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MOLECULAR PHYLOGENY OF THE HETEROTROPHIC DINOFLAGELLATES, *PROTOPERIDINIUM, DIPLOPSALIS* AND *PREPERIDINIUM* (DINOPHYCEAE), INFERRED FROM LARGE SUBUNIT rDNA[1]

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The genera *Protoperidinium* Bergh, *Diplopsalis* Bergh, and *Preperidinium* Mangin, comprised of species of marine, thecate, heterotrophic dinoflagellates in the family Protoperidinaceae Balech, have had a confused taxonomic history. To elucidate the validity of morphological groupings within the *Protoperidinium* and diplopsalids, and to determine the evolutionary relationships between these and other dinoflagellates, we undertook a study of molecular phylogeny using the D1–D3 domains of the large subunit (LSU) of the rDNA. Based on morphology, the 10 *Protoperidinium* species examined belonged to three subgenera and five morphological sections. Two diplopsalid species were also included. Single-cell PCR, cloning, and sequencing revealed a high degree of intraindividual sequence variability in the LSU rDNA. The genus *Protoperidinium* appeared to be recently divergent in all phylogenetic analyses. In maximum parsimony and neighbor joining analyses, *Protoperidinium* formed a monophyletic group, evolving from diplopsalid dinoflagellates. In maximum likelihood and Bayesian analyses, however, *Protoperidinium* was polyphyletic, as the lenticular, diplopsalid heterotroph, *Diplopsalis lenticula* Bergh, was inserted within the *Protoperidinium* clade as basal to *Protoperidinium excentricum* (Paulsen) Balech, and *Preperidinium meunieri* (Pavillard) Elbrächter fell within a separate clade as a sister to the Oceanica and *Protoperidinium steidingerae* Balech. In all analyses, the *Protoperidinium* were divided into two major clades, with members in the Oceanica group and subgenus *Testeria* in one clade, and the Excentrica, Conica, Pellucida, Pyriforme and Divergens sections in the other clade. The LSU rDNA molecular phylogeny supported the historical morphologically determined sections, but not a simple morphology based model of evolution based on thecal plate shape.

Key index words: ***Diplopsalis*; heterotrophic dinoflagellate; LSU rDNA; molecular phylogeny; *Preperidinium*; *Protoperidinium*; SEM; single-cell PCR; taxonomy**

Abbreviations: **DI, deionized water; FSW, filtered seawater; LSU, large subunit; ML, maximum likelihood; MP, maximum parsimony; NJ, neighbor-joining**

The genera *Protoperidinium* Bergh, *Diplopsalis* Bergh, and *Preperidinium* Mangin are comprised of species of marine, thecate, heterotrophic dinoflagellates in the family Protoperidinaceae Balech. As raptorial, often selective feeders on species of large phytoplankton, including diatoms, dinoflagellates (Jacobson and Anderson 1986, Naustvoll 2000), and even zooplankton eggs (Jeong 1996), these heterotrophs play an important, but poorly defined, role in planktonic trophic dynamics. These genera have had a confused taxonomic history, and until recently, no genetic information has been available to inform species identification or to resolve their evolutionary relationships with one another or with the other dinoflagellates.

Protoperidinium is a cosmopolitan genus of more than 200 morphologically defined species (Balech 1974). As summarized here from comprehensive historical reviews by Taylor (1976), Abé (1981), Dodge (1982), and references therein, the genus has a long and complex taxonomic history. The genus *Peridinium* Ehrenberg was established by Ehrenberg in 1830, and originally included not only species now in the *Protoperidinium* but also those in *Gymnodinium* Stein and *Ceratium* Schrank. Bergh erected the new genus *Protoperidinium* in 1881, including in it species with distinct sulcal lists, but leaving in the *Peridinium* species with antapical horns or without sulcal lists (Bergh 1881). Over the next few decades, many other workers divided the *Peridinium* into subgroups based largely on cell shape and girdle configuration until, in 1909, Kofoid provided the detailed thecal morphological descriptions upon which the current thecal classification is based (Kofoid 1909).

In 1912, Jörgensen proposed a system of subdivision of the *Peridinium* based upon the combinations of the thecal plate patterns of the ventral and dorsal epitheca (Jörgensen 1912). Species with two epithecal intercalary plates were placed in a new genus, *Archaeperidinium*, while *Peridinium* retained species with three intercalary plates. Based upon the shape of the first apical (1′) plate, two subgenera were erected, *Orthoperidinium* including species with ortho (four sided) 1′ plates and *Metaperidinium* including meta (five sided)

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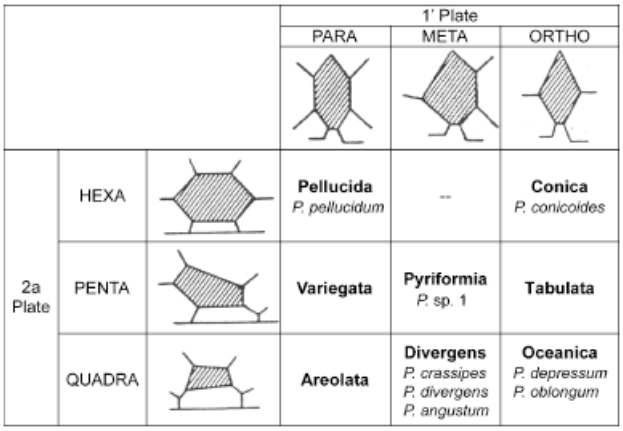


FIG. 1. Morphological sections of *Protoperidinium* as defined by Jörgensen (1912), based on structure of 1′ and 2a thecal plates. Species included in this study within each group are listed. Figure after Nicolaus Peters (1928).

and para (six sided) plates. Combined with the three shapes defined for the second intercalary plate, quadra (four sided), penta (five sided), and hexa (six sided), the *Orthoperidinium* was divided into three sections, and *Metaperidinium* into four sections. Jörgensen's system was generally accepted, with a few revisions. The *Paraperidinium* (six-sided 1′) was eventually made a separate group, divided into three sections, and the *Metaperidinium* was broken into two sections as shown in Figure 1. Paulsen (1931) followed Lebour's (1922) proposal that *Archaeperidinium* was a subgenus, but divided it into two sections: *Avellana* and *Excentrica*, with symmetrical or asymmetrical epithecae, respectively. Recently, Faust (2006) erected the subgenus *Testeria* for *Protoperidinium* with no apical pore and a single intercalary plate (Fig. 2).

Balech (1974) resurrected Bergh's genus, *Protoperidinium*, thereby separating marine, heterotrophic species with three cingular plates and a transitional

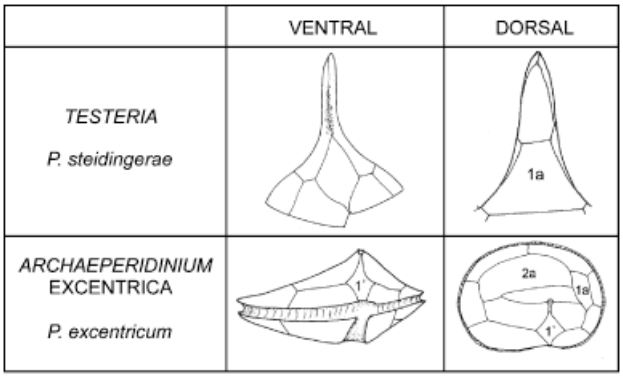


FIG. 2. Ventral and dorsal epithecal plate morphologies of *Protoperidinium* species with one or two intercalary plates that were included in this study. Illustrations of *Protoperidinium steidingerae* from Balech (1979) and of *Protoperidinium excentricum* from Dodge (1982).

plate from the *Peridinium,* phototrophic, freshwater species with five or six cingular plates plus a transitional plate. The characteristic plate formula of *Protoperidinium* is 4′, 3a, 7″, 3c, 5‴, 2⁗, though there are a few species with one, two, or four intercalary plates. An apical platelet surrounds the apical pore. The canal plate connects the apical pore and the first apical plate, a feature missing in some Peridinium species and in all Gonyalacoideae. A transition plate sits between the cingulum and sulcus, and the sulcus has six or seven plates. Balech and Abé greatly emphasized the structure of the sulcal plates in species identification. Given the difficulty in seeing these plates using transmitted light microscopy, and the frequent obscuration of the plates by pronounced sulcal lists, even when using SEM, these small plates have not been routinely used for identification.

The subfamily Diplopsaloideae Abé is composed of lenticular or globular thecate dinoflagellates. The cingulum in these species is equatorial, usually with prominent lists. A pronounced list usually outlines the left side of the sulcus. The large antapical flagellar pore is situated more medially in the diplopsalids than in the *Protoperidinium* and is difficult to observe due to the lists of the sulcal plates. Like the *Protoperidinium*, members of the Diplopsalis group have three cingular plates, but may have only one or two antapical plates, and have only one or two anterior intercalaries.

As with the *Protoperidinium*, the taxonomy of the Diplopsalis group has been greatly confused (Taylor 1976, Abé 1981, Dodge 1982 for full historical reviews). R. S. Bergh first established the genus *Diplopsalis* with his description of *Diplopsalis lenticula* (Bergh 1881), but Stein was the first to provide plate tabulation for the species, although his illustration of *D. lenticula* seems to have included more than one species (as discussed in Dodge 1982).

