

THE PHYLOGENETIC RELATIONSHIP OF *PFIESTERIA PISCICIDA*, CRYPTOPERIDINIOPSOID SP. *AMYLOODINUM OCELLATUM* AND A *PFIESTERIA*-LIKE DINOFLAGELLATE TO OTHER DINOFLAGELLATES AND APICOMPLEXANS¹

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The taxonomic relationship between heterotrophic and parasitic dinoflagellates has not been studied extensively at the molecular level. In order to investigate these taxonomic relationships, we sequenced the small subunit (SSU) ribosomal RNA gene of *Pfiesteria piscicida* (Steidinger et Burkholder), a *Pfiesteria*-like dinoflagellate, *Cryptoperidiniopsis* sp., and *Amyloodinium ocellatum* (Brown) and submitted those sequences to GenBank. *Pfiesteria piscicida* and *Cryptoperidiniopsis* sp. are heterotrophic dinoflagellates, purportedly pathogenic to fish, and *A. ocellatum*, a major fish pathogen, has caused extensive economic losses in both the aquarium and aquaculture industries. The pathogenicity of the *Pfiesteria*-like dinoflagellate is unknown at this time, but its growth characteristics and *in vitro* food preferences are similar to those of *P. piscicida*. The SSU sequences of these species were aligned with the other full-length dinoflagellate sequences, as well as those of representative apicomplexans and *Perkinsus* species, the groups most closely related to dinoflagellates. Phylogenetic analyses indicate that *Cryptoperidiniopsis* sp., *P. piscicida*, and the *Pfiesteria*-like dinoflagellate are closely related and group into the class Blastodiniphyceae, as does *A. ocellatum*. None of the species examined were closely re-

lated to the apicomplexans or to *Perkinsus marinus*, the parasite that causes “Dermo disease” in oysters. The overall phylogenetic analyses largely supported the current class and subclass groupings within the dinoflagellates.

Key index words: *Amyloodinium ocellatum*; *Cryptoperidiniopsis* sp.; dinoflagellate; evolution; parasite; *Pfiesteria piscicida*, Small Subunit (SSU) rRNA; 18S

Dinoflagellates are a diverse group of organisms that can be free-living phototrophs, heterotrophs, or mixotrophs; invertebrate endosymbionts; or obligate ecto or endoparasites (Taylor 1987, Schnepf and Elbrachter 1992). Some species have complex life cycles that include both amoeboid and free-swimming stages as well as environmentally resistant cysts. The diversity of their feeding strategies contrasts with the exclusively parasitic existence of the dinoflagellates' nearest relatives, the apicomplexans and *Perkinsus* species. Apicomplexans are responsible for diseases such as malaria, toxoplasmosis, cryptosporidiosis, coccidiosis, and theileriosis, and *Perkinsus marinus* causes “Dermo disease” in oysters. The evolutionary relationships between the dinoflagellates and apicomplexans (Gajadhar et al. 1991), and among mainly photosynthetic (Saunders et al. 1997) and endosymbiotic dinoflagellates (McNally et al. 1994), have been examined at the molecular level. However, nothing is known about the molecular taxonomy of parasitic dinoflagellates such as

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³ This new species is being described by K. Steidinger, manuscript in preparation.

Amyloodinium ocellatum. Therefore, we sequenced the small subunit (SSU) ribosomal RNA gene from Cryptoperidiniopsoid sp., *Pfiesteria piscicida*, a *Pfiesteria*-like dinoflagellate (PLD), and *A. ocellatum* and analyzed their phylogenetic relationship to the other known dinoflagellates and apicomplexans. These four species were also investigated because of their potential to cause economic or environmental damage.

Pfiesteria piscicida was discovered in the late 1980s by Smith et al. (1988) and was described as a new species in 1996 by Steidinger et al. (1996a). It has been associated with fish kills as well as certain epidemic skin ulcers in estuarine fish populations in North Carolina and elsewhere (Burkholder et al. 1992, Noga et al. 1996). *Pfiesteria piscicida* feeds heterotrophically on microalgae and is reportedly non-toxic unless it is exposed to unknown chemical cues associated with live fish (Burkholder and Glasgow 1997, Noga 1998). Once stimulated, *Pfiesteria* has been shown to kill fish and invertebrates in the laboratory (Smith et al. 1988, Burkholder et al. 1992). Currently, there is no evidence that *P. piscicida* functions as a parasite with prolonged attachment to healthy fish (Noga et al. 1993), as is the case with *A. ocellatum* (Lom and Lawler 1973). Instead, fish mortality is attributed to the release of potent ichthyotoxins (Smith et al. 1988, Burkholder et al. 1995a, b).

Cryptoperidiniopsoid sp. is a heterotrophic dinoflagellate that occupies the same habitat as *P. piscicida*, exhibits behavioral similarities, and is morphologically indistinguishable from *Pfiesteria* by light microscopy (Steidinger et al. 1996a, Environews Forum 1998; this new taxon is being described by Steidinger). Without careful examination via scanning electron microscopy, these dinoflagellate species can easily be confused. Cryptoperidiniopsoid sp. has been identified at a number of sites between Maryland and Florida where fish are noted to have skin lesions (Ahrenholz et al. 1987, Noga et al. 1991b). It co-occurs with *P. piscicida* at many fish-kill sites in Chesapeake Bay (Chicamacomico River and Pocomoke River, Maryland) and in Pamlico Sound (Neuse River and Pamlico Rivers, North Carolina) (Environmental Protection Agency 1998).

The PLD was isolated from a culture taken from the Pamlico River in 1991 at the site of a fish kill and maintained at the NC State University College of Veterinary Medicine. Cryptoperidiniopsoid sp. and the PLD are both morphologically similar to *P. piscicida*, have similar algal food preferences *in vitro*, and may prove to be ichthyotoxic.

Amyloodinium ocellatum is an obligate parasite responsible for amyloodiniosis, one of the most significant diseases of temperate and warm water marine fish (Noga and Levy 1995). It infects almost every species of teleost fish (Noga and Levy 1995) and causes epidemics over a wide range of salinities, from brackish water to full-strength seawater, and at

temperatures from 17° to 30° C (Paperna 1984, Sindermann 1990, Noga et al. 1991a, Landsberg et al. 1994). *Amyloodinium ocellatum* is responsible for major economic losses in both the aquarium and aquaculture industries.

