous in membrane-stripped, suture-swollen, and combination procedures. The peduncle extends from upper right sulcal area. No sulcal or cingular lists are present.

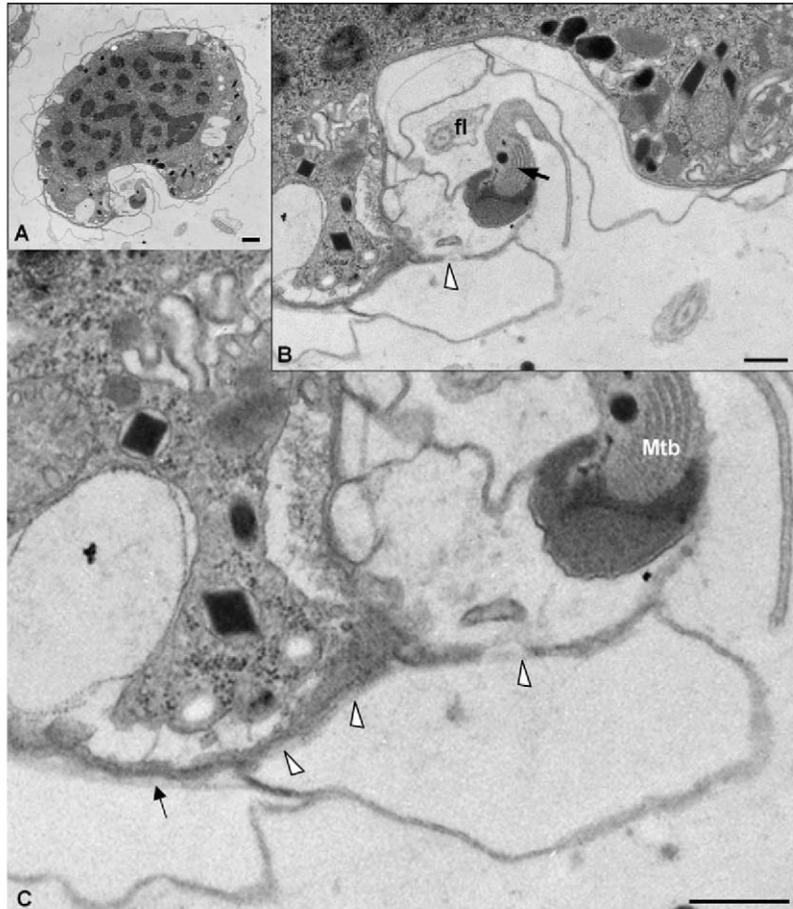


Fig. 4. Transmission electron micrographs (TEMs) of a flagellate cell of *Pfiesteria shumwayae* near upper sulcal region: (A) Entire cross-section of cell, for orientation. (B) Same cell at higher magnification, showing the longitudinal flagellum (fl), the microtubule basket of the peduncle (black arrow), and the peduncle cover plate (white arrow indicates plate line). (C) Higher magnification (peduncle cover plate – white arrows indicate plate line; adjacent plate, black arrow; Mtb, microtubule basket). Note that the adjacent plate is slightly overlying the peduncle cover plate. Scale bars: (A), 2  $\mu$ m and (B and C), 1  $\mu$ m.