Around the same time, Pouchet identified a lenticular species with an ascending and overhanging girdle as *Glenodinium lenticula*, but which likely belonged to the Globula group within the *Protoperidinium* (Pouchet 1883). *Glenodinium* Ehrenberg remained poorly defined, and species designated to this genus have largely been redefined over the years. Over the succeeding decades, there were several other descriptions of *D. lenticula* and of new species, variously placed into the *Diplopsalis* Meunier, *Peridinium*, *Glenodinium*, *Diplopsalopsis*, *Peridiniopsis* Lemmermann, *Zygabikodinium* Loeblich and Loeblich III, and *Preperidinium*, many of which were incompletely figured. The difficulty of the taxonomic history of the group is illustrated by the placement of approximately 20 species variously into 11 different genera (Dodge and Hermes 1981).

The subfamily Diplopsaloideae Abé now falls within the family Protoperidinaceae, and includes nine genera of extant, marine heterotrophs (Fensome et al. 1993), after *Zygabikodinium lenticulatum* Loeblich and Loeblich III was restored to its original species description as *Preperidinium meunieri* (Pavillard) Elbrächter (Elbrächter 1993). The group has been considered

ancestral to modern *Protoperidinium* based, in part, on cyst morphology (Matsuoka 1988).

Despite the more than 200 recorded species of *Protoperidinium* and the long history of investigation of both the *Protoperidinium* and the Diplopsaloideae, there remains a great deal of taxonomic uncertainty. The majority of species have been insufficiently figured and described. All species identifications have been from field samples, raising the distinct possibility that geographically separated strains of the same species may have been re-identified as separate species by different workers. Additionally, morphologically distinct life cycle stages may have been classified as separate species, as was the case with the small male gametes of *Ceratium* spp. which had been assigned to the subgenus *Tripoceratium* before the sexual cycle of *Ceratium* was understood (von Stoch 1964, 1973). The matching of morphology with molecular markers may thus provide more robust means for defining species.

Until recently, there has been a complete lack of information about the molecular phylogeny of these thecate heterotrophic dinoflagellates. Within the last few years, however, 11 *Protoperidinium* species have been added to dinoflagellate phylogenies based on small subunit (SSU) rDNA sequences (Saldarriaga et al. 2004, Yamaguchi and Horiguchi 2005). We do not yet have representatives from all major taxonomic groupings, nor any DNA sequence information about the presumably closely related diplopsalids of the genera *Diplopsalis* or *Preperidinium*.

To begin to remedy this taxonomic confusion, better elucidate the validity of morphological groupings within the *Protoperidinium* and diplopsalids, and determine the evolutionary relationships between these thecate heterotrophs and other dinoflagellates, we undertook a study of the molecular phylogeny of these heterotrophic dinoflagellates using the large subunit (LSU) of the rDNA. The LSU rDNA, having both highly conserved and highly variable regions, allows comparison across a range of taxonomic levels, from within species to between genera and families. Previous phylogenetic studies using the LSU rDNA provided sequences from other dinoflagellate species with which the sequences of *Protoperidinium* and diplopsalid species from this study could be compared (Daugbjerg et al. 2000, Hansen et al. 2000, 2003, Ellegaard et al. 2003).

In this study, we sequenced the partial LSU rDNA of 10 species of *Protoperidinium* from two subgenera, encompassing five described morphological sections and one undescribed section (Figs. 1 and 2), and two species of lenticular heterotrophic genera, *D. lenticula* Bergh and *Preperidinium meunieri*, and used these sequences to infer the phylogeny of the group. The molecular phylogeny was compared with the morphological groupings of these heterotrophic species.

MATERIALS AND METHODS

Culturing. *Protoperidinium* spp. were isolated from a variety of locations and grown in 0.2 μm-filtered, Teflon-autoclaved seawater from Vineyard Sound (30 psu, or amended to 35 psu by evaporation) and appropriate phytoplankton food species (Table 1). Cultures were contained in 70 mL untreated tissue culture flasks (Falcon 353009, Becton Dickinson, Franklin Lakes, NJ, USA) and rotated on a plankton wheel at 1–2 rpm at 15° C under low light (approximately 50 μmol photons $\cdot m^{-2} \cdot s^{-1}$) on a 14:10 light:dark (LD) cycle. Cultures were transferred every 4–5 days by pouring approximately two-thirds the volume of the old culture into a new flask containing fresh sterile seawater and phytoplankton prey.

Dinoflagellate cultures used as food for the heterotrophic dinoflagellates were maintained at 15° C or 20° C in tubes with 25 mL of f/2 nutrient medium minus silicate (Guillard 1975), except for *Lingulodinum polyedrum* (Stein) Dodge which was grown in ES medium (Kokinos and Anderson 1995). Diatom cultures were maintained in tubes with 25 mL of f/2 nutrient medium plus silicate at 15° C. All prey cultures were kept at a irradiance of approximately 100 μmol photons $\cdot m^{-2} \cdot s^{-1}$, on a 14:10 LD cycle.

TABLE 1. Culture designation, type, origin, food species, and GenBank accession number for LSU rDNA sequence of thecate, heterotrophic dinoflagellates species studied.

Species	Culture designation	Clonal (C)/unialgal (U)/field (F)	Origin	Food species	Accession number
Diplopsalis lenticula Bergh	M2	U	Gulf of Mexico, FL	*Db*, *Ca*	DQ444226
Preperidinium meunerii (Pavillard) Elbrächter	NA	F	Salt Pond, MA	NA	DQ444232
Protoperidinium angustum (Dangeard) Balech	M30	U	Gulf of Mexico, FL	*Lp*	DQ444237
Protoperidinium conicoides (Paulsen) Balech	NA	F	Salt Pond, MA	NA	DQ444227
Protoperidinium crassipes (Kofoid) Balech	24(B)15	C	SW Coast of Ireland	*Lp*	DQ444234
Protoperidinium crassipes (Kofoid) Balech	M065-PC-Cl7	C	Gulf of Mexico, FL	*Lp*	DQ444224
Protoperidinium depressum (Bailey) Balech	PDIR1A-Cl1	C	SW Coast of Ireland	*Db*, *Ca*	DQ444228
Protoperidinium divergens (Ehrenberg) Balech	24(4)	C	SW Coast of Ireland	*Lp*	DQ444236
Protoperidinium divergens (Ehrenberg) Balech	24B(14)	C	SW Coast of Ireland	*Lp*	DQ444235
Protoperidinium excentricum (Paulsen) Balech	NA	F	Vineyard Sound, MA	NA	DQ444229
Protoperidinium oblongum (Aurivillius) Parke and Dodge	24C(5)-PO-1	C	SW Coast of Ireland	*Db*, *Ca*	DQ444230
Protoperidinium pellucidum Bergh	24C(6)	U	SW Coast of Ireland	*Db*, *Ca*	DQ444233
Protoperidinium steidingerae Balech	MV0923-PO-Cl7	C	Vineyard Sound, MA	*Db*, *Ca*	DQ444231
Protoperidinium sp. 1 Bergh	M064-sm-1	U	Gulf of Mexico, FL	NA	DQ444225

Lp, *Lingulodinium polyedrum*; *Db*, *Ditylum brightwellii* (West) Grunow; and *Ca*, *Chaetocerous affinis* Lauder. NA, not applicable.

SEM. Morphological species identification was confirmed by examination of thecal plate structure using SEM. Samples were preserved with borate-buffered formalin (5% final concentration) and stored at 4° C at least overnight. Subsamples were centrifuged, aspirated to 1 mL, and brought up to 4 mL with filtered seawater. Several hundred *Protoperidinium* cells were isolated by micropipette from phytoplankton prey in the sample, and were deposited into 2 mL cryovials with 5% formalin in filtered seawater and stored at 4° C overnight. Samples were drawn down onto filters (Nucleopore track-etched membrane, 13 mm, 5 μm pore size), and washed with filtered sea water and then with distilled, deionized water to remove fixatives and salts. Samples were dehydrated in a series of ethanol washes of increasing concentration, critical point dried (Tousimis Samdri-780A, Tousimis Research Corp., Rockville, MD, USA), sputter coasted with gold palladium (Tousimis Samsputter-28, Tousimis), and examined on a scanning electron microscope (JEOL JSM-840 Tokyo, Japan).

Calcofluor white. As an alternative to SEM, thecal plates were examined by staining with Calcofluor White (Polysciences, Warrington, PA, USA) (Fritz and Triemer 1985). Cultures and field samples were preserved with formalin (5% final concentration) and stored at 4° C until analysis. Samples were centrifuged, aspirated to a pellet, resuspended in 1 mL filtered seawater and 5 μL of a 1.0 mg · mL^{-1} solution of Calcofluor White MR2, which stains cellulose thecal plates. After staining for 10 min, each sample was aspirated to a pellet and then resuspended in 2–10 mL of filtered seawater for analysis. Subsamples of up to 1 mL were examined at × 100–200 on a Zeiss Axioskop microscope (Zeiss, Jena, Germany) with a 100 W mercury lamp and a Zeiss #2 filter set (excitation 365 nm, emission 420 nm). Images were taken with a Zeiss MC 100 digital camera system. Species identifications were as in Abé (1981), Dodge (1982), and Elbrächter (1993).