The results of this study showed that *P. piscicida*, the PLD, and Cryptoperidiniopsoid sp. are closely related to each other and belong to the class Blastodiniphyceae along with *A. ocellatum*. The association with the more ancestral Blastodiniales indicates that *P. piscicida*, Cryptoperidiniopsoid sp., *A. ocellatum*, and the PLD represent a relatively ancestral group of heterotrophic dinoflagellates. The phylogenetic results support the major subclass groupings within the class Dinophyceae.

MATERIALS AND METHODS

Culture. *Amyloodinium ocellatum* was isolated in 1986 and has been grown continuously in cell culture on a fish-gill cell line in a modified artificial seawater solution according to Noga (1987). For propagation, the infective stage (dinospores) was added to cell cultures. The dinospores then attached to the gill cells and differentiated into the trophonts. The trophonts fed for approximately 3 days, at which time they detached and differentiated into tomonts (the reproductive stage). The medium containing the detached tomonts was gently pipetted to 24-well tissue culture plate wells containing IO2/HBSS. The tomonts were dense and slightly adherent to the wells. The cell debris could, therefore, be removed by gently swirling the 24-well plate and pipetting off the supernatant. New medium was added and the procedure was repeated several times until the tomonts were clean. The isolated tomonts sporulated and began releasing dinospores 3 days later. The dinospores were collected by gently removing the supernatant and concentrating the cells by centrifugation at $500 \times g$ for 30 min. Fresh IO2/HBSS was added to the plate, and newly hatched dinospores were collected again on days 4 and 5. The concentrated dinospores were frozen at -80°C .

Cryptoperidiniopsoid sp. was isolated in July 1992 by Tester and Guo (Guo et al. 1994) at the site of fish kills on the Pamlico River, North Carolina. The PLD was isolated from a culture derived from a water sample taken in the Pamlico River, North Carolina, in 1991 and maintained at the NC State University College of Veterinary Medicine. *Pfiesteria piscicida* was isolated in September 1997 from the site of a fish kill in the Chicamacomico River, Maryland and was provided by Dr. Karen Steidinger (Florida Fish and Wildlife Conservation Commission, Marine Research Institute).

Before beginning the molecular studies, all cultures except *A. ocellatum* were isolated by dilution series and grown on the cryptophyte *Rhodomonas* sp. WT651 (CCMP 767) at 10^5 cells·mL⁻¹. Cells were concentrated by gentle filtration through either a 3 or 5 μm Nucleopore (Costar[®]) filter. Filtered cells were rinsed with isotonic, sterilized seawater and frozen at -80°C . The taxonomic identities of *Cryptoperidiniopsoid* sp., *P. piscicida*, and PLD cultures was confirmed by Steidinger using the SEM methods of Steidinger et al. (1996b) and Truby (1997).

Strategy for sequencing SSU genes. Dinoflagellate DNA is unlike that of other known taxa, in that 12%–75% of the thymine is replaced by 5-hydroxymethyluracil (Rae 1976, Rae and Steel 1978). These modified bases are not randomly distributed throughout the genome. There is also evidence from *Cryptocodinium cohnii* that cytosines in the SSU genes of dinoflagellates are highly methylated, which is unusual for most eukaryotes (Steel and Rae 1980). These modified bases may be the reason why the amplification of dinoflagellate SSU rDNA with Taq polymerase is sometimes difficult. To overcome this problem, Saunders et al. (1997) found it necessary to amplify the SSU genes of photosynthetic dinoflagellates in 2–4 overlapping segments and then clone and sequence these segments independently. In this

study, the multiple segment approach was problematic because the *Rhodomonas* food source also amplified with the same primers used for the dinoflagellate SSU gene. Therefore, we chose an alternative approach, utilizing SSU RNA transcripts. Briefly, the species were grown on *Rhodomonas* and allowed to deplete the food cells. The cultures were then filtered, and total RNA was isolated and reverse transcribed into cDNA suitable for PCR amplification. The PCR products were then cloned and sequenced.

RNA isolation. Approximately 5×10^5 *A. ocellatum* dinospores were ground with a pestle to lyse the cells. Total RNA was immediately isolated, following the Trizol Procedure (Gibco BRLTM). Frozen filters containing either $5\text{--}10 \times 10^4$ Cryptoperidiniopsis sp., *P. piscicida*, or PLD cells were lysed in RLT buffer (QiagenTM) in a Tenbroeck tissue grinder. After grinding, the Nucleopore filters were largely intact, so the supernatant was removed and used to isolate total RNA following the RNeasy protocol (QiagenTM).

Reverse transcription. Approximately 60 ng of total RNA were diluted to 14 μL with sterile deionized water and combined with 1 μL (10 ng) primer B (5' GCCGGGATCCAAGCTTGA-TCCCTTTCGACGGTTCACCTAC 3') located at the 3' end of the rRNA gene (Medlin et al. 1988). The diluted RNA samples were heated to 90° C in a 400 mL water bath to disrupt the RNA secondary structure and allowed to cool gradually to 70° C before snap cooling in an ice-water bath. A reverse transcription (RT) master mix was prepared by combining 2 μL of 0.1 M DTT, 4 μL of 5 \times single strand buffer (Gibco BRLTM), and 1 μL of 10 mM dNTPs per sample. Thirteen-microliter aliquots of RNA and seven-microliter aliquots of RT master mix were transferred into 0.2- μL amplification tubes and heated to 42° C in a Perkin-Elmer 9600 Thermocycler. One microliter of reverse transcriptase (Gibco BRLTM) was added to each tube and the RT reaction was incubated at 42° C for 1 h and subsequently stored at -80° C until used for PCR.

Because reverse transcriptase has a slightly higher error rate than Taq polymerase, three separate RT reactions were done from each of the four dinoflagellate total-RNA samples. Each of these transcripts was, in turn, amplified, cloned, and sequenced separately to resolve any sequence ambiguities.