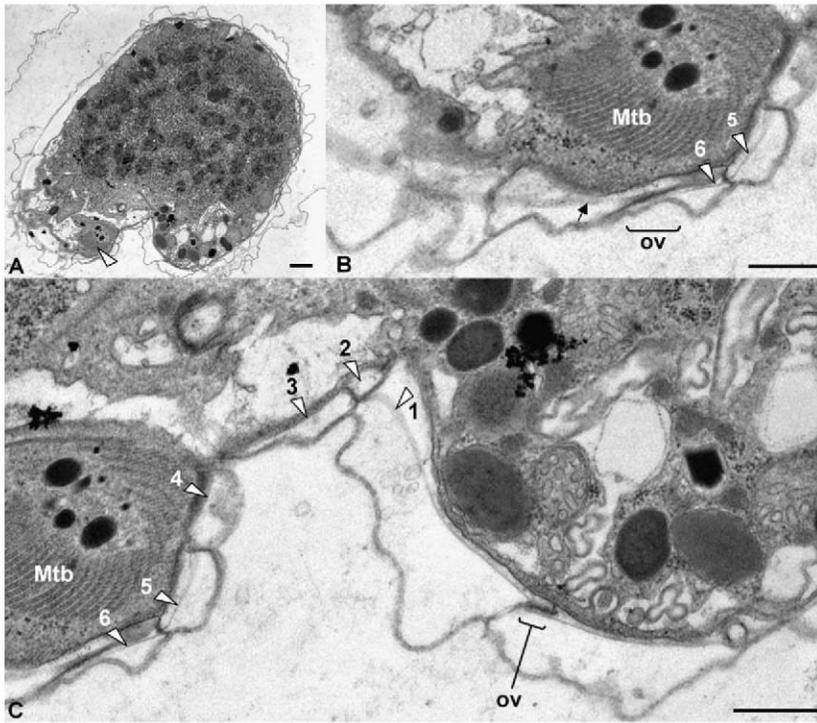


Fig. 5. (A) TEM through a flagellate cell of *Pfiesteria shumwayae*, including the microtubule basket of the peduncle (A, arrow) and the sulcal region. Scale bar, 2  $\mu\text{m}$ . (B and C) Same cell at higher magnification: Mtb, the microtubule basket of the peduncle; some sulcal plates are arbitrarily designated from 1 to 6 (white arrows point to plate lines within amphiesmal membranes); ov, examples of areas of overlap of a plate inside the sulcus with a plate outside the sulcus (in B, black arrow indicates plate line; plate identity uncertain because of uncertainty in the exact location and angle of the cross-section). Scale bars, 1  $\mu\text{m}$ .

The material used originally to designate the holotype of *P. shumwayae* was isolated from the New River Estuary, North Carolina, and was cultured using both fish and algae as nutritional sources (as in Burkholder et al., 2001a, Burkholder et al., 2001b and Glasgow et al., 2001). This brackish/marine coastal organism is widely distributed along the Atlantic seaboard and Gulf Coast of the U.S. (Rhode Island to Florida), and also has been found globally based upon reports from Norway, New Zealand, and Australia (Burkholder et al., 2001b, Jakobsen et al., 2002, Rhodes et al., 2002, Rublee et al., 1999, Rublee et al., 2001 and Rublee et al., 2005b). Its habitat spans oligohaline and mesohaline estuarine waters, extending to coastal marine waters.

