Protist, Vol. 155, 347–359, September 2004
http://www.elsevier.de/protist
Published online 14 September 2004

Protist

ORIGINAL PAPER

Highly Divergent SSU rRNA Genes Found in the Marine Ciliates *Myrionecta rubra* and *Mesodinium pulex*

Matthew D. Johnson[a,1], Torstein Tengs[b,2], David W. Oldach[b], Charles F. Delwiche[c], and Diane K. Stoecker[a]

[a] Horn Point Laboratory, University of Maryland, Center for Environmental Science, PO Box 775, Cambridge, MD 21613, USA
[b] Institute of Human Virology, University of Maryland, School of Medicine, Baltimore, MD, USA
[c] Department of Cell Biology and Molecular Genetics/Plant Biology, University of Maryland, College Park, MD, USA

Submitted April 2, 2004; Accepted May 30, 2004
Monitoring Editor: Mitchell L. Sogin

***Myrionecta rubra* and *Mesodinium pulex* are among the most commonly encountered planktonic ciliates in coastal marine and estuarine regions throughout the world. Despite their widespread distribution, both ciliates have received little attention by taxonomists. In order to better understand the phylogenetic position of these ciliates, we determined the SSU rRNA gene sequence from cultures of *M. rubra* and *M. pulex*. Partial sequence data were also generated from isolated cells of *M. rubra* from Chesapeake Bay. The *M. rubra* and *M. pulex* sequences were very divergent from all other ciliates, but shared a branch with 100% bootstrap support. Both species had numerous deletions and substitutions in their SSU rRNA gene, resulting in a long branch for the clade. This made the sequences prone to spurious phylogenetic affiliations when using simple phylogenetic methods. Maximum likelihood analysis placed *M. rubra* and *M. pulex* on the basal ciliate branch, following removal of ambiguously aligned regions. Fluorescent in situ hybridization probes were used with confocal laser scanning microscopy to confirm that these divergent sequences were both expressed in the cytoplasm and nucleolus of *M. rubra* and *M. pulex*. We found that our sequence data matched several recently discovered unidentified eukaryotes in Genbank from diverse marine habitats, all of which had apparently been misattributed to highly divergent amoeboid organisms.**

Introduction

The phototrophic ciliate *Myrionecta rubra* (= *Mesodinium rubrum*) (Lohmann 1908, Jankowski 1976) (Mesodiniidae, Litostomatea) is nearly ubiquitous in coastal marine and estuarine habitats and has long been a curiosity to evolutionary biologists, perhaps beginning with Darwin (1839). The important ecological role of this ciliate is periodically made conspicuous by massive non-toxic red tides in coastal and estuarine regions throughout the world, some of which may exceed 100 square miles (Jiménez and Intriago 1987; Ryther 1967). *M. rubra* is well documented to possess organelles of cryptophycean origin, including plastids, mitochondria (Taylor et al. 1969, 1971), and nuclei (Hibberd 1977; Oakley and Taylor 1978). While early studies debated whether

[1] Corresponding author;
e-mail mjohnson@hpl.umces.edu
[2] Present address: Dana-Farber Cancer Institute, 44 Binney St, Boston, MA 02115, USA

1434-4610/04/155/03-347 $ 30.00/0

these organelles represented a true symbiosis or were the result of sequestration from living prey (e.g. Taylor et al. 1969), more recent studies have provided evidence for the latter, showing that growth and photosynthesis in *M. rubra* are dependent upon ingesting free-living cryptomonads (Gustafson et al. 2000).

Mesodinium (Stein 1863) (Mesodiniidae, Litostomatea) is a commonly encountered genus of non-pigmented ciliates found in coastal marine, estuarine, and fresh water systems (Foissner et al. 1999). *M. pulex* is a heterotrophic ciliate that feeds upon bacteria, flagellates, algae, and ciliates (Dolan and Coats 1991; Foissner et al. 1999). Although there are few described species in the genus besides *M. pulex*, it is often confused with *M. acarus* and *M. fimbriatum* (Foissner et al. 1999).