Single-cell PCR amplification, cloning, and sequencing. Single-cell PCR was used to amplify the target sequence. Single dinoflagellate cells were isolated from culture or field samples by micropipette. Each cell was washed two to three times in sterile filtered seawater and one to two times in sterile deionized (DI) water before being deposited individually into a PCR tube in approximately 10 μL of sterile DI water. The PCR tubes with isolated cells were frozen at − 80° C overnight to enhance cell lysis. To further improve lysis, isolated cells in PCR tubes, immersed in an ice bath, were subjected to a sonification bath at 40 A for approximately 30 s immediately before PCR.

The single cells were used directly as template to amplify approximately 1430 bp of the LSU rDNA containing the variable domains D1–D6, using the primers D1R (Scholin et al. 1994) and 28-1483R (Daugbjerg et al. 2000). The 50 μL PCR reaction mixture contained 2.5 units *Pfu*, a proofreading DNA polymerase (Stratagene, La Jolla, CA, USA), 5 μL 10 × buffer (1 × final concentration), 0.3 μM of each primer, and 200 μM dNTPs (Takara, Shiga, Japan). Thermal cycling was conducted using an initial denaturation at 95° C for 5 min, 30 cycles of 95° C for 1 min, 50° C for 1 min, and 72° C for 2 min, followed by a final elongation step of 72° C for 10 min.

Between 25 and 30 μL of PCR product was run on a 1% agarose gel. Positive bands were excised and the product purified and concentrated using a MinElute Gel Extraction Kit (Quiagen, Valencia, CA, USA). For each species, from one to eight purified PCR products were cloned separately using the Zero Blunt TOPO PCR Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA). Primers T3, T7, and an appropriate internal primer (Table 2) were used for sequencing between 12 and 96 clones for each species. Sequencing was done on an Applied Biosystems 3730XL capillary sequencer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analyses. Partial LSU rDNA sequences from *Protoperidinium* spp., *D. lenticula*, and *Preperidinium meunieri* were edited using Sequencher 4.5. Using Clustal X, these sequences were aligned with sequences from GenBank of 21 other dinoflagellate species and of two ciliates and one apicomplexan as an outgroup (Table 3). Only the D1–D3 domains of the LSU rDNA were available for most dinoflagellates species in GenBank, so we included only these regions in alignments and analyses. Alignments produced by Clustal X were edited manually. Bases 427–737 were too divergent for accurate alignment and were excluded from phylogenetic analyses, leaving a total of 775 bases.

TABLE 2. Primers used for PCR and sequencing of *Protoperidinium*, *Diplopsalis*, and *Preperidinium* spp.

Primer	Sequence	Source
D1R	ACCCGCTGAATTTAAGCATA	1
D2C	CCTTGGTCCCGTGTTTCAAGA	1
D3B	TCGGAGGGAACCAGCTACTA	2
D3BPR	TCGGAGGTAACCAGCTACCA	3
D3SP	AGGTAAAGCGAATGATTAG	3
D3PE	CTTTGGAGAGAACCAGCTACT	3
M064SMD3	TCATTTGCTTTACCTGATGAAA	3
28-1483R	GCTACTACCACCAAGATCTGC	4
T3	ATTAACCCTCACTAAAGGGA	5
T7	TAATACGACTCACTATAGGG	5

1. Scholin et al. (1994).
2. Nunn et al. (1996).
3. This study.
4. Daugbjerg et al. (2000).
5. Invitrogen Zero Blunt TOPO PCR Cloning Kit for Sequencing.

The alignment was subjected to neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) analyses using PAUP* 4.0b10 (Swofford 2002) and to Bayesian analysis using Mr. Bayes 3.1.1 (Ronquist and Huelsenbeck 2003). For NJ, ML, and Bayesian analyses, the parameters of the most appropriate model of DNA substitution were determined using Modeltest 3.06 (Posada and Crandall 1998). A general time reversible model (GTR + I + G) with four rate categories, a proportion of invariable sites of 0.1324, and a gamma distribution of 0.9597 best fit the data. In all analyses, gaps were treated as missing data. Phylogenetic trees calculated by MP and ML (ML using the GTR + I + G model described above) were determined with starting trees obtained by stepwise addition and a heuristic search with 10 replicates using random addition of sequences. Branch swapping was by tree-bisection-reconnection. Bootstrap values were determined for NJ (1000 reps), MP (1000 reps) and ML (100 reps). Bayesian analysis was run for 1,000,000 generations with four chains, beginning from a random starting tree. A GTR + I + G model was used in Bayesian analysis, with the proportion of invariable sites estimated from the data and four rate categories to approximate the gamma distribution.

RESULTS

Morphological analyses. Based on thecal plate patterns, the *Protoperidinium* species examined belonged to three subgenera and five defined morphological sections, with some sections represented by only one species.

Members of the Divergens section, characterized by a meta 1′ plate and a quadra 2a plate, included two strains of *Protoperidinium divergens* (Ehrenberg) Balech,

TABLE 3. GenBank accession numbers of large subunit rDNA sequences for dinoflagellate, ciliate, and apicomplexan species examined in this study.

Species	Accession number
Akashiwo sanguinea (Hirasaka) G. Hansen and Moestrup	AF260397
Alexandrium margalefii Balech	AY154958
Alexandrium pseudogonyaulax (Biecheler) Horiguchi ex Kita and Fukuyo	AY154957
Amphidinium carterae Hulburt	AY455669
Amphidinium semilunatum Herdman	AY455678
Amphidinium steinii Lemmermann	AY455673
Ceratium lineatum (Ehrenberg) Cleve	AF260391
Euplotes aediculatus Pierson	AF223571
Fragilidium sublglobosum (von Stoch) Loeblich III	AF260387
Gonyaulax baltica Ellegaard, Lewis and Harding	AY144962
Gonyaulax cf. *spinifera* Diesing	AY154960
Gymnodinium pellucidum (Herdman) Jørgensen and Murray	AY455681
Heterocapsa sp. Stein	AF260399
Heterocapsa triquetra (Ehrenberg) F. Stein	AF260401
Karenia brevis (Davis) G. Hansen and Moestrup	AF200677
Peridinium cinctum Ehrenberg	AF260385
Peridinium palatinum Lauterbom	AF260394
Peridinium willei Huitfeld-Kaas	AF260384
Polarella glacialis Montresor, Procaccini and Stoecker	AY036081
Pyrodinium bahemense var. *compressum* (Böhm) Steidinger, Tester and Taylor	AY154959
Symbiodinium sp. Freudenthal	AJ291512
Tetrahymena thermophila Furgason	X54512
Toxoplasma gondii Nicolle and Manceaux	X75429
Woloszynskia pseudopalustris (Woloszynska) Kisselew	AF260402

two strains of *Protoperidinium crassipes* (Kofoid) Balech, and one strain of *Protoperidinium angustum* (Dangeard) Balech (Fig. 3). The plate patterns were the same in these species, so they were distinguished by differences in gross morphology. *P. divergens* was slightly longer than wide (length 90 μm, width 75 μm), while *P. crassipes* was of approximately equal length and width (length 90 μm, width 90 μm). The sulcal lists of both species projected anteriorly, making the antapical horns appear to end in multiple spines. In *P. divergens*, the suture dividing the two antapical plates was shifted slightly to the left of center in the Irish strains examined here (Fig. 3, A–C). In *P. crassipes* strains from both Ireland and from the Gulf of Mexico (Fig. 3D–I), the two antapical plates were more symmetrically divided, with the suture meeting the middle of the third postcingular plate (3‴). The cingulum of *P. crassipes* formed a slight proximal arch on the left side to meet the excavated sulcus, which widened posteriorly. The degree of concavity of the epitheca appeared variable in culture. The cingulum of *P. divergens* was not offset. *P. angustum* (Fig. 3, J–L) was a smaller species (length 70 μm, width 65 μm) with a more rounded epitheca and hypotheca. The ventral hypotheca was deeply excavated, tapering to very small antapical horns.