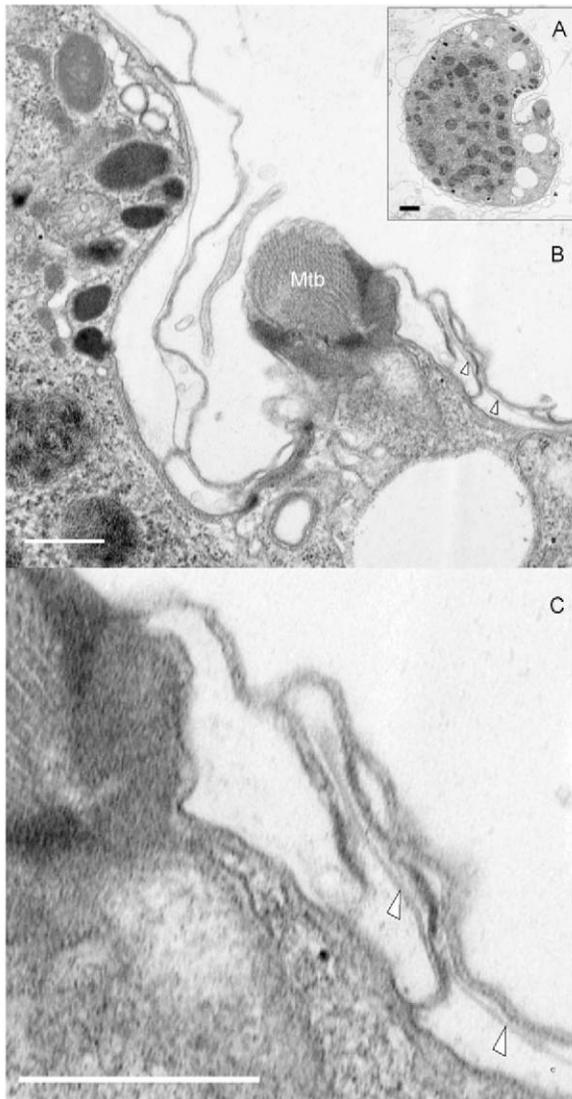


Fig. 6. TEMs of a flagellate cell of *Pfiesteria shumwayae* in sulcal region: (A) entire cross-section of cell, for orientation; (B and C) same cell at higher magnification, indicating the microtubule basket (Mtb) of the peduncle, and overlying, layered plates (arrows indicate plate lines). Scale bars: (A), 2  $\mu\text{m}$  and (B and C), 1  $\mu\text{m}$ .

#### 4.2. Sequence analysis of an amoeboid morph

Regarding evidence from this study for other active, non-flagellate *Pfiesteria* morphs, the 208 bp consensus sequence (from two forward primer and two reverse primer reads) from the clonal amoeba culture isolated from estuarine sediments (GenBank accession DQ417655) had 100% identity with the corresponding region of sequence data for all *Pfiesteria piscicida* GenBank submissions that had been obtained from flagellate stages. The culture was lost before further sequencing could be completed.

#### 4.3. Re-analysis of phylogenetic relationships for *Pfiesteria* and “*pfiesteria-like*” taxa

The new rooted consensus trees showed considerably higher bootstrap support for the grouping of *Pfiesteria piscicida* together with *Pfiesteria shumwayae* than that reported by Litaker et al. (2005) (Fig. 7 and Fig. 8). Both phylogenies in Fig. 7 and Fig. 8, as well as that proposed by Litaker et al. (2005), rely upon sequences obtained from one strain of each taxon. Examination of the 5.8S region of 19 strains of *P. piscicida* and *P. shumwayae* for intraspecific genetic variation revealed no difference within this region among the strains of the two species (Fig. 9), except for 1 bp difference in 1 *P. piscicida* strain (DQ344041, from New Zealand). The phylogeny shown in Fig. 9 shares the same topology as the consensus cladogram. The other most parsimonious trees primarily differ in branch configurations within the *P. piscicida/P. shumwayae* clade, due to the highly

conserved sequence data reported thus far within the genus *Pfiesteria*. The sequence alignment also reveals numerous derived nucleotide substitutions within the *Pfiesteria* clade inferred by the branch length leading to the *Pfiesteria* node shared by the two *Pfiesteria* species, which are not associated with the “pfiesteria-like” species (Fig. 9). The tree shown in Fig. 9 also provides support for placement of the *Pfiesteria*/“pfiesteria-like” clade within the Order Peridinales, as first suggested by Parrow et al. (2002), based on a small number of base pairs (~159 bp) in a conserved region.

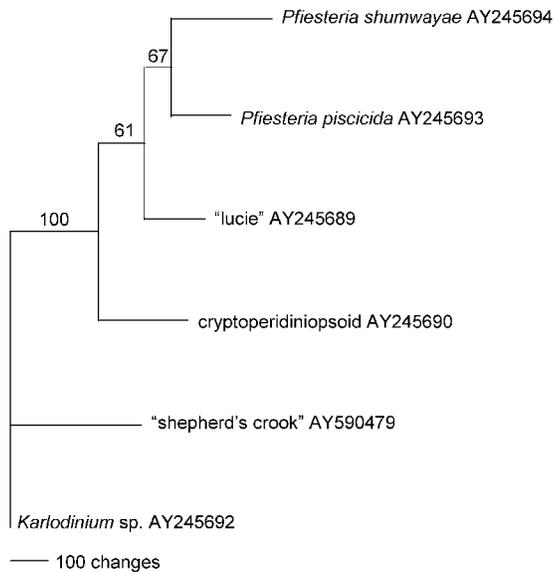
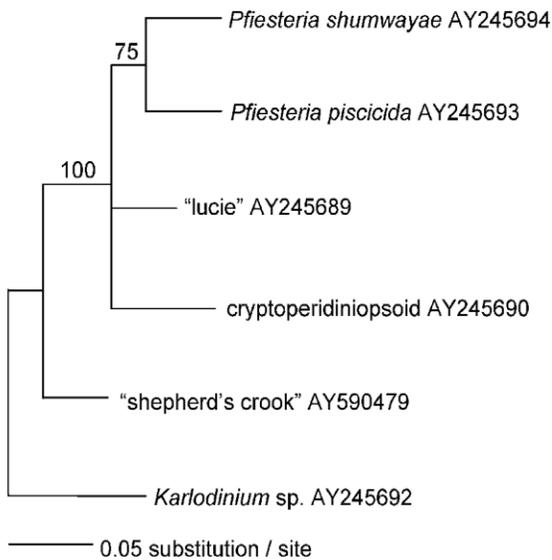