Currently no sequence data are available for the Mesodinidae, and only recently have efforts been made to determine small subunit (SSU) ribosomal RNA sequences for other familiar pelagic marine ciliates (e.g. Snoeyenbos-West et al. 2002, Strueder-Kypke and Lynn 2003). Several recent PCR-based studies to assess microbial eukaryotic diversity in the world's oceans have led to the discovery of numerous unidentified SSU rRNA sequences, some of which constitute new branches on familiar phylogenetic lineages (e.g. López-Garcia et al. 2001). Many of these new sequences can be attributed to well described protistian groups such as alveolates (apicomplexans, ciliates, colpodellids, dinoflagellates, perkinsids), while some are sequences with uncertain phylogenetic affiliation. However, many of the rRNA genes of recognized marine protists have yet to be sequenced, suggesting that some of the new sequences may not be novel taxa. Recently Leander et al. (2003) identified some of these novel sequences as belonging to the colpodellids. In the present study we present evidence that several newly described unidentified eukaryotic sequences with uncertain taxonomic affiliation also belong to a familiar lineage of alveolates. Herein we present sequence data that show *Myrionecta* and *Mesodinium* share similar and highly divergent SSU rDNA sequences that suggest they are an early branching lineage of ciliates, and use in situ hybridization to verify that these sequences are present and expressed.

Results

SSU rRNA Gene Characteristics

The rDNA genes amplified for the Antarctic and Chesapeake Bay *M. rubra* and the Chesapeake Bay *M. pulex* were highly divergent compared to other ciliates and alveolates in general. The rDNA sequences for both *Myrionecta* and *Mesodinium* were relatively short, 1548 and 1543 bp respectively, compared to an average for alveolates of about 1750. This was primarily due to coincident deletions in the gene, for both taxa, in helices 10 (variable region [V] 2) (~20bp), 11 (~9 bp), E23-1-7 (V4) (~35 bp), E23-14 (V4) (absent), and 43 (V7) (~23 bp) (based on Wuyts et al. 2000 model). Furthermore both taxa have numerous substitutions in helices 8, 16, 18, 25, and 26 compared with all other alveolate taxa.

In situ Hybridization Analysis

Due to the highly unusual nature of the *Myrionecta* and *Mesodinium* sequences we used fluorescent *in situ* hybridization (FISH) to determine whether these sequences originated from the ciliates in question. In addition, confocal laser scanning microscopy (CLSM) was used to localize probe binding to the nucleolus, in order to verify that the probes were hybridizing to the targeted genome. An oligonucleotide probe (Myr2) was designed from a variable region of the SSU rRNA molecule common to both *Myrionecta* and *Mesodinium*, but with numerous substitutions in other alveolates and distantly related taxa (Table 2). We found that the Myr2 probe always bound to rRNA within the ciliate cytoplasm and the nucleolus of the ciliate nuclei (Figs 1D3, 2D3), and never to RNA in other taxa (data not shown). Many *M. rubra* cells also possessed nuclei of their cryptophyte prey, *Teleaulax acuta*, to which no binding of the Myr2 probe was observed (data not shown). The macronuclei of both ciliates were found to have a single large nucleolus or sphere filled with rRNA that comprised much of the volume of each macronucleus (Figs 1B3,D3). The universal eukaryotic positive control probe uniC, labeled rRNA throughout the cell cytoplasm and within the nucleoi of all nuclei, including those of cryptophyte prey when present (Figs 1B3, 2B3). No binding of any probes to nuclear DNA within the ciliates could be detected, nor of any negative control probes to any portion of the cell. Negative control probes consisted of antisense probes for both the universal eukaryote RNA probe (uniR) (Figs 1A1–4, 2A1–4), and the *Myrionecta*/*Mesodinium* probe (Myr2-neg) (Figs 1C1–4, 2C1–4).