PCR amplification, cloning, and sequencing. The PCR reactions were optimized using the Opti-Prime procedure (StratageneTM). The following 50 μL reactions were found to produce a specific amplification product of approximately 1800 bp: 10 mM Tris-HCl (pH 8.8), 0.5 mM MgCl₂, 25 mM KCl (10 \times dilution Stratagene Buffer 5), 6% DMSO, 10 mM dNTPs, 500 ng each of GCG18SF and GCG18SR primer, and 0.5 μL Taq (PromegaTM). The GCG18SF (5' CTGGTTGATCCTGCCAGTAGTC 3', positions 4-25) and GCG18SR (5' CAAGGTTTCCGTAGGTGAACCT 3', positions 1773-1795) primers were selected by the GCG Prime Program as the primers at the 5' and 3' end of the SSU rRNA gene with the lowest annealing score (Wisconsin Package Version 9.1 1999). The PCR conditions were one cycle at 94° C for 3 min, 35 cycles at 94° C for 45 s, 60° C for 45 s, and 72° C for 1.54 min, followed by 72° C for 7 min.

The PCR reactions were diluted with 35 μL TE and size fractionated on a Chromaspin-1000 column (ClontechTM) to remove primer dimers and reduce the abundance of smaller nonspecific bands that were sometimes present in the reaction. Two microliters from each size-fractionated PCR reaction were cloned using the TOPO TA kit (InvitrogenTM). Plasmid minipreps were done on overnight cultures using the QIAprep Spin Plasmid Kit (QiagenTM). Plasmids were digested with *Eco*RI and run on 1.2% TAE agarose gel. Because of a conserved *Eco*RI site at the 3' end of the gene, plasmids containing an SSU gene could be identified by characteristic ~1600 and 200 bp bands.

Purified DNA from each independent RT reaction was sequenced on an ABI 373A DNA sequencer using the Taq Dye-DeoxyTM Terminator Cycle Sequencing Kit (Applied Biosystems-ABITM). The various internal and external primer sequences are listed below. All primers are listed 5' to 3' and unless specified, 10 pM of primer were added to each sequencing reaction. Forward primers were: M13F (GTTTTCCCGTCACGAC), G03 (GTCTGTGCCAGCAGCCGCGG, 100 pM), G17 (ATACCGTCTAGTCT-TAACCC), G19 (CATCTAAGGAAGGCAGCAGG), G20 (AGTTTCT-

GACCTATCAGCTT), and G22 (TGGTGGAGTGATTTGTCTGG). The reverse primers were M13R (CAGGAAACAGCTATGAC), G10 (CCGCGGCAGCTGGCACCAGAC), G18 (GCATCACAGACCT-GTTATTG), and G23 (TTCAGCCTTGCAGCATAAC). Primers G03 and G10 were designed by Saunders. All clones were sequenced in both directions. The sequences from independent clones agreed well with each other.

Sequence alignment and phylogenetic analysis. An ideal data set for phylogenetic reconstruction of any taxonomic group would contain an even distribution of species within the taxonomic group and equal divergence (branch lengths) between all adjacent taxa in the phylogenetic tree. Equal branch lengths reduce the probability of "long-branch attraction," an artifact wherein divergent taxa group together based on long-branch lengths rather than taxonomic affinity. Unfortunately, the existing dinoflagellate SSU data set is still relatively small with a few orders being over-represented and other crucial, ancestral orders being either under-represented or missing. Further, there are a number of taxa in the available data set that are separated by long branches. This, in part, results from the rapid divergence of modern dinoflagellate groups about 230-260 million years ago (Fensome et al. 1993), which has led to a paucity of intermediate species that could help resolve the phylogenetic tree by equalizing branch lengths between taxa. Given these constraints, a maximum-likelihood analysis was chosen over maximum parsimony or distance approaches. The reason is that analyses of data sets, as well as simulation studies, have shown that maximum-likelihood approaches are generally more likely to recover correct phylogenies when branch lengths are unequal (Cunningham et al. 1998). In this analysis, the PAUP* program was used to estimate which maximum-likelihood model best fits the data (Swafford 1999). PAUP* is computationally more efficient than previous maximum-likelihood programs and allows for a rigorous statistical analysis of successively more complex maximum-likelihood models.

The specific phylogenetic analyses were performed according to the approach outlined in Cunningham et al. (1998) and Moncalvo et al. (1999). First, the dinoflagellate and apicomplexan sequences were aligned using pileup (Wisconsin Package 1999). Variable regions were further aligned manually using the SEQPUP editor (Gilbert 1996) and regions that could not be aligned unambiguously were excluded from the analysis. An initial equally weighted parsimony analysis was undertaken using PAUP* with the following options in effect: steepest descent off, branches allowed to collapse creating polytomies if maximum branch length = 0, tree bisection rearrangements in effect, all character states unordered, and 1000 random additions. Indels or groups of indels that aligned unambiguously were scored as a "fifth state" and ambiguously aligned gaps in nonexcluded regions were treated as missing.

Unweighted parsimony analysis found four most parsimonious trees of length 2882. All trees were very similar except for one or two minor variations in terminal branch order. Tree 2 was used to estimate the best maximum-likelihood model (Table 1). The procedure consisted of evaluating increasingly complex models including JC (Jukes and Cantor 1969), F81 (Felsenstein 1981), KP2 Kimura-2-parameter (Kimura 1980), HKY (Hasegawa et al. 1985), GTR general time reversible model (Lanave et al. 1984), INV invariable sites method (Hasegawa et al. 1985), and GAM discrete gamma distribution, four categories (Yang 1994, 1996). The parameters that were sequentially added included base composition and numbers of substitution classes, as well as incorporation of among-site variations, including the INV method (Hasegawa et al. 1985) and the four-category discrete gamma distribution (Yang 1994, 1996). As additional parameters were added to the analysis, a likelihood-ratio test (LRT), which follows a chi-squared distribution, was performed to determine when increasingly-complex models could be rejected (Goldman 1993).