Fig. 7. Maximum likelihood analysis of *Pfiesteria shumwayae*, *Pfiesteria piscicida*, two informally described “pfiesteria-like” taxa including a cryptoperidiniopsoid species, an informally named taxon called “lucie” (Steidinger et al., 2001; Litaker et al., 2005), and an informally described taxon called “Shepherd’s crook” (Steidinger et al., 2001; Litaker et al., 2005) using the complete 5.8S, ITS1, ITS2, and partial 18S and 28S sequence data (sequence data from Litaker et al., 2003, 2005). The results are presented as a phylogram rooted with *Karlodinium* sp. as an outgroup, showing bootstrap values >50.

Fig. 8. Maximum parsimony analysis of *Pfiesteria shumwayae*, *Pfiesteria piscicida*, two informally described “pfiesteria-like” taxa including a cryptoperidiniopsoid species and an informally described taxon called “lucie” (Steidinger et al., 2001; Litaker et al., 2005), and an informally described taxon called “Shepherd’s crook” (Steidinger et al., 2001; Litaker et al., 2005) using the complete 5.8S, ITS1, ITS2, and partial 18S and 28S sequence data (sequence data from Litaker et al., 2003, 2005). The results are presented as a phylogram rooted with *Karlodinium* sp. as an outgroup, showing bootstrap values >50.