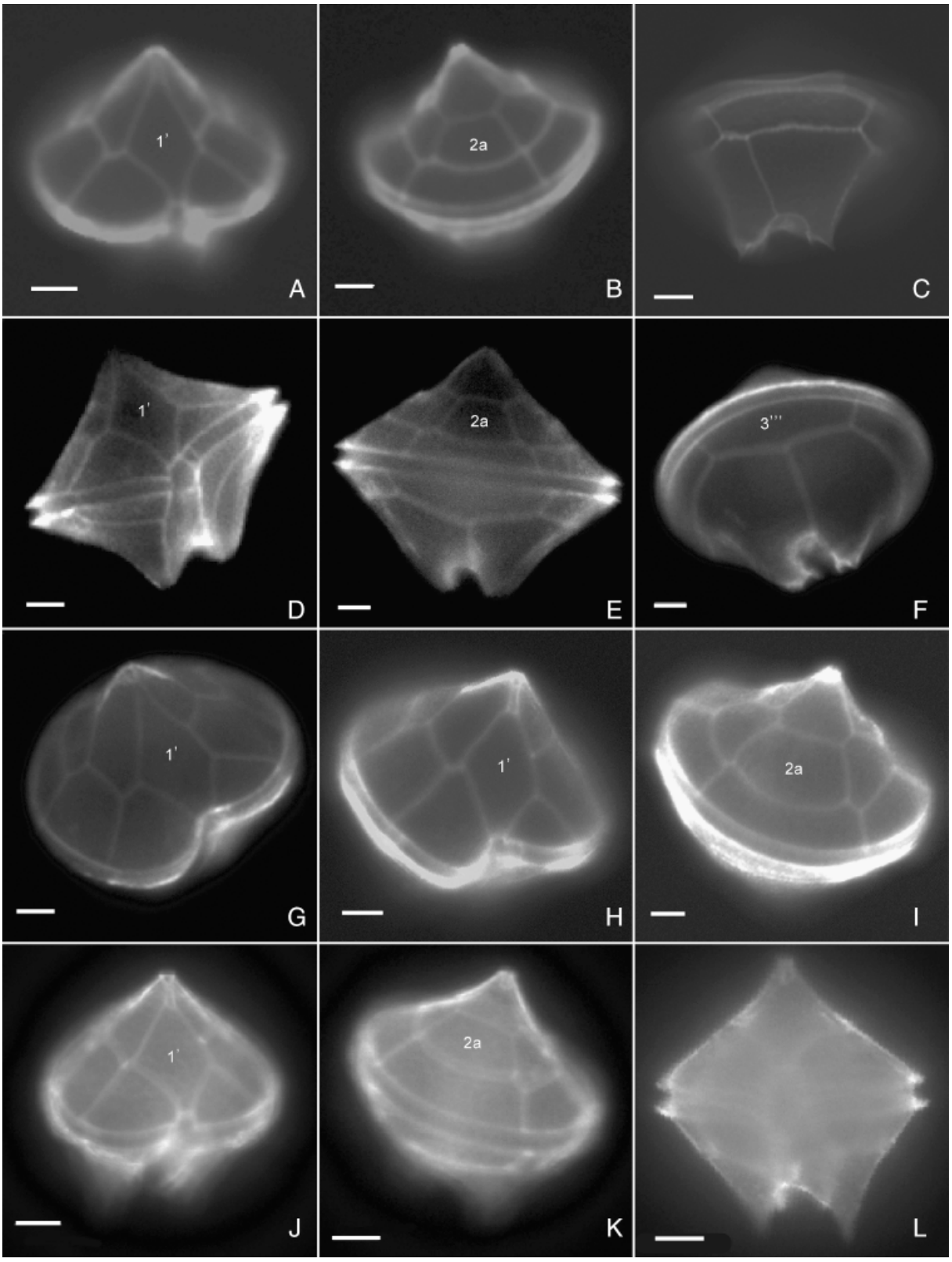
Several *Protoperidinium* morphological groups were represented by a single species. *Protoperidinium pellucidum* Bergh, with a para 1′ plate and a hexa 2a plate was in the Pellucida group (Fig. 4, A–C). The Pyriforme group, with a meta 1′ plate and a penta 2a plate was represented by *Protoperidinium* sp. 1 (Fig. 4, D–F), with a plate pattern and shape similar to that of *Protoperidinium pyriforme* (Paulsen) Balech, but whose small size precluded that species designation. A full taxonomic description of *Protoperidinium* sp. 1 will be presented elsewhere. *Protoperidinium conicoides* (Paulsen) Balech had an ortho 1′ plate and a hexa 2a plate, placing it in the Conica group (Fig. 4, G–H). In the subgenus *Archaeperidinium*, members of which have only two antapical plates, was *Protoperidinium excentricum* (Paulsen) Balech (Fig 4, I–L). This species was anterior-posteriorly compressed, with an apical horn displaced to the ventral side of the cell, and the sulcus extending to the center of the hypotheca. *P. excentricum* had one small six-sided intercalary plate on the left side of the epitheca, and a second, large intercalary plate covering the majority of the dorsal side of the cell. The asymmetrical epitheca of the species placed it within the section Excentrica.

Two species, *Protoperidinium depressum* (Bailey) Balech and *Protoperidinium oblongum* (Aurivillius) Parke and Dodge, both from Ireland, were in the Oceanica section, with ortho 1′ and quadra 2a plates (Fig. 5,A–C, F–H). Both species were large and had the same thecal plate pattern but different gross morphology. *P. oblongum* was long and dorso-ventrally compressed. *P. depressum* was broad and round at cingulum, with the concavity of the ventral apex lending the distinctive "tilt" to the cell.

A *P. oblongum*-like species, *Protoperidinium steidingerae* Balech, was isolated from Vineyard Sound, Woods Hole, MA (Fig. 5, D and E). *P. steidingerae* had one intercalary plate and a single ortho apical plate on the ventral side that did not reach the apex of the apical horn (Fig. 5E). This species falls within the recently defined subgenus *Testeria*, given that it has only one apical plate and lacks an apical pore (Faust 2006).

The two lenticular species studied, *Preperidinium meunieri* (syn. *Zygabikodinium lenticulatum*) and *D. lenticula*, both had a pronounced list on the left side of the sulcus, an equatorial girdle, three apical plates with an ortho 1′ apical plate in which the anterior triangle was longer than the posterior triangle, and a single antapical plate (Fig. 6, A–H). In *D. lenticula*, there were three apical plates, with a relatively wide 1′ plate (Fig. 6, A). The relative sizes of the 2′ and 3′ plates varied, ranging from equal in size to unequal, so that the 2′ plate was one-half the size of the 3′ plate (Fig. 6, C and D). There was a single, narrow, long intercalary plate, and six narrow precingular plates. *Preperidinium meunieri* (Fig. 6, E–H) was more globular in shape and

had a thinner 1′ plate than did *D. lenticula*. A small, four-sided intercalary plate (1a) was on the left side of *Preperidinium meunieri*, a second, large intercalary (2a) plate occupied the dorsal half of the epitheca, nearly contacting the apical pore, and there were seven precingular plates.



Sequence variability. In attempting to sequence directly from PCR products, we found high intraindividual variability in the LSU rDNA, requiring sequencing from cloned PCR products. The sequence used for phylogenetic analysis of a given species was the most abundant sequence in the clone library from multiple single-cell PCR reactions for that species. Results regarding intraindividual sequence variability will be presented separately.

Interestingly, the site of the D3B primer, useful in many other studies of dinoflagellate phylogeny, was variable within the *Protoperidinium* and diplopsalid dinoflagellates in this study, necessitating the development of new internal primers for sequencing most species (Table 2).

Dinoflagellate phylogeny. Two dinoflagellate phylogenies that include the *Protoperidinium*, *Preperidinium*, and *Diplopsalis* are presented. Figure 7 provides the results of MP analysis, with bootstrap support from MP and NJ analyses. Figure 8 gives a tree from ML analysis, supported by bootstrap values from ML and posterior probability values from Bayesian analyses.

The phylogenetic trees from MP and ML analyses shared many features. Uncertainties at particular nodes in a given tree, indicated by low bootstrap support, were often supported by higher bootstrap values in the other tree. In both trees, the major groupings of dinoflagellates were present. As is the case with many LSU and SSU rDNA phylogenies of the dinoflagellates, the branching order between these groups was poorly defined, with low bootstrap support for many deeper branches, and with branching order dependent upon the model of evolution and the method of analyses used. The Gonyaulacoides formed a monophyletic group with high support in all analyses. As in LSU and SSU rDNA phylogenies constructed by others, the Gymnodinales were scattered throughout the tree. *Amphidinium carterae* Hulburt and *Amphidinium steinii* Lemmermann diverged early, while *Amphidinium semilunatum* Herdman was placed more recently. *Symbiodinium* sp. Freudenthal fell into a clade with two gymnodinales, *Woloszynskia pseudopalustris* (Woloszynska) Kisselew and *Polarella glacialis* Montresor, Procaccini and Stoecker. Placement of *Fragilidium subglobosum* (von Stoch) Loeblich III within the Gonyaulacoides varied depending upon analysis, although its position within the *Alexandrium* Halim clade in the ML tree was most likely, given the support by Bayesian analysis, and lack of high bootstrap support for placement with *Gonyaulax* spp. Diesing and *Ceratium lineatum* (Ehrenberg) Cleve in the MP tree.

The thecate, heterotrophic, marine *Protoperidinium*, *Diplopsalis*, and *Preperidinium* diverged from the primarily freshwater, phototrophic *Peridinium* spp. in all analyses; a result well supported in both ML and Bayesian analyses, but not in MP or NJ analyses. The other Peridinaceae species included in the study, *Heterocapsa* spp. Stein, were basal to the *Peridinium* spp.

Phylogeny within the thecate heterotrophic dinoflagellates. The *Protoperidinium* appeared to be recently divergent in all analyses. In MP and NJ analysis, the *Protoperidinium* formed a monophyletic group, albeit with low bootstrap support, evolving from the diplopsalid dinoflagellates. In ML and Bayesian analyses, however, the *Protoperidinium* were paraphyletic, as the diplopsalid, *D. lenticula*, was inserted, with low bootstrap support, within the *Protoperidinium* clade as basal to *P. excentricum*, and *Preperidinium meunieri* fell, with low bootstrap support, within a separate clade as a sister to the Oceanica and *Testeria*. A partial MP tree, showing just the *Peridinium* spp., *Protoperidinium* spp. and the diplopsalids is shown in Figure 9.