The same data set was also used to estimate the best tree using the neighbor-joining method (Saitou and Nei 1987). The heuristic search settings were optimality criterion = distance, negative branch lengths allowed, but set to zero for tree-score calculation, distance measure = uncorrected, branch-swapping algorithm = tree bisection reconnection (TBR), steepest descent option not

TABLE 1. Alternative evolutionary models used to estimate likelihoods scores ($-\ln L$) and to calculate the likelihood ratios tests for the most-parsimonious unweighted parsimony tree (UP) and the neighbor-joining tree (NJ) described in the text. The maximum-likelihood model based on evaluating the maximum parsimony score always gave consistently lower likelihood scores than the best distance tree.

Evolution model ^a	Number of substitution variable types	Assumed base rate frequencies	Among-sites rate variation			UP		NJ	
			Invariables sites	Rates of sites ^b	Number of categories	$-\ln L$	Probability ^c	$-\ln L$	Probability ^c
JC	1	Equal				22,665.382	—	22,782.912	—
F81	1	Estimated ^d				22,608.416	<0.001	22,739.609	<0.001
K2P	2	Equal				22,100.114	<0.001	22,193.347	<0.001
HKY85	2	Estimated ^d				22,007.424	<0.001	22,113.324	<0.001
GTR-3	3	Equal				22,046.768	<0.001	22,121.267	NS
GTR-4	4	Equal				22,035.130	<0.001	22,081.361	<0.001
GTR-5	5	Equal				21,982.176	<0.001	22,076.829	<0.001
GTR-6	6	Equal				21,977.360	<0.01	22,071.238	<0.001
GTR-6	6	Equal	Estimated ^e	Equal		20,414.935	<0.001	20,477.483	<0.001
GTR-5	5	Equal	0	Estimated ^e	4	19,723.757	<0.001	19,787.447	<0.001
GTR-6	6	Equal	Estimated ^e	Estimated ^e	4	19,678.471	<0.001	19,731.949	<0.001
GTR-6	6	Estimated ^d	Set	Set ^f	4	19,678.471	NS	19,688.660	<0.001
GTR-6	6	Estimated ^d	Set ^f	Set ^f	5	19,666.286	<0.001	19,676.792	<0.001
GTR-6	6	Estimated ^d	Set ^f	Set ^f	6	19,659.256	<0.001	19,668.288	<0.001
GTR-6	6	Estimated ^d	Set ^f	Set ^f	7	19,654.742	<0.001	19,663.309	<0.01
GTR-6	6	Estimated ^d	Set ^f	Set ^f	8	19,651.601	<0.05	19,659.758	<0.05
GTR-6	6	Estimated ^d	Set ^f	Set ^f	9	19,649.282	<0.05	19,657.117	<0.05
GTR-6	6	Estimated ^d	Set ^f	Set ^f	10	19,647.300	<0.05	19,656.094	NS
GTR-6	6	Estimated ^d	Set ^f	Set ^f	11	19,646.088	NS	19,655.061	NS

^a The various models listed in order of complexity as described in the text.

^b Variable sites assumed to follow a gamma distribution with a gamma shape parameter.

^c Compared with best value previously calculated, likelihood was significantly improved or not significantly improved (NS).

^d Base frequencies estimated while other parameters held constant.

^e Likelihood estimated from data.

^f Set as estimated above.

in effect, max tree allowed to increase as necessary, zero-length branches not collapsed, topological constraints not enforced, and trees unrooted. The resulting neighbor-joining tree was also evaluated using the procedure outlined in Table 1.

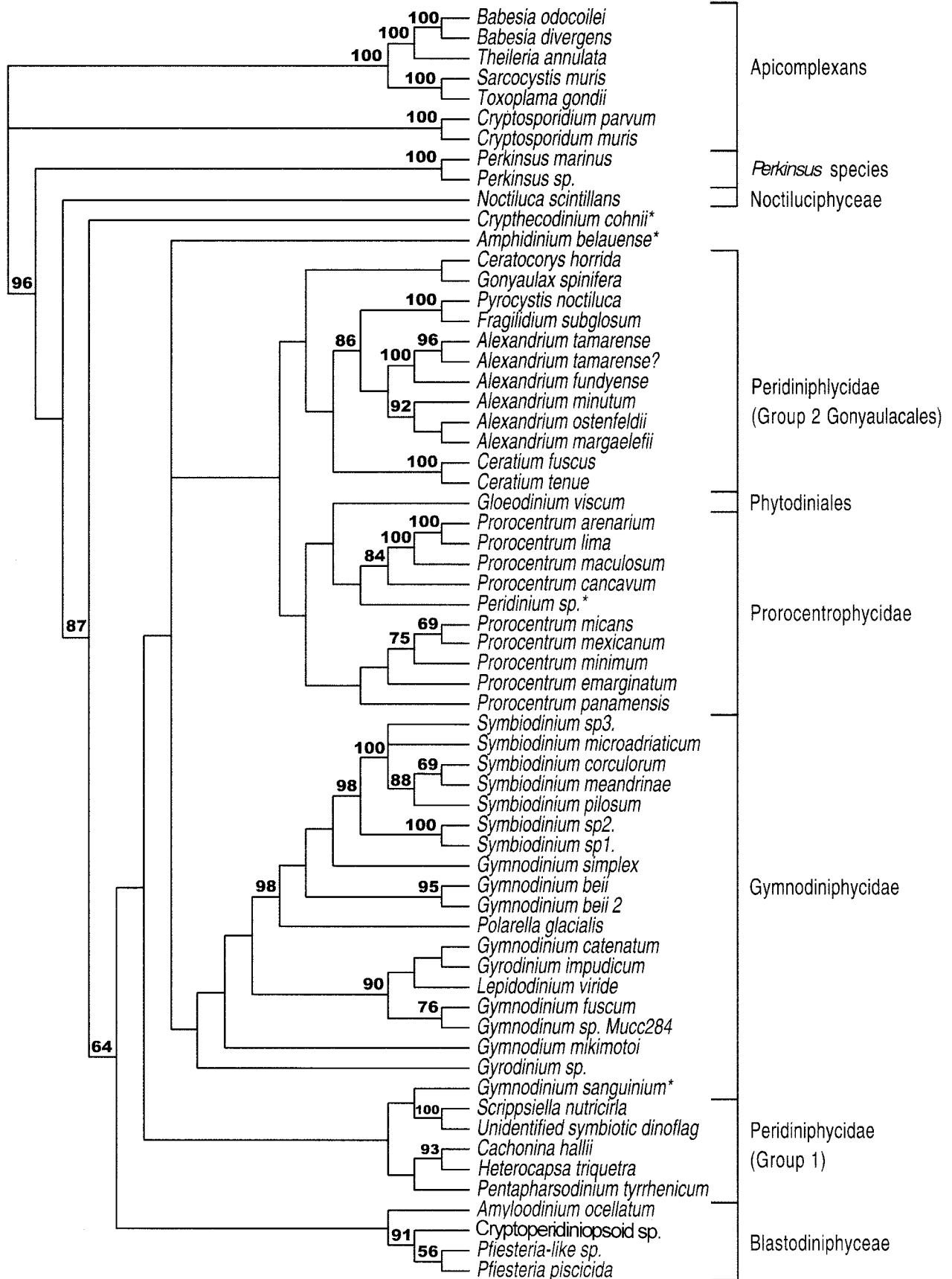
Once the maximum-likelihood model was established as described above, the same most parsimonious tree was used for a maximum-likelihood analysis employing the nearest neighbor interchange (NNI) branch swapping algorithm to determine if a better arrangement of the terminal branches could be found. The best tree from the NNI search was then used to perform an equivalent maximum-likelihood analysis using the TBR option to examine rearrangements at the deeper branches. Support for each of the phylogenetic groups was estimated by doing 100 bootstrap (Felsenstein 1985) replicates. The maximum-likelihood settings