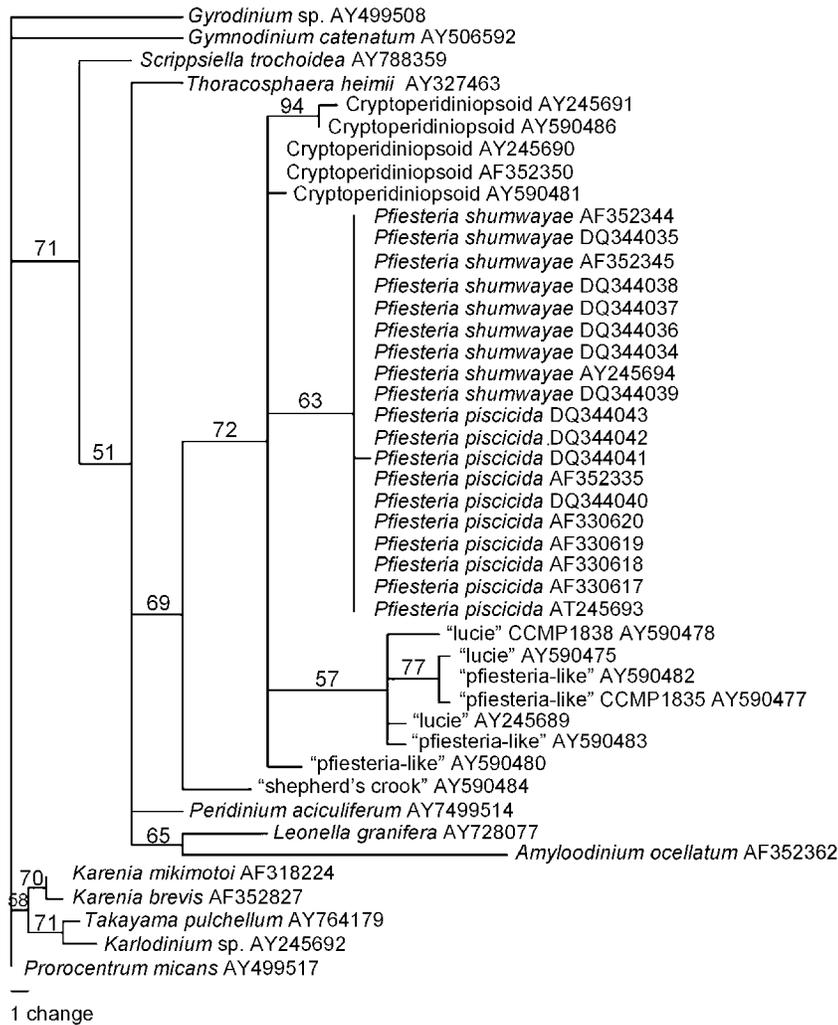


Fig. 9. Phylogram of maximum parsimony analysis of *Pfiesteria shumwayae*, *Pfiesteria piscicida*, "pfiesteria-like" species, and other dinoflagellates using the 159 bp 5.8S sequence data. Bootstrap values  $\geq 50$  are indicated. The tree shown is one of 84 minimum-length maximum parsimony trees that were developed (length = 93, CI = 0.667, RI = 0.8502).

## 5. Discussion

The available data do not support assignment of the organism originally named as *Pfiesteria shumwayae* to a separate genus, based on morphological and molecular information as well as other taxonomic considerations, as follows.

### 5.1. Morphological considerations

Historically, species differing by only one or a few Kofoidian plates are commonly included within the same genus, especially in smaller genera with few species (Fensome et al., 1993 and Steidinger and Tangen, 1997). Taxonomic rules invoked by Litaker et al. (2005, p. 650), requiring separation of organisms differing by one Kofoidian plate into different genera, were not found. Approximately one-third (16 of 49) of the presently accepted genera considered by Steidinger and Tangen (1997) included species that differ by one Kofoidian plate, and ~20% (11 of 49) of the genera included species that differ by two or more plates. As an example, most (>200 taxa) *Protoperidinium* species have either two or three apical intercalary plates.

As other examples, *Protoperidinium latidorsale* (Dangeard) Balech (Dangeard, 1927) and a newly described subgenus *Testeria* within *Protoperidinium* each have one anterior intercalary plate (Faust and Tester, 2006). In addition, the subgenus *Testeria* has no apical pore plate or X plate, usually present in *Protoperidinium* species (Steidinger and Tangen, 1997). *Protoperidinium americanum* (Gran et Braarud) Balech has four anterior intercalary plates (see also Lewis and Dodge, 1987). The genera *Protoperidinium*, *Glochidinium*, *Peridinium*, and *Scrippsiella* were described by Litaker et al. (2005) as having nearly identical Kofoidian plate tabulations,

with placement into separate genera based upon differing numbers of cingular plates. These genera have been described to differ, however, by two or more plates (*Protoperidinium*: 2–4a, 4c, 6s plates; *Glochidinium*: 0a, 3c, 4s plates; *Peridinium*: 2–3a, 5–6c, 5–6s plates; *Scrippsiella*: 3a, 6c, 4–5s plates) (Lewis and Dodge, 1987, Steidinger and Tangen, 1997, Boltovskoy, 1999 and Steidinger et al., 2001). The genus *Amphidiniopsis* contains species that differ in the number of precingular plates and cingular plates (6–8 and 5(4)–8, respectively; Hoppenrath, 2000), as does the genus *Fragilidium* (7–9 precingular plates, 9–11 cingular plates; Steidinger and Tangen, 1997).

Considerable variability in plate number is known, as well, for some species from field samples and from clonal cultures: vegetative cells and – (“female”) gametes of *Pyrophacus steinii* from the same clone have 6–8 apical plates, 10–13 precingular plates, 12 cingular plates, 11–13 postcingular plates, and 3 posterior intercalary plates. In contrast, + (“male”) gametes have 6 apical plates, 8–9 precingular plates, 8–9 cingular plates, 9 postcingular plates, and 2 posterior intercalary plates (Pholpunthin et al., 1999). In addition, in clonal cultures, the number (e.g. Morrill and Loeblich, 1981) as well as the shape and contact of some plates (e.g. Elbrächter and Meyer, 2001) can vary substantially (also see Matsuoka, 1985 and Montresor and Marino, 1994; description of *Scrippsiella* in Steidinger and Tangen, 1997). If rules required the splitting of taxa based upon a difference of one in plate number, as asserted by Litaker et al. (2005), cells within the same clone showing such variability would have to be categorized as separate taxa – which is unacceptable for specimens of the same clone.