Phylogenetic Analysis

The *Myrionecta*/*Mesodinium* SSU rDNA sequences share high sequence similarity (7% sequence difference, Table 1) in addition to the above-mentioned

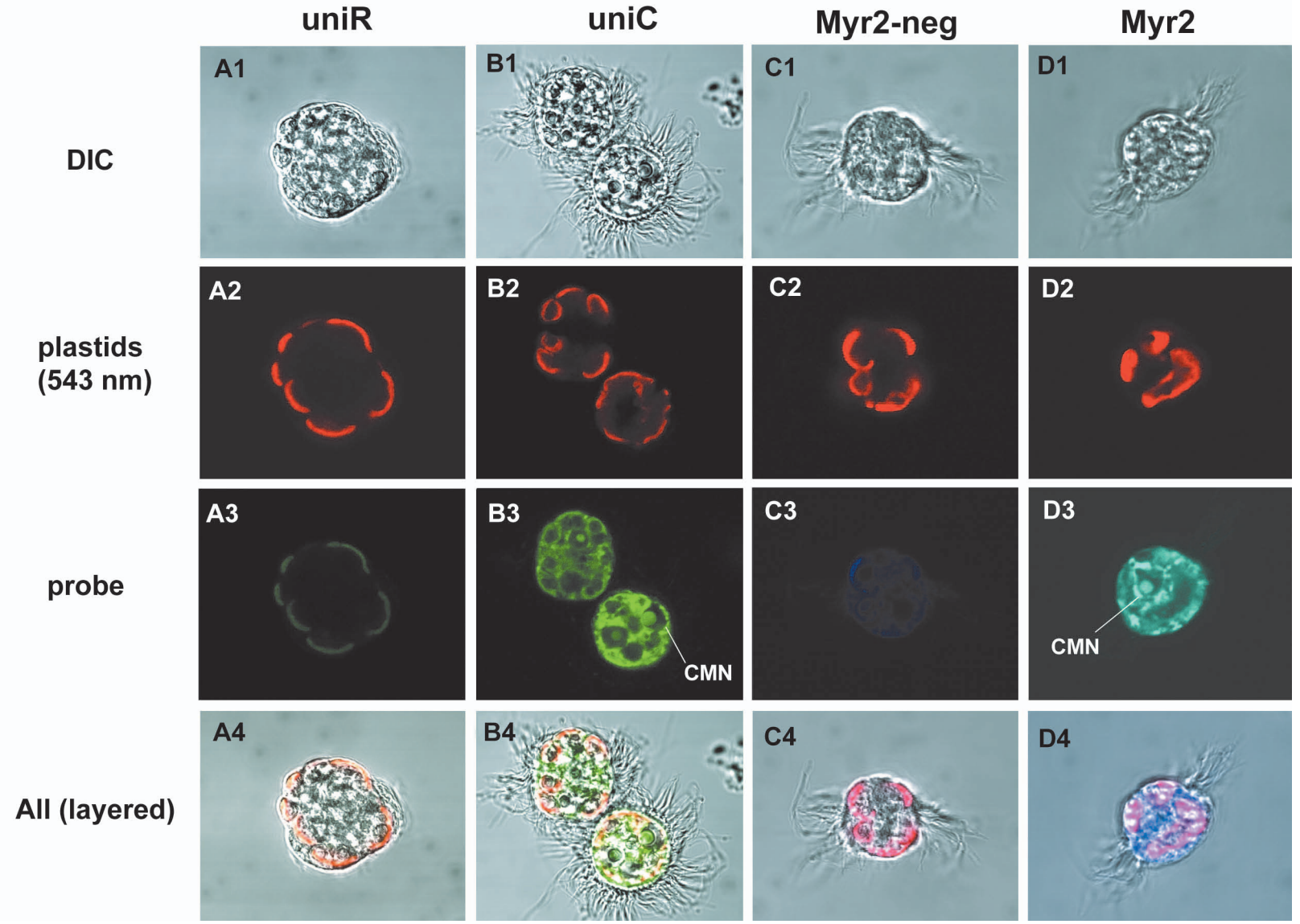


Figure 1. Confocal laser scanning micrographs of several typical rRNA probe hybridizations to *Myrionecta rubra* cells. Figure description: **column A:** eukaryotic antisense negative control probe (uniR); **column B:** universal-eukaryotic rRNA positive control probe (uniC); **column C:** *Myrionecta*/*Mesodinium* antisense negative control probe (Myr2-neg); **column D:** *Myrionecta*/*Mesodinium* probe for SSU rRNA gene (Myr2); images in each column are the same cell. Row 1: differential interference contrast (DIC) images; row 2 autofluorescence of plastids (em 543 nm); row 3: probe-specific fluorescence (ex 488 or 633); row 4: rows 1–3 layered. CMN = ciliate macronucleus.