were: “fast stepwise addition” with no branch swapping, MUL-PARS off, and steepest descent = no. Groups retained in 50% or more of the trees are indicated on the final maximum-likelihood tree. The fast procedure slightly underestimates branch support relative to the more computationally intensive TBR methods (Moncalvo et al. 1999).

RESULTS

The apicomplexans and the dinoflagellates form distinct groups, with the *Perkinsus* species being intermediate in the final phylogenetic analysis (Figs. 1, 2). Within the dinoflagellates, the most ancestral

FIG. 1. Cladogram of best maximum-likelihood tree showing the major taxonomic groupings and % boot strap support for various branches. Bootstrap values less than 50% were not included. The species included in the analysis are given below. The GenBank accession number for the SSU gene of each species is noted in parentheses. Apicomplexans—*Babesia divergens* (U16370), *Babesia odocoilei* (U16369), *Cryptosporidium muris* (X64342), *Cryptosporidium parvum* (L16997), *Plasmodium cynomolgi* (L08242), *Plasmodium malariae* (M54897), *Sarcocystis muris* (M64244), *Theileria annulata* (M64243), and *Toxoplasma gondii* (U03070), Perkinsus species—*Perkinsus marinus* (X75762), *Perkinsus* sp. (L07375), and Dinoflagellates—*Alexandrium fundyense* (U09048), *Alexandrium margalefii* (U27498), *Alexandrium minutum* (U27499), *Alexandrium ostenfeldii* (U27500), *Alexandrium tamarense* (X54946), *Alexandrium tamarense?* (AF022191), *Amphidinium belauense* (L13719), *Amyloodinium ocellatum* (AF080096), *Cachonina hallii* (AF033865), *Ceratium fuscum* (AF022153), *Ceratium tenue* (AF022192), *Ceratocorys horrida* (AF022154), *Crypthecodinium cohnii* (M64245 M34847), *Cryptoperidiniopsis tyrrheni* (AF080097), *Fragilidium subglobosum* (AF033869), *Gleodinium viscum* (L13716), *Gonyaulax spinifera* (AF022155), *Gymnodinium beii* (U37406), *Gymnodinium beii* (U41087), *Gymnodinium catenatum* (AF022193), *Gymnodinium fuscum* (AF022194), *Gymnodinium mikimotoi* (AF022195), *Gymnodinium sanguinum* (U41085), *Gymnodinium simplex* (U41086), *Gymnodinium* sp. MUCC284 (AF022196), *Gyrodinium impudicum* (AF022197), *Heterocapsa triquetra* (AF022198), *Lepidodinium viride* (AF022199), *Noctiluca scintillans* (AF022200), *Pentaptharsodinium tyrrheni* (AF022201), *Peridinium foliaceum* (M88517), *Peridinium* sp. (AF022202), *Pfiesteria piscicida* (AF149793 AF077055), *Pfiesteria*-like dinoflagellate (AF080098), *Polarella glacialis* (AF099183), *Prorocentrum arenarium* (Y16234), *Prorocentrum concavum* (Y16237), *Prorocentrum emarginatum* (Y16239), *Prorocentrum lima* (Y16235), *Prorocentrum maculosum* (Y16236), *Prorocentrum mexicanum* (Y16232), *Prorocentrum micans* (M14649), *Prorocentrum minimum* (Y16238), *Prorocentrum panamensis* (Y16233), *Pyrocystis noctiluca* (AF022156), *Scrippsiella nutricula* (U52357), *Symbiodinium corculorum* (L13717), *Symbiodinium meandrinae* (L13718), *Symbiodinium microadriaticum* (M88521), *Symbiodinium pilosum* (X62650), *Symbiodinium* sp. (M88509), *Symbiodinium* sp. (U10893), and Unidentified symbiotic dinoflagellate from *Collozoum caudatum* (U52354). * indicates species that fall outside the current subclass to which they are assigned (Fensome et al. 1993).