## 5.2. Molecular information

Among eight previous phylogenetic analyses of *Pfiesteria* and “pfiesteria-like” taxa, only one study (Litaker et al., 2005) asserted that molecular phylogenetic data supported reassignment of *Pfiesteria shumwayae* to a new genus. That analysis did not consider, however, the critical question of root position in the phylogenetic tree of *Pfiesteria* and “pfiesteria-like” sequences. All seven other previous studies, using various phylogenetic methods, presented a largely congruent evaluation of *Pfiesteria* / “pfiesteria-like” phylogeny, with the following features: first, most taxonomic groupings previously defined from morphology are supported by molecular data. Second, the order of divergence among the major dinoflagellate groupings is poorly resolved due to short internal branch lengths and, consequently, weak bootstrap support. The topology of the major dinoflagellate groups is star-like and suggests a rapid initial radiation from a common ancestor. Third, *Pfiesteria piscicida* and *Pfiesteria shumwayae* consistently are indicated as sister taxa with weak to moderate support, while the informally named cryptoperidiniopsoids, “shepherd's crook” and “lucie” isolates appear to represent early-diverging taxa within the “*Pfiesteria*/pfiesteria-like” lineage. However, the *cob* trees (Zhang et al., 2005) produce a different weakly supported topology for the “*Pfiesteria*/pfiesteria-like” lineage.

The relevance of branch lengths in a phylogenetic tree is dependent in part upon the time since divergence, but also upon differences in substitution rates among lineages. Thus, unless statistical tests reject rate variability and in essence support a “molecular clock,” it cannot be argued that branch lengths are proportional to phylogenetic divergence (Li, 1993). Finally, it should be noted that trees from individual genes may not represent the true species tree due to lineage sorting, hybridization, and possibly horizontal gene transfer. The existing data are based heavily upon SSU rDNA sequences, and inclusion of other genes may lead to different conclusions (as in Zhang et al., 2005). Murray et al. (2005) found evidence of non-independent evolving sites in dinoflagellate rDNA sequences which may confound phylogenetic analyses. That finding underscores the need to use sequences from multiple genes when possible.

The trend in classification over the past two decades has been to use phylogenetic systematics, wherein named classifications have been inferred from monophyletic groups (Judd et al., 2002). Assessment of monophyly requires a reliable, rooted phylogenetic tree topology (Felsenstein, 2004). The preponderance of previous research suggests that *Pfiesteria piscicida* and *Pfiesteria shumwayae* are sister species, at least among the species described, and they apparently form a monophyletic group. Since these are the only formally described species of *Pfiesteria*, reclassifying one or the other would result in two genera that are trivially monophyletic. Beyond the issue of monophyly, the criteria for what tree nodes define genera, families, etc. are basically arbitrary; moreover, Litaker et al.'s (2005) phylogenetic analysis based on relative branch lengths is misleading

because the authors provided no evidence for the root location in their tree. The phylogenetic trees contributed by the present analysis showed considerably higher bootstrap support for the grouping of *Pfiesteria piscicida* together with *Pfiesteria shumwayae* than that reported by Litaker et al. (2005), and support placement of the two species within the same genus. The collective evidence for the root position is weak at this time, however, thus requiring emphasis on morphological data.

### 5.3. Considerations at the order level

Steidinger et al. (1996a) assigned *Pfiesteria* to the order Dinamoebales based on amoeboid stages observed in cultures of some strains. According to Fensome et al. (1993, p. 164), however, the order Dinamoebales was uncertain: “The name Dinamoebales is based on a dominantly amoeboid genus that may be part of the life-cycle of another, “coccoid” genus. Moreover, amoeboid forms are not typical of the order ....” Amoeboid cells have been described in some dinoflagellate taxa since the early 1900s (Pascher, 1916), mostly in ecto- and endoparasites and some mixotrophic predaceous species (e.g. Pfiester and Popovský, 1979, Buckland-Nicks et al., 1990, Buckland-Nicks et al., 1997, Buckland-Nicks and Reimchen, 1995 and Appleton and Vickerman, 1998). Popovský and Pfiester (1990, p. 50) asserted that “many, possibly most, dinoflagellates exhibit amoeboid stages or tendencies such as the formation of pseudopodia during phagocytosis at some stage in their life histories.” Nevertheless, in dinoflagellates including *Pfiesteria*, photographic (tracking one cell) or video sequences of transformations involving amoeboid cells have not been obtained (Burkholder and Glasgow, 2002 and Elbrächter, 2003).