Whether monophyletic or polyphyletic, the *Protoperidinium* spp. branched into two major clades, one containing the Oceanica section and *P. steidingerae*, and the other clade containing all other morphological sections, including the subgenus *Archaeperidinium*. In the first major clade, *P. steidingerae*, in the subgenus *Testeria*, was a sister to the Oceanica species, *P. depressum* and *P. oblongum*. The second major clade, in which *P. excentricum* was the basal-most species, contained all other morphological sections of *Protoperidinium*. Single representatives from major morphological groupings each branched separately—*P. conicoides* in the Conicum section was basal to *Protoperidinium* sp. 1 in the Pyriforme group, which in turn was basal to *P. pellucidum* of the Pellucida group. Members of the Divergens section, including *P. angustum*, *P. crassipes* and *P. divergens*, were most the derived. Two strains of *P. divergens* were included, both from the same region of Ireland and both with the same sequence. Two strains of *P. crassipes*, one from Ireland the other from the Gulf of Mexico, were more closely related to one another than to *P. divergens*. *P. angustum* was most basal in the Divergens section.

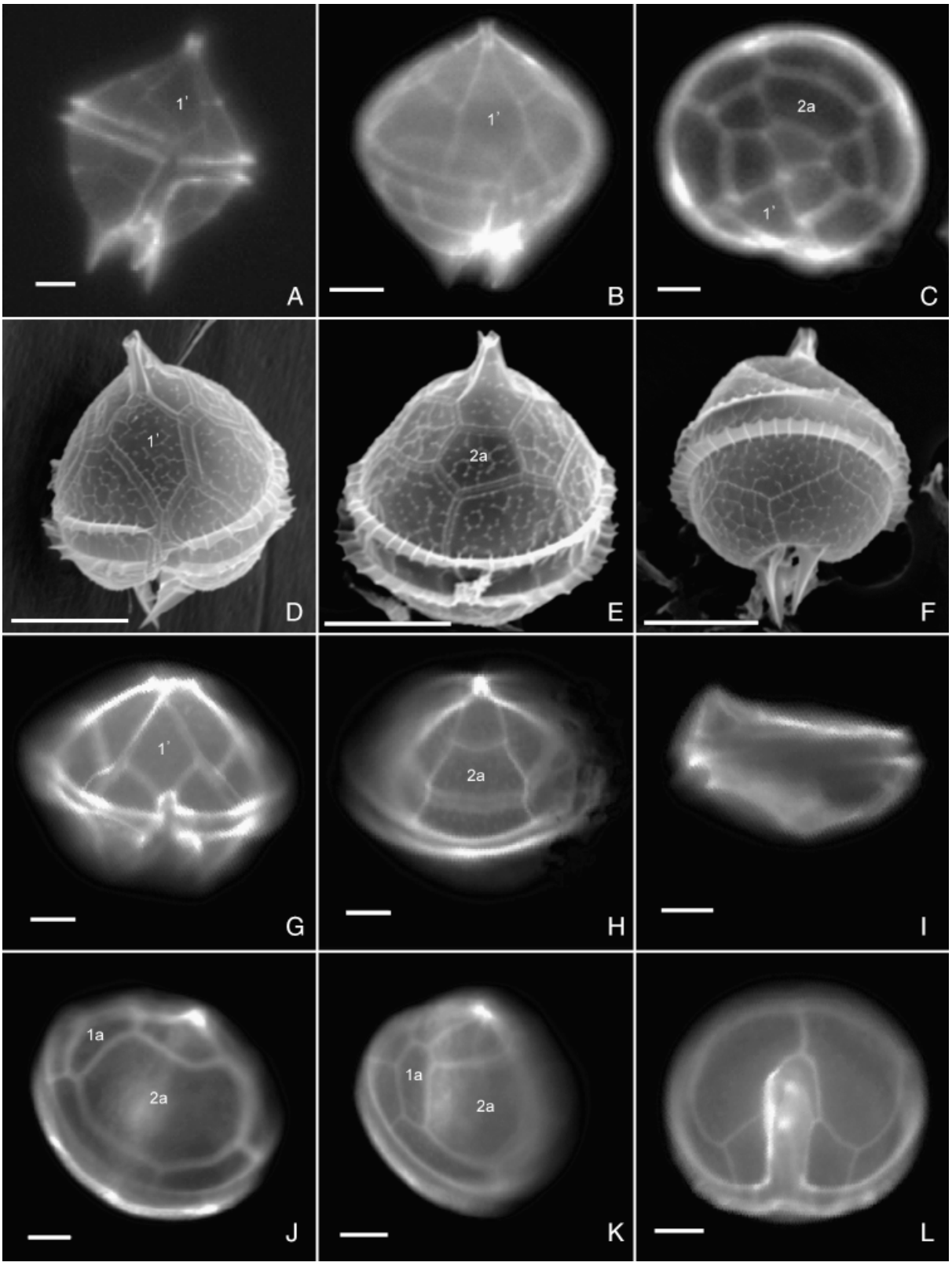
DISCUSSION

This study helped to elucidate the relationship between molecular evolution and morphology in the thecate heterotrophic dinoflagellates, in an effort to sort out the confused taxonomies of the *Protoperidinium* and diplopsalids. While the historical morphological

FIG. 3. Fluorescence micrographs of Calcofluor White-stained cells of *Protoperidinium* spp. in the Divergens section. Scale bars, 10 μm. (A–C) *Protoperidinium divergens* from western Ireland: (A) Ventral epitheca, showing meta 1′ plate, cingulum not displaced. (B) Dorsal epitheca, showing quadra 2a plate. (C) Dorsal hypotheca with suture between antapical plates offset to the left. Antapical horns ending in multiple spines. (D–G) *Protoperidinium crassipes* from the Gulf of Mexico, FL: (D) Ventral side, with meta 1′, displaced cingulum, and sulcus widening antapically. (E) Dorsal side, with quadra 2a plate. (F) Dorsal hypotheca, with centered suture between anapical plates and antapical horns ending in multiple spines. (G) Epitheca, with meta 1′ plate. (H–I) *Protoperidinium crassipes* from western Ireland: (H) Ventral epitheca, with meta 1′ plate. (I) Dorsal epitheca with quadra 2a plate. (J–L) *Protoperidinium angustum*: (J) Ventral epitheca with meta 1′ plate. (K) Dorsal epitheca with quadra 2a. (L) Dorsal side, showing cell shape, cingular and sulcal lists, and pointed antapical horns.

sections defined for these groups were evolutionarily germane, the lineage of those groups was not so straightforward.

Single-cell PCR. In the past few years, single-cell PCR has become more commonly used in molecular genetic investigations of dinoflagellates (Bolch 2001,



Sebastián and O'Ryan 2001, Godhe et al. 2002, Rehnstam-Holm et al. 2002, Yamaguchi and Horiguchi 2005). In this study, we performed PCR reactions directly on single-cells, without DNA extraction. Given that even washing in DI water did not always rupture the thecate cells, we increased the number of successful PCR reactions by freezing isolated cells in DI water overnight at -80° C and then sonifying in a water bath to enhance cell lysis.

Single-cell PCR allowed the heterotrophic dinoflagellates to be separated from their phytoplankton prey, which are often the same size as the heterotrophs, and thus not easily removed. Single-cell PCR also allowed isolation of these difficult-to-culture species directly from field samples, as was done for three species in this study. Working directly from field samples meant we could obtain sequence data in cases where we did not want to culture (*P. conicoides* and *Preperidinium meunieri*) or were unable to culture (*P. excentricum*) the heterotrophic dinoflagellates. The majority of thecate heterotrophs investigated were cultured, however, to confirm species identifications and to examine the range of morphological variability within a given species.

Species identification. Accurate verification of the thecal plate morphology for each species and ample replication of PCR reactions was possible because most species examined were maintained in culture. Cultures also allowed us to better distinguish interspecific from intraspecific morphological and sequence variability. For example, the relative sizes of the 2′ and 3′ plates varied widely in *D. lenticula*. In *P. divergens*, the suture between the antapical plates was shifted left of center in the strains analyzed, a trait not described in the literature. Many of the *Protoperidinium* species studied here exhibited a variety of morphologies throughout their life cycles, including isogamous gametes and a reduction to approximately half to three-quarters of the original size at the time of cell division. *P. oblongum* cells in clonal culture, for example, ranged in length from 60 to 150 μm in culture, and cells in clonal cultures of *P. depressum* similarly ranged in width from 40 to 120 μm. *P. steidingerae* varied from 35 μm long gametes to 140 μm long vegetative cells. If such morphologically distinct life stages were isolated from field samples, they would likely be mis-identified as separate species.

Although longer sequences or data from multiple genes would likely have improved the accuracy of the tree topology for the *Protoperidinium* and diplopsalids, because of the taxonomic uncertainty in these heterotrophic groups, we could not concatenate the SSU sequences from separate strains examined in previous studies (Saldarriaga et al. 2004, Yamaguchi and Horiguchi 2005) with the LSU rDNA data generated in this study. Despite the similarity of names of some species in the three studies, including *P. pellucidum*, *P. crassipes*, *P. divergens*, and *P. excentricum* in this study and either one or both of the other two studies, we may have been dealing with different species or genetically distinct strains of the same species. *P. crassipes* and *P. curtipes*, for example, have been viewed by some as synonymous, while others separated the two, choosing one name or the other (Taylor 1976, Abé 1981, Dodge 1982).