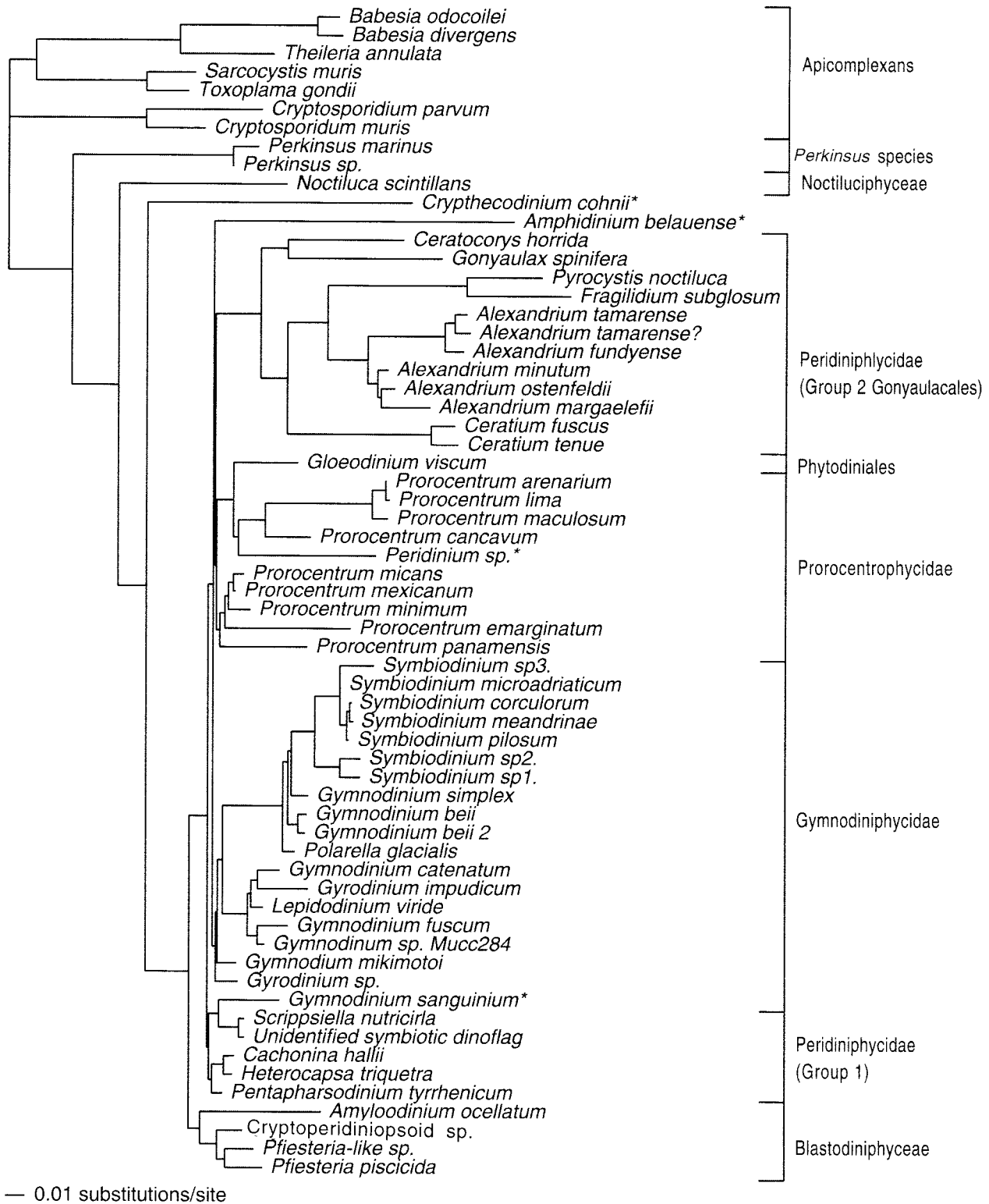


FIG. 2. Phylogram of the best maximum-likelihood tree showing relative branch lengths.

species was *Noctiluca scintillans* (Noctiluciphyceae) followed by *C. cohnii*, currently classified within the Dinophyceae (Peridiniphyceae). The next most ancestral group was composed of *P. piscicida*, the PLD,

Cryptoperidiniopsoid sp., and *A. ocellatum* (Blastodiniphyceae).

The remaining species in the study are all classified as members of the Dinophyceae. Members of

TABLE 2. Taxonomic scheme presented in Fensome et al. (1993) for the species included in this study. An * indicates a species that is in a significantly different place in the molecular scheme than would be predicted by the current taxonomic scheme.

Subdivision Dinokaryota				
Class	Subclass	Order	Family	Genus
Noctiluciphyceae		Noctilucales	Noctilucaceae	<i>Noctiluca</i>
Blastodiniphyceae		Blastodinales	Oodiniaceae	<i>Amyloodinium</i>
		Unknown		<i>Cryptoperidiniopsis</i>
				<i>Pfiesteria</i>
				<i>Pfiesteria</i> -like species
Dinophyceae	Gymnodiniphycidae	Gymnodinales	Gymnodiniaceae	<i>Amphidinium</i> *
				<i>Gymnodinium</i>
				<i>Gyrodinium</i>
				<i>Lepidodinium</i>
				<i>Polarella</i>
		Suessiales	Suessiaceae	<i>Symbiodinium</i>
			Symbiodiniaceae	<i>Ceratium</i>
	Peridiniphycidae	Gonyaulacales	Ceratiaceae	<i>Ceratocorys</i>
			Ceratocoryaceae	<i>Crypthecodinium</i> *
			Crypthecodiniaceae	<i>Gonyaulax</i>
			Gonyaulaceae	<i>Alexandrium</i>
			Goniodomaceae	<i>Fragilidium</i>
				<i>Pyrocystis</i>
		Peridinales	Heterocapsaceae	<i>Cachonina</i>
				<i>Heterocapsa</i>
			Peridiniaceae	<i>Pentaparsodinium</i>
				<i>Peridinium</i> *
				<i>Scrippsiella</i>
	Uncertain	Phytodinales	Gloeodiniaceae	<i>Gloeodinium</i>
	Prorocentrophycidae	Prorocentrales	Prorocentraceae	<i>Prorocentrum</i>

this group generally fell into well-defined subclasses, including the Gymnodiniphycidae, Peridiniphycidae, and the Prorocentrophycidae (Figs. 1, 2; Table 2). Members of the Dinophysiphycidae and Syndiniophyceae subclasses were not included in the study because SSU sequences are not presently available. The SSU phylogeny agrees well with most aspects of the classical scheme proposed by Fensome et al. (1993) although the deep branches resolving the relative relationships between the divergence of the Gymnodiniphycidae, Peridiniphycidae, and the Prorocentrophycidae were not significantly resolved (Table 2). Both the Peridiniphycidae and Prorocentrophycidae fell into two distinct lineages (Fig. 1). Only *Amphidinium belauense*, *Peridinium* sp., and *Gymnodinium sanguinium* did not fall into groups where they currently are classified. The initial unweighted maximum parsimony tree used to evaluate the maximum-likelihood model had a tree length = 2882, consistency index = 0.448, and retention index = 0.607. The final maximum-likelihood model that best fit the data was a general-time-reversible model (Yang 1994) with rate heterogeneity. The specific conditions were as follows: nucleotide frequencies were estimated via maximum likelihood, six substitution types, proportion of sites assumed to be invariable = 0.33759, rates for variable sites assumed to follow a discrete gamma distribution with shape parameter = 0.64953, number of rate categories = 10, average rate for each category represented by mean and the molecular clock not enforced (Table 1). The final tree was obtained upon stopping the

TBR analysis after 1510 rearrangements. The best tree score at this point was 19,545.367.