Stages reported by Litaker et al. (2005) in the isolates they examined (in culture for 6–14 years) were similar to those described for *Pfiesteria shumwayae* by Parrow and Burkholder (2003), who worked with isolates that were 2–3 years old. Seaborn et al. (1999), Marshall et al. (2000), Burkholder et al. (2001c) and Glasgow et al. (2001) found amoeboid cells in some clonal strains of *Pfiesteria* spp. and “pfiesteria-like” dinoflagellates, but generally within the first year of isolation. Burkholder et al. (2001c) and Burkholder and Glasgow (2002) reported that many strains examined since the early work of Steidinger et al. (1996a) have not formed amoeboid cells. We therefore support the recommendation by Parrow et al. (2002) to move the genus *Pfiesteria* to the order Peridiniales based on plate tabulation of flagellate stages, which is the basis for the taxonomy of other thecate dinoflagellates, and also is based upon the consistent occurrence of flagellate stages in cultures.

### 5.4. Summary

The consistent morphological analyses showing a difference of only one precingular plate in *Pfiesteria piscicida* versus *Pfiesteria shumwayae*; the common practice of placement of organisms differing by one or more plates (including precingular plates) within the same genus, particularly in small genera; and the preponderance of molecular data support placement of these two species within the same genus. In our view, at the present state of knowledge, the erection of a separate monospecific genus for *P. shumwayae* is not justified, given the small differences between *P. piscicida* and *P. shumwayae*. Placement of *P. piscicida* and *P. shumwayae* within one genus will prevent formation of two trivially monophyletic genera, and contribute to the nomenclatural stability of organisms of interest to natural resource and public health managers.

Although molecular methods have become important tools for phylogenetic analysis (genospecies), they have not supplanted classical approaches (morphospecies), especially in taxa such as the dinoflagellates where sequence data on a range of genes are relatively scarce. Thus, it is important to use all relevant information when assessing phylogenetic relationships. Future work with multiple clones over extended time in culture may reveal more polymorphism in the plate structure of *Pfiesteria* spp., as has been shown for various other thecate dinoflagellate species. As additional species are found and/or described within this genus, further molecular studies involving more sequence data will strengthen insights about phylogenetic relationships among *Pfiesteria* spp. and closely related taxa.

Recalling the data presented and discussed above, we propose the following classification of *Pfiesteria*:

Class: Dinophyceae Pascher 1914

Order: Peridinales Haeckel 1894

Genus: *Pfiesteria* Steidinger et Burkholder in Steidinger et al. (1996)

Thus far, two species are formally assigned to the genus: *Pfiesteria piscicida* Steidinger et Burkholder in Steidinger et al. (1996a) and *Pfiesteria shumwayae* Glasgow et Burkholder in Glasgow et al. (2001), as characterized above.

Based upon present knowledge, we regard it as inappropriate to assign *Pfiesteria* to a family at this time. Apparently it is closely related to other, informally named “pfiesteria-like” organisms with unresolved tabulation patterns (Steidinger et al., 2001 and Seaborn et al., 2006). Recent phylogenetic analyses (Saldarriaga et al., 2001, Saldarriaga et al., 2004, Gottshling et al., 2005 and Kremp et al., 2005) indicate that *Pfiesteria* is closely related to calcareous dinoflagellates such as *Thoracosphaera*, suggesting that the suprageneric classification will require fundamental revision that is beyond the scope of this paper. All of these taxa will have to be placed together within the same family; thus, as the nomenclature and classification of calcareous dinoflagellates are under revision (Elbrächter et al. in preparation), at present we abstain from assigning *Pfiesteria* to a family.

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