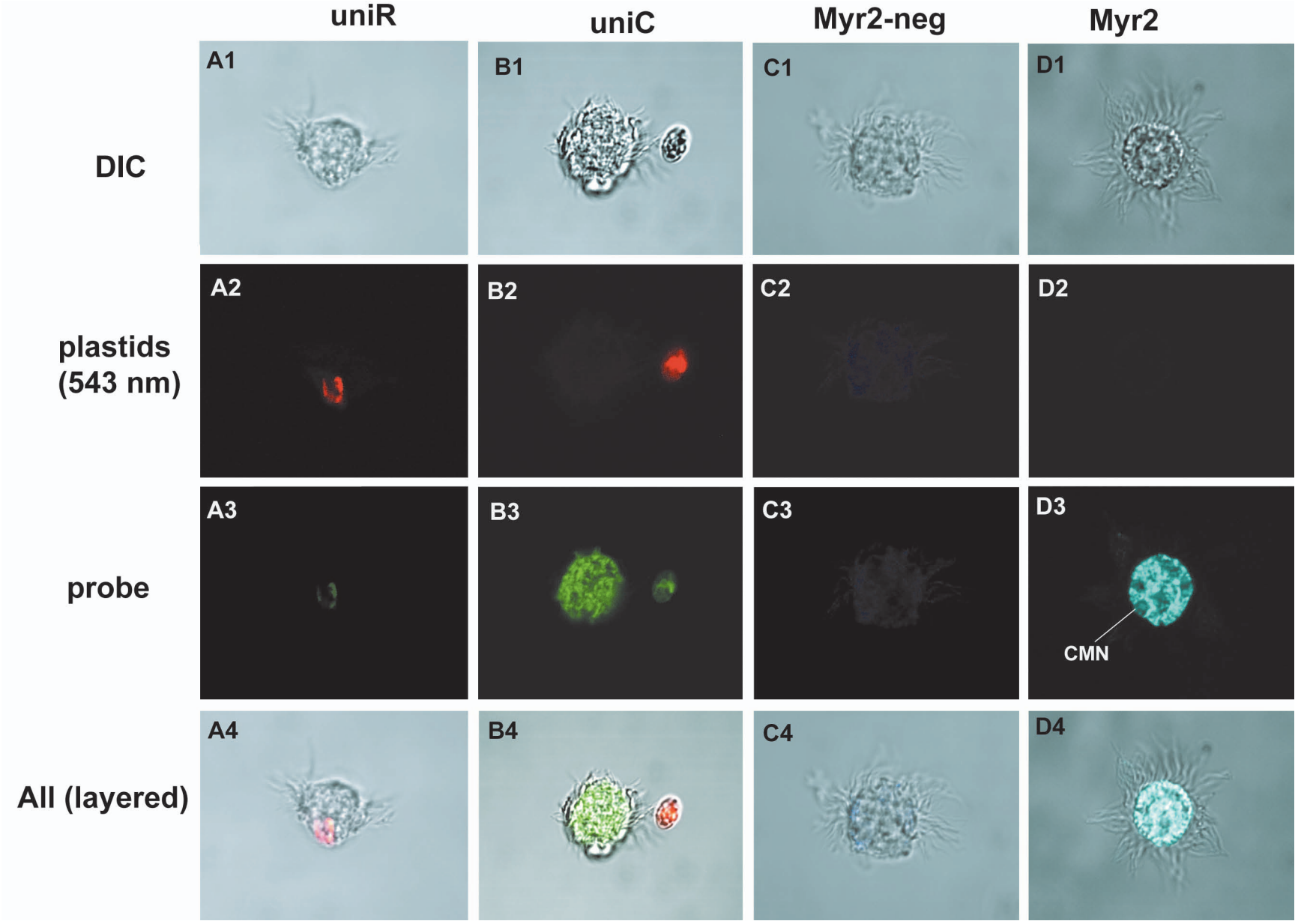


Figure 2. Confocal laser scanning micrographs of several typical rRNA probe hybridizations to *Mesodinium pulex* cells. Figure description: **column A:** eukaryotic antisense negative control probe (uniR); c**olumn B:** universal-eukaryotic rRNA positive control probe (uniC); **column C:** *Myrionecta/Mesodinium* antisense negative control probe (Myr2-neg); **column D:** *Myrionecta/Mesodinium* probe for SSU rRNA gene (Myr2); images in each column are the same cell. Row 1: differential interference contrast (DIC) images; row 2 autofluorescence of cryptophyte prey (em 543 nm); row 3: probe-specific fluorescence (em 488 or 633); row 4: rows 1–3 layered. CMN = ciliate macronucleus.