Sequence variability. Yamaguchi and Horiguchi (2005) reported intraspecific variability in the SSU rDNA for *Protoperidinium* spp. collected from the same area. Such diversity of the slowly evolving SSU rDNA within a population is surprising, unless there has been importation of the same species from another area. Cloning the PCR products of the SSU rDNA and sequencing the clones, rather than the PCR products, might have revealed that the sequence variability was actually intracellular, in the form of pseudogenes in SSU rDNA, as we have found in the LSU rDNA (unpublished data). Pseudogenes, multiple, non-functional copies of both SSU and LSU rDNA genes, have been found in other dinoflagellates species (Scholin et al. 1993, Yeung et al. 1996, Santos et al. 2003). These different gene copies have generally been found in approximately equal abundance. Randomly preferential PCR amplification of one copy over another, as could be more likely when starting from the low template concentrations found in single-cell PCR, could give results that appear to be intraspecific variability at a single field location.

The sequence at the site of the D3B primer varied among the thecate heterotrophic species examined in this study, requiring development of new primers for sequencing on nearly a species-by-species basis. The D3B primer, designed for studies of the Isopoda (Nunn et al. 1996) has been used successfully in phylogenetic investigations of a range of other dinoflagellate taxa, including *Alexandrium*, *Gymnodinium*, *Dinophysis* Ehrenberg, and *Polykrikos* Kofoid and Swezy (Hansen et al. 2000, 2003, Bolch 2001) and is considered to lie within a relatively well-conserved region. The variability of this site in these thecate heterotrophs may have

FIG. 4. Fluorescence micrographs of Calcofluor-white stained cells and SEM micrographs of *Protoperidinium* spp. in the Pellucida, Pyriforme, Conica, and Excentricum sections. Scale bars, 10 μm. (A–C) *Protoperidinium pellucidum*: (A) Ventral side showing para 1′ plate, slight displacement of cingulum, solid antapical spines and large sulcal list on left side. (B) Expanded view of ventral epitheca. (C) Top view of epitheca, showing hexa 2a plate. (D-E) *Protoperidinium* sp. 1: (D) Ventral epitheca, with meta 1′ plate and cingular lists supported by spines. (E) Dorsal epitheca showing penta 2a plate. (F) Dorsal hypotheca, showing two antapical plates and solid, winged antapical horns. (G–H) *Protoperidinium conicoides*: (G) Ventral side of cell, with ortho 1′ plate and deeply excavated sulcus. (H) Dorsal epitheca, showing para 2a plate. (I–L) *Protoperidinium excentricum*: (I) Side view, showing cell shape with equilatoral cingulum and apical horn displaced to ventral side of cell. (J) Dorsal epitheca, showing small 3′ plate, portion of 1a plate, and very large 2a plate extending across most of dorsal epitheca. (K) Left side of epitheca, showing 1a plate. (L) Hypotheca, with sulcus extending to center of hypotheca, two antapical plates, and absence of antapical horns.

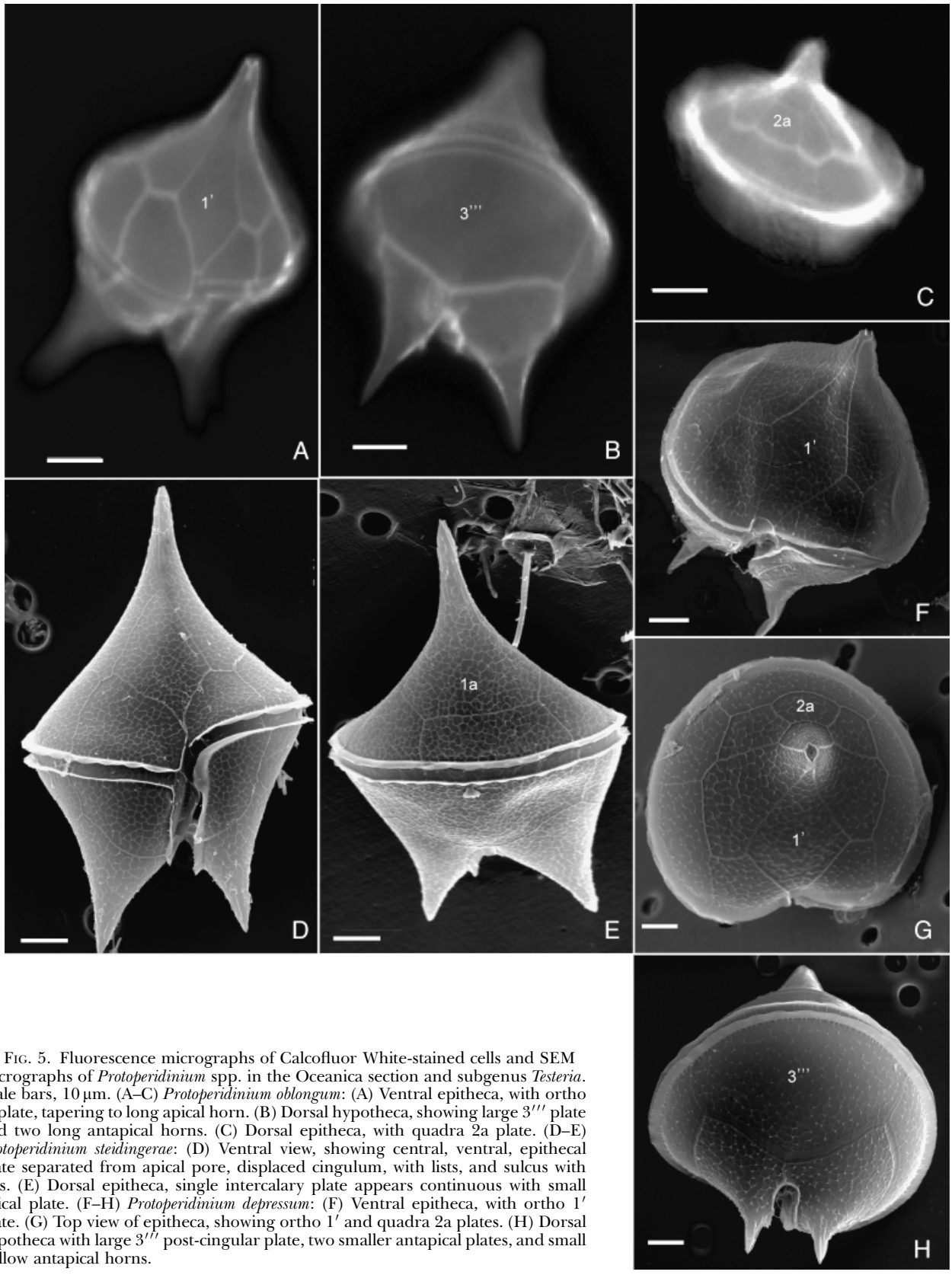


FIG. 5. Fluorescence micrographs of Calcofluor White-stained cells and SEM micrographs of *Protoperidinium* spp. in the Oceanica section and subgenus *Testeria*. Scale bars, 10 µm. (A–C) *Protoperidinium oblongum*: (A) Ventral epitheca, with ortho 1′ plate, tapering to long apical horn. (B) Dorsal hypotheca, showing large 3′′′ plate and two long antapical horns. (C) Dorsal epitheca, with quadra 2a plate. (D–E) *Protoperidinium steidingerae*: (D) Ventral view, showing central, ventral, epithecal plate separated from apical pore, displaced cingulum, with lists, and sulcus with lists. (E) Dorsal epitheca, single intercalary plate appears continuous with small apical plate. (F–H) *Protoperidinium depressum*: (F) Ventral epitheca, with ortho 1′ plate. (G) Top view of epitheca, showing ortho 1′ and quadra 2a plates. (H) Dorsal hypotheca with large 3′′′ post-cingular plate, two smaller antapical plates, and small hollow antapical horns.