DISCUSSION

Phylogenetic analyses confirmed that the heterotrophic dinoflagellates *P. piscicida*, the PLD, and *Cryptoperidiniopsis* sp. are all closely related and diverged relatively early in dinoflagellate evolution. The current SSU phylogenetic analysis assigns these species to the Blastodinales, the only recognized order in the Blastodiniphyceae (Fensome et al. 1993; Table 2). Species currently assigned to the Blastodinales are highly variable, mostly marine, extracellular parasites that generally have a complicated life cycle that includes a sporogenetic stage which culminates in a motile, infective dinospore stage. The life cycle may also include one or more cyst stages (Cachon and Cachon 1987). The large diversity of species in Blastodiniphyceae suggests that the group may be polyphyletic (Fensome et al. 1993). Besides parasitism and the presence of specific life-cycle stages, the primary characteristic that allows assignment of a species to the Blastodiniphycidae is the absence of the typical dinokaryon in one or more life-cycle stages. The dinokaryon is a nuclear organization unique to the Dinoflagellates characterized by permanently condensed, banded chromosomes, where only certain loops of DNA extending out from the condensed chromosomes are transcriptionally active (Sigeo 1984). The dinokaryon is considered a derived characteristic relative to the

dispersed chromosomes of the more typical eukaryotic nucleus.

Pfiesteria piscicida is reported to have a dinokaryotic nucleus in the dinospore stage and a typical eukaryotic nucleus in the amoebae stage (Steidinger et al. 1996a) supporting inclusion in the Blastodinales. Further, the Kofoidian plate tabulation series from the dinospore stages of both *P. piscicida* (Steidinger et al. 1996a) and of *A. ocellatum* (Landsberg et al. 1994) are typical of the Peridiniales, whose representative members belong to the Peridiniphycean Group 1 that emerged in the SSU tree just after the Blastodiniphyceae.

Pfiesteria piscicida, the PLD, and Cryptoperidiniopsis sp., however, are unlike most of the described Blastodinales in that they are not modified for a parasitic existence as is *A. ocellatum*. The *Pfiesteria* group may therefore form a distinct lineage within the Blastodiniphyceae. Further support for the divergence comes from the ribosomal ITS regions of *A. ocellatum*, *P. piscicida*, and Cryptoperidiniopsis sp.. *Amyloodinium ocellatum* has a substantially larger and more divergent ITS region than *P. piscicida* or Cryptoperidiniopsis sp., which have similar ITS sequences. The *Pfiesteria* group may therefore represent either a new, as yet undescribed order within the Blastodiniphyceae, or constitute a novel group if the Blastodiniphyceae are indeed polyphyletic.

It should be noted that our understanding of the Blastodiniphyceae is based on a very few parasitic species (Cahon and Cahon 1987). More species need to be examined to determine if the eukaryotic nucleus in one or more of the life-cycle stages is a truly uniform characteristic of the Blastodiniphyceae. Confirming this distinction is particularly important in understanding when species belong to the Blastodinales or the Phytodinales (subclass currently unknown). Many Phytodinialian species are morphologically indistinguishable from members of the Blastodinales and have similarly complex life cycles. The only definitive criterion currently used to distinguish these two groups is that Phytodinales retain a dinokaryon in all life-cycle stages. This presents a problem in many cases because not all life-cycle stages are known. Hence, when a few known stages only have a dinokaryon, and other stages are missing, taxonomic assignment becomes problematic (Timpano and Pfister 1985, Buckland-Nicks et al. 1990). The SSU sequence data may prove helpful in resolving these taxonomic affiliations because the Phytodinales, as represented by *Gloeodinium viscum*, form a separate and more derived group than the Blastodinales (Figs. 1, 2). The SSU sequence divergence between the Phytodinales and Blastodinales also implies that the morphological similarities between members of these two groups are due to convergent evolution. In addition to taxonomic assignment, sequences from the SSU, and other variable regions within the ribosomal genes, are likely to

prove useful in confirming when different life stages belong to the same species.

Having the SSU sequences for these species makes it possible to determine if other *Pfiesteria*-like heterotrophs are truly *P. piscicida*, the PLD, Cryptoperidiniopsis sp., or other members of the *Pfiesteria* complex. Unambiguously identified clonal cultures will allow future work on the ecology, physiology, and taxonomy of these species to proceed more rapidly. Identification of SSU sequence differences between these species is essential for the development of diagnostic PCR assays for analyzing field samples.

Comparison of molecular and classical taxonomy. The best phylogenetic tree was consistent with other studies showing the apicomplexans form a distinct, but closely related group to the dinoflagellates (Gajadhar et al. 1991, Cavalier-Smith 1993). This tree also placed the *Perkinsus* species in an intermediate group between the dinoflagellates and apicomplexans, yet could not distinguish whether they represent an intermediate group between the apicomplexans and the dinoflagellates (Fong et al. 1993, Goggin and Barker 1993, Flores et al. 1996, Reece et al. 1997), or are primitive dinoflagellates (Siddall et al. 1997).

The SSU phylogeny of the Dinokaryota is largely consistent with the current dinoflagellate classification by Fensome et al. (1993) based primarily on plate counts, and other cellular structures, including the presence or absence of the dinokaryon. Both schemes concur that the two most ancestral classes in the Dinokaryota are the Noctiluciphyceae, that retain a dinokaryon only in the gametes (Soyer 1972, Fensome et al. 1993), followed by the Blastodiniphyceae, that retain a dinokaryotic nucleus for a larger portion of the life cycle.