Table 1. Environmental clones in Genbank that are closely related to cultures of *Myrionecta rubra* and *Mesodinium pulex*.

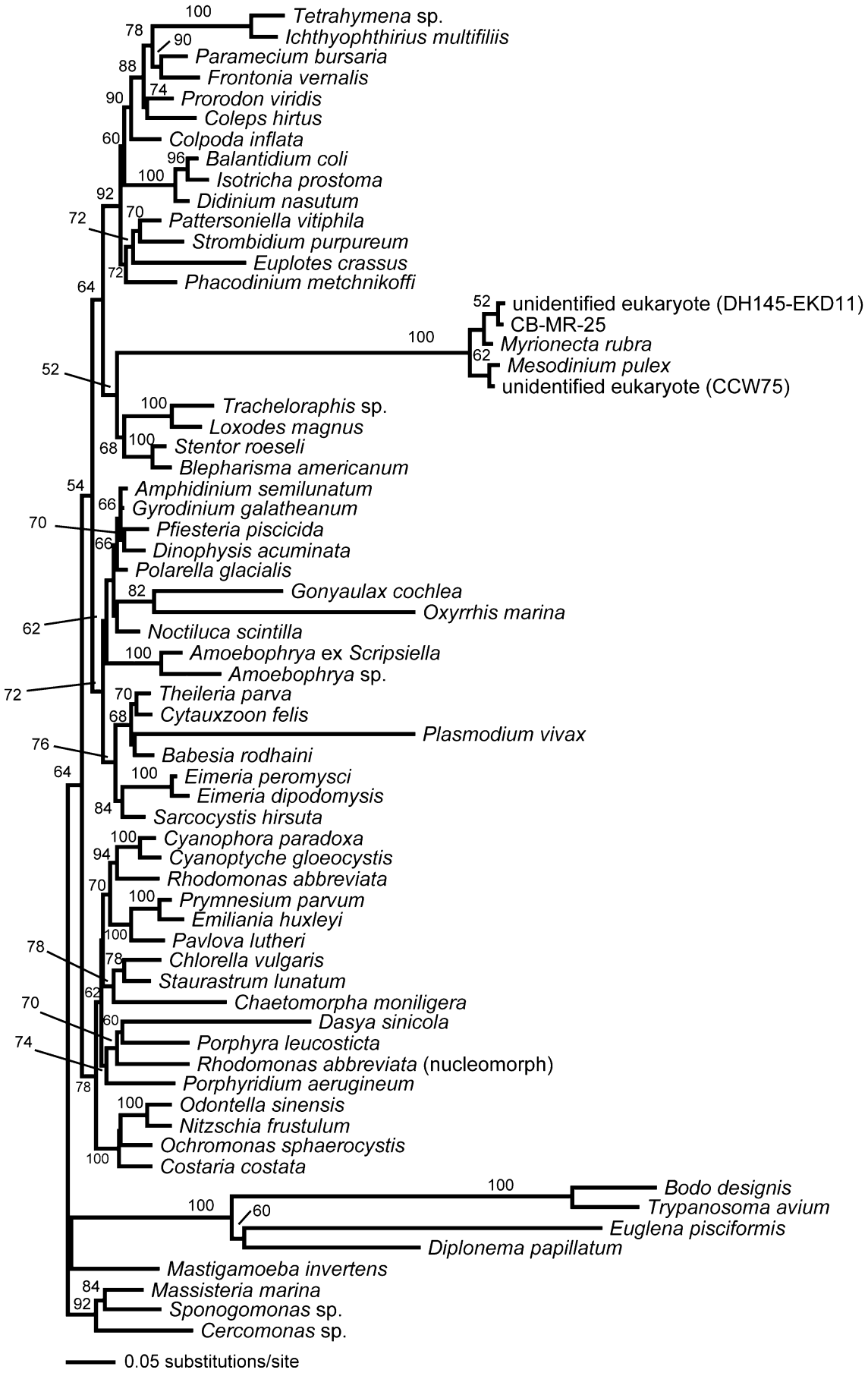
Clone or culture	Accession number	Base pairs	S′[a]		Total gaps		% identity	
			MR[b]	MP[b]	MR	MP	MR	MP
Myrionecta rubra	AY587129	1543	–	–	–	28	–	93
Mesodinium pulex	AY587130	1548	–	–	28	–	93	–
CB-MR-25[c]	AY587131	662	–	–	10	22	97	90
DH145-EKD11	AF290065[d]	1474	1398	1052	7	14	98	91
CCW100	AY180041[e]	1518	1320	1046	9	14	96	91
CCW75	AY180032[e]	1519	1146	1357	10	11	92	98
M43	AY331778[f]	1137	937	640	1	16	98	92
M112	AY331777[f]	1173	1109	803	2	17	98	92
M110	AY331783[f]	1141	1028	759	5	20	98	91