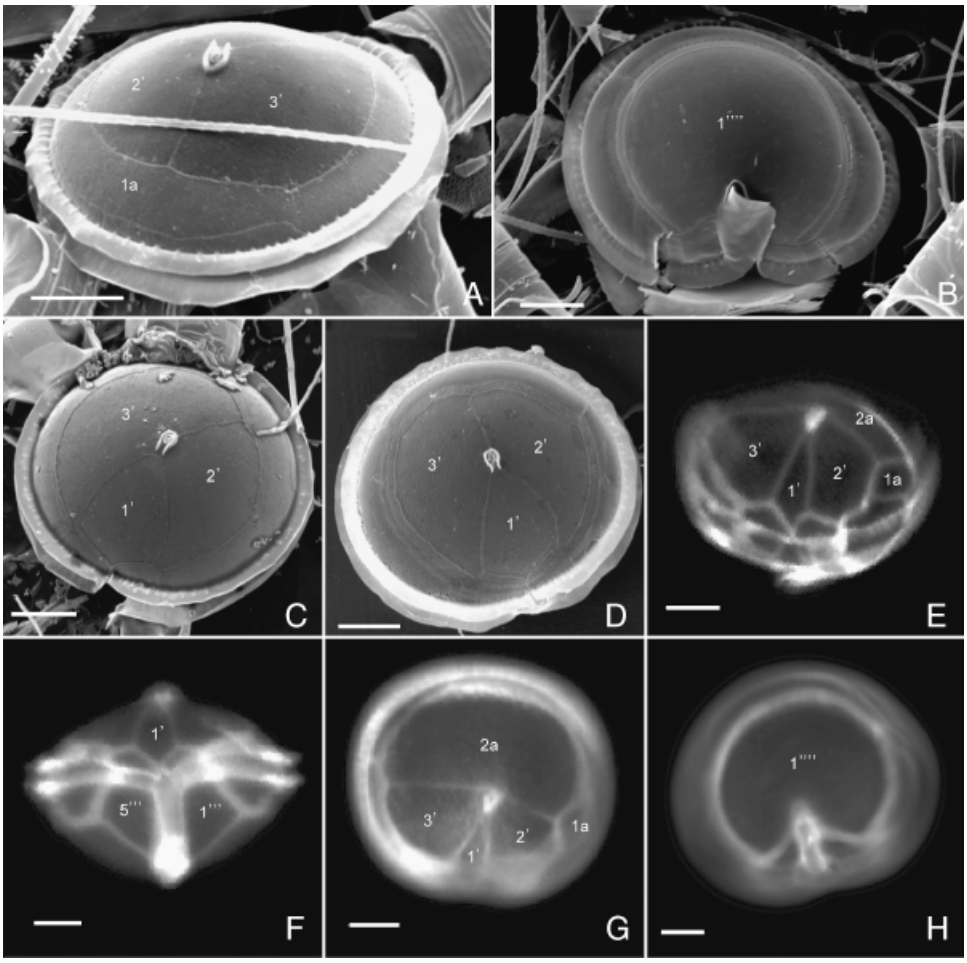


FIG. 6. Fluorescence micrographs of Calcofluor White-stained cells and SEM micrographs of diplopsalid heterotrophic dinoflagellates. Scale bars, 10 μm. (A–D) *Diplopsalis lenticula*: (A) Dorsal epitheca, showing single, wide intercalary plate. (B) Hypotheca, with single antapical plate and pronounced list on left side of sulcus. (C and D) Epitheca, showing ortho 1′ plate with extended upper triangle and shortened lower triangle. In (C), epitheca divided unequally, with 2′ plate half the size of the 3′ plate. In (D), 2′ and 3′ plates are the same size. (E–H) *Preperidinium meunieri*: (E) Ventral epitheca, with narrow, long, ortho 1′ plate and diamond-shaped 1a plate. (F) Ventral view of slightly globular cell with equilateral cingulum. Post-cingular plates 1‴ and 5‴ extending nearly to bottom of sulcus. (G) Top view of epitheca, showing three apical plates on ventral side of cell, diamond shaped 1a plate, and large 2a plate extending across dorsal side of epitheca. (H) Hypotheca, with single antapical plate.

contributed to the difficulty others have reported in obtaining PCR products from *Protoperidinium* using the D3B primer (Bolch 2001).

Phylogeny of the Protoperidinium, Diplopsalis*, and* Preperidinium. Results from this study indicate that the Peridinaceae gave rise first to the Diplopsaloideae, from which the *Protoperidinium* then arose. These findings differed slightly from the hypothetical, parsimonious, morphology-based model of evolution proposed by Taylor (2004), in which a plate-reduction model predicted evolution from the *Peridinium*, with three intercalary plates and five or six cingular plates, to the *Protoperidinium*, and to the *Protoperidinium* having then given rise to the diplopsalids, which have only one or two antapical plates and one or two intercalary plates. Similarity in thecal plate morphology supported the close evolutionary relationship between the *Protoperidinium* and *Peridinium* that we found, which differed from the two other dinoflagellates phylogenies that included *Protoperidinium* to date (Saldarriaga et al. 2004, Yamaguchi and Horiguchi 2005). These previous works were both based on SSU rDNA sequences, and suggested that the *Protoperidinium* were not closely related to the *Peridinium*. These previous studies also argued that the *Protoperidinium* is a monophyletic genus within the dinoflagellates (Saldarriaga et al. 2004, Yamaguchi and Horiguchi 2005), in agreement with our MP results. Our ML results indicate that the *Protoperidinium* may be polyphyletic, however. The inclusion in this study of diplopsalid species and of a wider range of morphological sections of the *Protoperidinium*, in particular members of the Oceanica and the subgenus *Testeria*, likely lead to the disparity

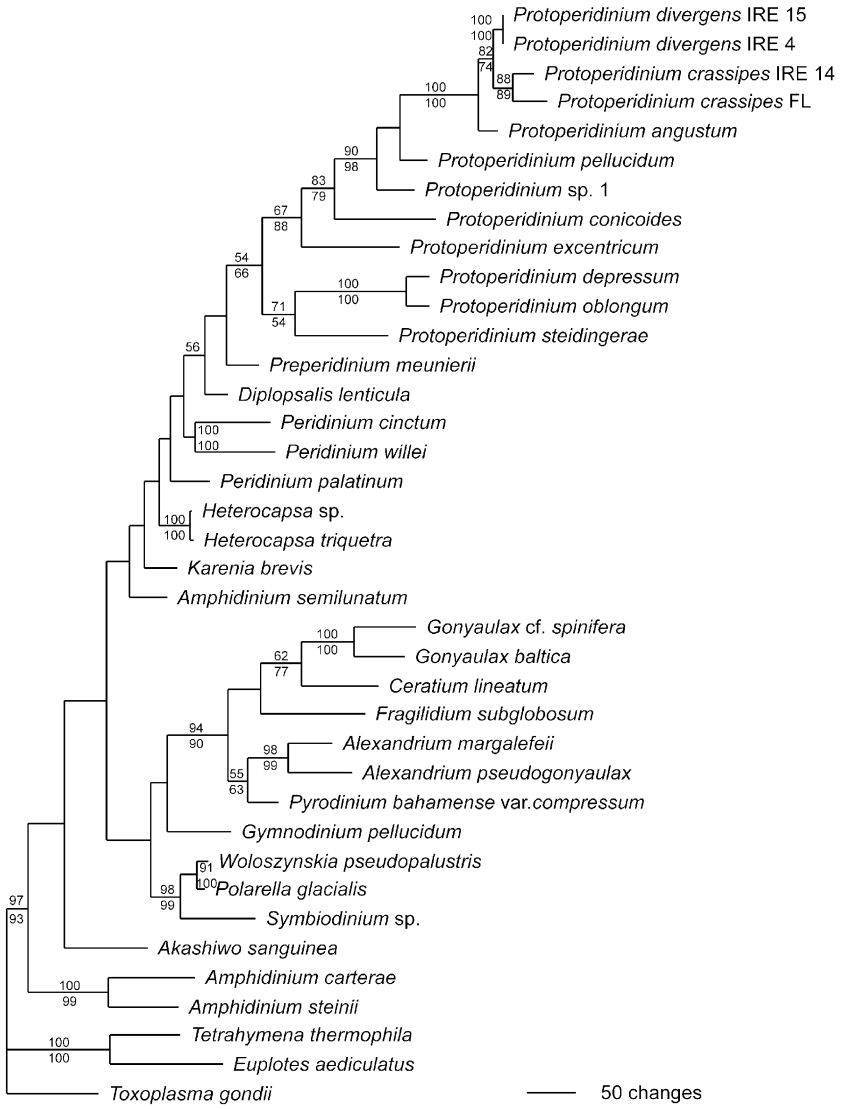


FIG. 7. Maximum parsimony (MP) tree based on domains D1–D3 of the large subunit rDNA gene. Bootstrap values greater than 50% are shown, with values from MP bootstrap (1000 replicates) above the node and values from neighbor joining bootstrap (1000 replicates) below the node. Two ciliates and one apicomplexan were used to root the tree.

between the studies. The Oceanica and *P. steidingerae* formed a sister group to the rest of the *Protoperidinium*, and the diplopsalids appear to be basal to *Protoperidinium* spp., so including these groups may have provided a stronger link to the common ancestor of the *Peridinium* and the *Protoperidinium*.

Thecal plate morphology appears to have relevance to the intrageneric molecular phylogeny, as the *Protoperidinium* spp. grouped strongly by morphological section as determined from thecal plate patterns (Fig. 9). There is not, however, a straightforward evolutionary lineage in the shape of either the 1′ or the 2a plate. Species with para or meta 1′ plates seem to have evolved from species with ortho 1′ plates. The Orthoperidinium is not monophyletic, however, as *P. conicoides* and *P. excentricum* branch separately from the Oceanica clade containing *P. depressum* and *P. oblongum*.