Interestingly, *C. cohnii* comes out as intermediate between the Noctiluciphycidae and Blastodiniphycidae, which is inconsistent with the nuclear structure and may be because of long branch attraction problems in this analysis (Fig. 1). Studies by Saunders et al. (1997) using 31 dinoflagellate species and by Grzebyk et al. (1998) similarly found that *C. cohnii* diverged early in dinoflagellate evolution. These studies, however, placed *C. cohnii* as diverging with or near *A. belauense*. Fensome et al. (1993), in contrast, assigned *Crypthecodinium* to its own family within the Gonyaulacales, a position not supported by this study. Other species are needed to determine the precise phylogenetic placement of *C. cohnii*.

The remaining species in the study belong to the class Dinophyceae and should retain a dinokaryotic nucleus throughout the entire life cycle. The molecular data supports the Gymnodiniphycidae, Peridiniphycidae, and Prorocentrophycidae (GPP) subclass divisions presented in Fensome et al. (1993) with the exception of a relatively small number of species shown in Figure 2. The order and family distinctions are also reasonably consistent except where noted in Table 2. Certain genera appear

monophyletic (e.g. *Alexandrium*), whereas others are not (e.g. *Gymnodinium*; Figs. 1, 2), indicating some genera are more complex than previously assumed.

Saunders et al. (1997) found similar support for the GPP subclasses except that they proposed the Gonyaulacales constitute a separate order not within the Peridiniphyceae. This conclusion was based on the fact that all the Peridiniphyceid species fell into a group that was distinctly different from *Peridinium* sp., the type genus represented in the study. (Fig. 1, Saunders et al. 1997). In this analysis, the Gonyaulacales, which form the Peridiniphyceid Group 2, form a distinct group that appear to have diverged later than the other Peridiniphyceid Group 1 (Figs. 1, 2). The molecular phylogeny therefore supports the contention that the Gonyaulacales are at least a more recently derived group within the Peridiniphyceae, or form a group that diverged from a common ancestor leading to the Gonyaulacales and Prorocentrophycidae (Figs. 1, 2, Table 2).

It should be noted that the support for the deep branches within the GPP complex was low, and hence inferences must be made with caution. Nevertheless, the major branching patterns do suggest the hypothesis that the Peridiniphyceid Group 1 species arose relatively early in dinoflagellate evolution and were followed by the divergence of the Gymnodiniphyceae, Prorocentrophycidae, and Gonyaulacales (Peridiniphycean Group 2) lineages (Figs. 1, 2). The early emergence of the Peridiniphyceid is again supported by the the Peridiniphycean-type plate tabulations of the Blastodiniphyceid, the group that diverged just prior to the Peridiniphyceid Group 1 (Landsberg et al. 1994, Steidinger et al. 1996a). Sequences from other genes that have evolved more slowly than the SSU genes may also prove useful in evaluating the deep branches within the Dinophycidae.

In this study, *Peridinium* sp. grouped within the Prorocentrophycidae and not with the other two distinct Peridiniphycean lineages as expected. Other analyses have shown similar enigmatic positions for the same *Peridinium* sp. (Saunders et al. 1997, Grzebyk et al. 1998). More *Peridinium* species are needed to determine if the *Peridinium* represent their own group or are members of the lineage leading to the Prorocentrum/Gonyaulacales (Peridiniphycean group 2; Fig. 1).

Saunders et al. (1997) proposed that the Sussiales (*Symbiodinium* group) should not necessarily be placed in the Gymnodiniphyceae. This analysis, instead, shows the Sussiales as a distinct lineage belonging to the Gymnodiniphyceae. It should also be emphasized that the *Symbiodinium* species, along with *A. belauense*, and the unidentified symbiotic dinoflagellate, all represent organisms that have formed endosymbiotic relationships with invertebrate hosts. The distinctly different phylogenetic placement of these species is consistent with dino-

flagellates undergoing a number of independent symbiotic events throughout evolutionary history.

The order phytodiniales, represented by *Hemidinium* (= *Gloeodinium*, life-cycle stage of *Hemidinium*) viscum previously has not been assigned to a subclass (Fensome et al. 1993). In this analysis *Gloeodinium* was tentatively placed on an outer branch within Prorocentrophycidae, but was not significantly supported by bootstrap analysis. Based on plate tabulations, *Hemidinium*, was assigned to the Peridiniphyceid, though that classification was problematic because *Hemidinium* poses an incomplete cingulum (Fensome et al. 1993). Again more sequence data from other representative Phytodiniales will be needed to determine if this group represents divergent Peridiniphyceid or Prorocentrophycidae, or are in an intermediate subclass between the two. In either case, the SSU phylogeny confirm that the Phytodiniales diverged subsequent to the Blastodiniales.

All current analyses show *A. belauense* diverging early in the evolution of the Dinophyceae and not falling into the Gymnodiniphyceid where it currently is assigned (Fensome et al. 1993, Saunders et al. 1997, Grzebyk et al. 1998). McNalley et al. (1994) instead suggested that this species represent a separate lineage outside the Gymnodiniales, a contention supported by this SSU phylogeny.

Pfiesteria piscicida, the PLD, Cryptoperidiniopoid sp., and *A. ocellatum* are closely related and represent a relatively ancestral group of heterotrophic dinoflagellates belonging to the Blastodiniphyceid. None of these species was closely related to the exclusively parasitic apicomplexans or *Perkinsus* species. The overall SSU phylogeny estimates using maximum-likelihood techniques were largely consistent with the current taxonomies based primarily on plate structure, though exceptions did occur. This indicates that ribosomal gene sequences will be useful in helping to resolve many of the phylogenetic relationships within the dinoflagellates. Species representing intermediate stages in the early evolution of the dinoflagellates, however, will be needed before deep branches in the tree can be adequately resolved. Sequences from variable portions of the ribosomal genes may prove useful not only for phylogenetic studies, but also in identifying life-cycle stages. It should be emphasized that we examined only 54 dinoflagellate species from 14 of the more than 60 known dinoflagellate families and that there is still much to resolve concerning the evolution of the Dinoflagellata.

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