[a] bit score from BLAST search, [b] *Myrionecta rubra* and *Mesodinium pulex*, [c] Chesapeake Bay, *Myrionecta rubra* clone (partial sequences), [d] Lòpez-Garcia et al. 2001, [e] Stoeck and Epstein 2003, [f] Savin et al. 2004

Table 2. Probe and target sequence for *Myrionecta rubra* and *Mesodinium pulex* and comparisons to other taxa; target region in bold and mismatches between target taxa and other taxa highlighted in gray.

Species	Sequence
Outgroup taxa	
Mastigamoeba invertens	5′..GAAATTC**TTGGATTTATTAAAGAT**GAACTA..3′
Diplonema papillatum	5′..GAAATTC**TTAGATCGTAGGAAGAC**GAACTT..3′
Echinamoeba therma	5′..GAAATTC**TAGGATTAACTGAAAAC**AAACTA..3′
Other alveolates	
Dinophysis acuminata	5′..GAAATTC**TTGGATTTGTTAAAGAC**GGACTA..3′
Plasmodium vivax	5′..GAAATTC**TTAGATTTTCTGGAGAC**AAACAA..3′
Other ciliates	
Didinium nasutum	5′..GAAATTC**TTGGATTTATTAAAGAC**TAACGT..3′
Euplotes crassus	5′..GAAATTC**TTTGAAATATTAAAGAC**TAACTT..3′
Stentor roeseli	5′..GAAATTC**TATGATTTATTAAAGAC**GAACTT..3′
Loxodes magnus	5′..GAAATTC**TTGGATTTACTGAAGAC**CAACTA..3′
Target taxa	
Myrionecta rubra	5′..GAAATTC**TTGGACCGGACGAAGAC**GACCAG..3′
Mesodinium pulex	5′..GAAATTC**TTGGACCGGACGAAGAC**GATCAG..3′
Probe	
Myr2 probe	3′..-------**TTGGACCGGACGAAGAC**-------..5′

common deletions, and formed a well-supported clade in all analyses (100% bootstrap support, Figs 3, 4). In preliminary phylogenetic analyses of these sequences using distance and maximum parsimony methods, the *Myrionecta*/*Mesodinium* clade consistently grouped with other highly divergent sequences (e.g. *Plasmodium vivax*, *Oxyrrhis marina*), a result that we attribute to branch-length effects (data not shown). In order to eliminate these effects, we removed ambiguously aligned regions of the data set and proceeded with maximum likelihood methods.