Cyst morphology may also be correlated with molecular phylogeny, although the cyst-theca relationships are not known for all species in this study. Like most diplopsalids, *D. lenticula* and *Preperidinium meunieri* have unornamented, spherical brown cysts, in which the archeopyle forms a slit without a detached operculum (Wall and Dale 1968, Matsuoka 1988). In the Oceanica and *Testeria* clade, both *P. oblongum* and *P. steidingerae* have smooth-walled, rhomboidal to chordate, dorso-ventrally compressed cysts with intercalary archeopyles and apical horns that vary from long and narrow to short and rounded (Wall and Dale 1968, K. Gribble, unpublished data). No cyst has ever been reported for *P. depressum*, despite the species' cosmopolitan nature and easily recognized morphology, so it may be that not all species in the Oceanica clade have cysts. In the other major clade of *Protoperidinium*, both *P. excentricum* and *P. conicoides* have round, unornamented, brown cysts, compressed in either a polar or dorso-ventral direction, respectively. No cysts have been reported for *P. pellucidum* or for any of the species included in this study in the Divergens clade. Additional cyst-forming heterotrophic dinoflagellate

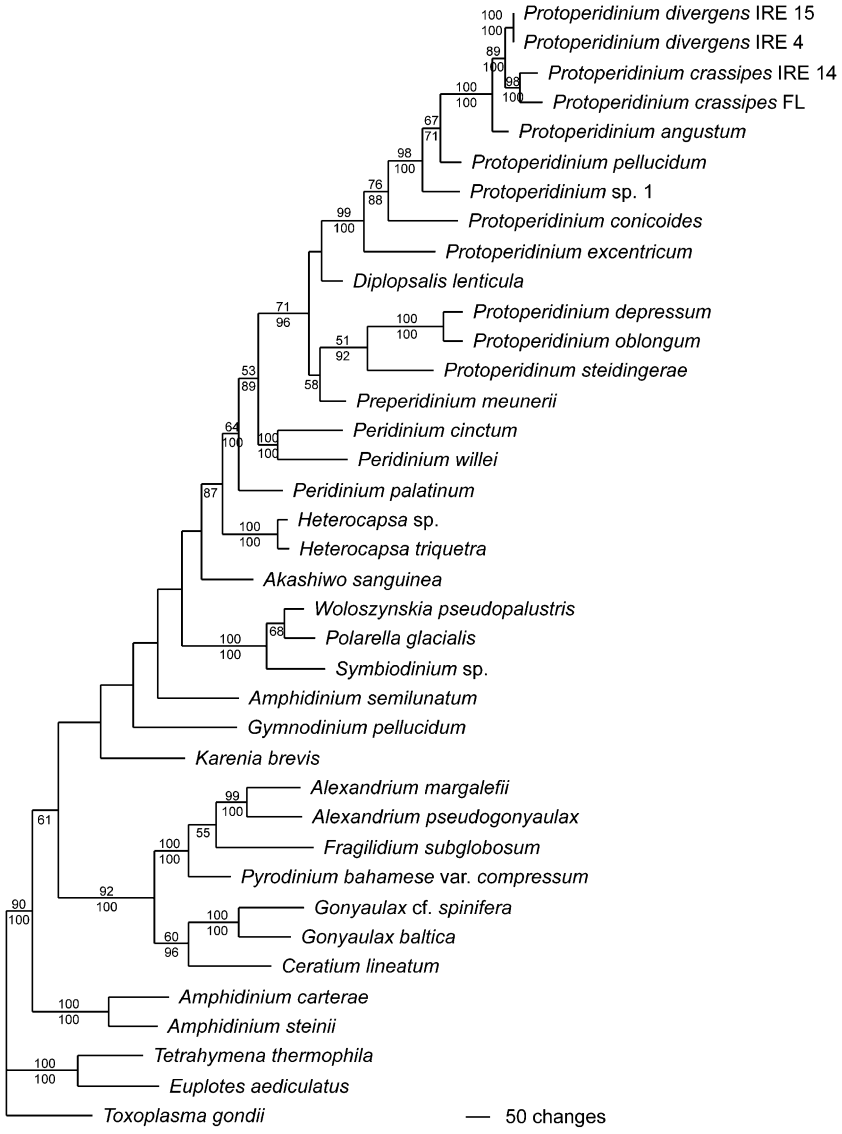


FIG. 8. Maximum likelihood (ML) tree based on domains D1–D3 of the large subunit rDNA gene. Bootstrap values from ML (100 replicates) greater than 50% are shown above the node. Posterior probabilities from Bayesian analysis (1,000,000 generations), given as a percent, are shown below the node. Two ciliates and one apicomplexan were used to root the tree.

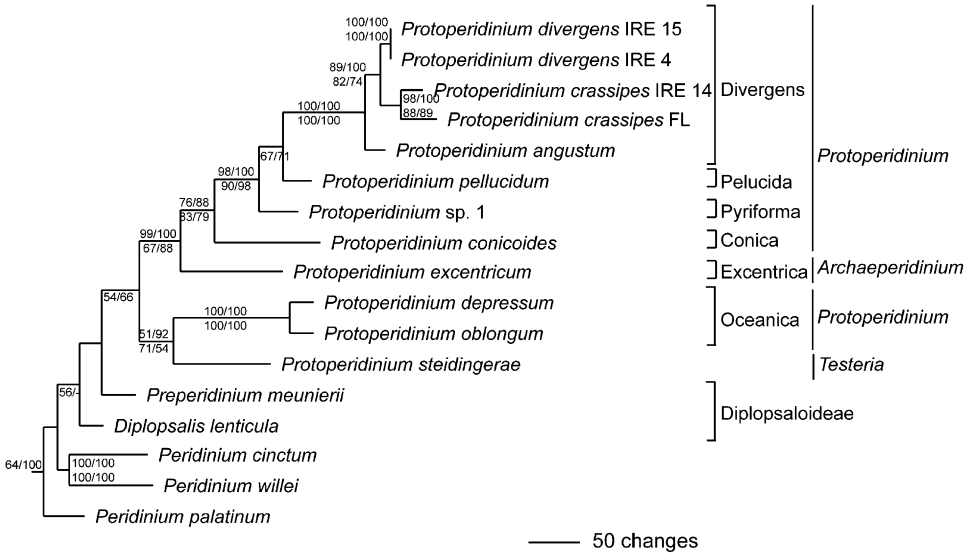


FIG. 9. Partial maximum parsimony (MP) tree based on domains D1–D3 of the large subunit rDNA gene. Bootstrap values greater than 50% are shown. Values above the node are bootstrap support from maximum likelihood/posterior probabilities from Bayesian analysis and values below the node are bootstrap support from MP/neighbor-joining.

species from a variety of morphological sections would need to be added to validate the relationship between molecular phylogeny and cyst morphology.

P. excentricum, a species in the subgenus *Archaeperidinium* and the section Excentrica, was most basal within the larger clade of *Protoperidinium* species, making the subgenus *Protoperidinium* polyphyletic. Based on morphology, the placement of *P. excentricum*, which had only two intercalary plates and a compressed shape unlike that of any other *Protoperidinium* species, was unexpected. In a SSU rDNA phylogeny of the *Protoperidinium*, the Avellena section within the *Archaeperidinium* appeared to have evolved from, or perhaps even fell in within the same clade as, the Conica (Yamaguchi and Horiguchi 2005). A single phylogeny including both the Avellana and the Excentricum is required to determine the unity of the *Archaeperidinium*. As additional species are sequenced, the distinction of the *Archaeperidinium* as a subgenus or the placement of the Oceanica within the subgenus *Protoperidinium* may have to be reconsidered.

The Divergens section formed a single clade, diverging from the Pellucida section as was also observed in a phylogeny based on SSU rDNA (Yamaguchi and Horiguchi 2005). Taxonomy and species classification within the Divergens section has always been problematic. *P. crassipes* and *P. curtipes*, for example, have been separated as two species and united as a single species (called variously *P. crassipes* or *P. curtipes*) repeatedly to the present day. We are unable to make judgments about the species designations of *P. crassipes* from *P. curtipes* based on the sequences and level of morphological examination of the two strains studied here. We did see a range in cell widths from 65 to 90 μm in cultured *P. crassipes* from the Gulf of Mexico, however, and a range of coloration (used by some to distinguish the species) from pale yellow to pale pink to dark pink. Based on the relatively modest concavity of the apical and antapical horns, and the slight displacement of the cingulum, similar to Kofoid's original description (Kofoid 1907), we have designated these two strains as *P. crassipes*. Additional strains of *P. crassipes* must be brought into culture to observe the variability in concavity of the epitheca and the degree of arch to the cingulum, and to obtain sequences to determine if *P. crassipes* and *P. curtipes* are separate species.

This work and other dinoflagellate molecular phylogenies have barely scratched the surface of the diversity of the *Protoperidinium* and Diplopsaloideae. In this survey, we examined only two species of diplopsalids and only 10 species of *Protoperidinium* in three subgenera and five morphological sections, out of the more than 200 described *Protoperidinium* species. Additional species in the Diplopsaloideae need to be included, to determine whether this group is evolutionarily coherent, or if these species do indeed fall within the Protoperidinaceae. Working from cultures, though time-consuming, provides the opportunity for more thorough investigation of the importance of gross morphology, including size, shape, and the absence or presence and nature of apical and antapical horns, to phylogeny. Many more species of *Protoperidinium* must be incorporated into future analyses, including those from other taxonomic sections and additional members of the morphological groups represented here. This will help to determine whether the morphologically defined classifications are truly evolutionarily relevant for this important group of organisms.

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