Tetrahymena sp.
Ichthyophthirius multifiliis
Paramecium bursaria
Frontonia vernalis
Prorodon viridis
Coleps hirtus
Colpoda inflata
Balantidium coli
Isotricha prostoma
Didinium nasutum
Pattersoniella vitiphila
Strombidium purpureum
Euplotes crassus
Phacodinium metchnikoffi
unidentified eukaryote (DH145-EKD11)
CB-MR-25
Myrionecta rubra
Mesodinium pulex
unidentified eukaryote (CCW75)
Tracheloraphis sp.
Loxodes magnus
Stentor roeseli
Blepharisma americanum
Amphidinium semilunatum
Gyrodinium galatheanum
Pfiesteria piscicida
Dinophysis acuminata
Polarella glacialis
Gonyaulax cochlea
Oxyrrhis marina
Noctiluca scintilla
Amoebophrya ex Scripsiella
Amoebophrya sp.
Theileria parva
Cytauxzoon felis
Plasmodium vivax
Babesia rodhaini
Eimeria peromysci
Eimeria dipodomysis
Sarcocystis hirsuta
Cyanophora paradoxa
Cyanoptyche gloeocystis
Rhodomonas abbreviata
Prymnesium parvum
Emiliania huxleyi
Pavlova lutheri
Chlorella vulgaris
Staurastrum lunatum
Chaetomorpha moniligera
Dasya sinicola
Porphyra leucosticta
Rhodomonas abbreviata (nucleomorph)
Porphyridium aerugineum
Odontella sinensis
Nitzschia frustulum
Ochromonas sphaerocystis
Costaria costata
Bodo designis
Trypanosoma avium
Euglena pisciformis
Diplonema papillatum
Mastigamoeba invertens
Massisteria marina
Sponogomonas sp.
Cercomonas sp.
0.05 substitutions/site

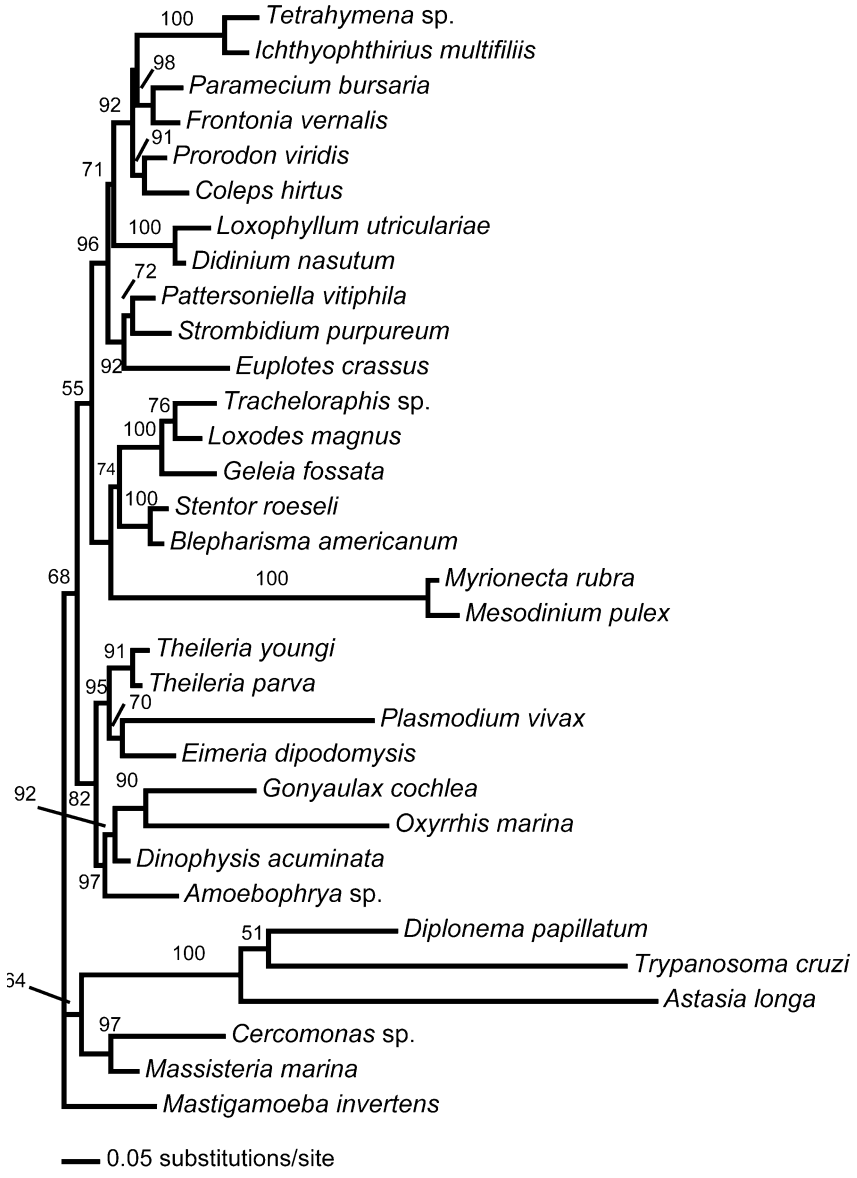


Figure 4. Gamma-corrected (Γ = 0.5444) maximum likelihood (-lnL = 15334.84); GTR model) tree with proportion of invariable sites (0.14153) using a small subunit rDNA alignment of 1479 sites. Base frequencies and substitution rates were estimated with a distance-maximum likelihood search. Tree topology was found using stepwise addition and10× heuristic searches with TBR branch swapping and random-addition sequences. Numbers on branches correspond to bootstrap values (100× with stepwise addition and a heuristic search with 2× random addition and TBR branch swapping) for this ML tree.

◀ **Figure 3.** Gamma-corrected (Γ = 0.5576) distance-maximum likelihood (ML) (GTR model) tree (minimum evolution) with proportion of invariable sites (0.1079), using a small subunit rDNA alignment of 1474 sites. ML parameters were estimated with Modeltest (Posada and Crandall 1998). Tree topology was found using stepwise addition and 25× heuristic searches with TBR branch swapping and random-addition sequences. Numbers on branches correspond to bootstrap values (100× with stepwise addition and heuristic searches with 2× random addition and TBR branch swapping).

A matrix of 65 assorted protist taxa of 1474 characters and about 1300 nucleotides (923 were parsimony informative) was used for a Γ-corrected distance-maximum likelihood (DML) search (general time reversible model) with proportion of invariable sites. In this analysis *Myrionecta*/*Mesodinium* clade branched within the ciliates, albeit with low bootstrap support (64%) (Fig. 3). Included in this analysis were several unidentified eukaryote clones with high sequence similarity to our *Myrionecta*/*Mesodinium* sequences, and our Chesapeake Bay *M. rubra* sequence (Table 1). These environmental taxa grouped strongly with our *Myrionecta* and *Mesodinium* cultures (100% bootstrap support), revealing the likely source of these clones (Fig. 3). All previous analyses of these environmental clones, in the absence of the *Myrionecta*/*Mesodinium* sequences, had placed them outside of the alveolates with divergent amoeboid taxa. Of the six environmental clones that grouped with *Myrionecta* and *Mesodinium*, five appeared to be closely related to *M. rubra*, with high % sequence identity (96–98%) and alignment bit score (S'), while one (CCW75) was most similar to *M. pulex* (98%) (Table 1).

A full ML analysis was then conducted on a nearly identical matrix (1479 bp) greatly reduced in taxa (32 taxa) and composed mostly of alveolate taxa. This Γ-corrected ML (GTR model) analysis with pinvar also revealed low bootstrap support to group the *Myrionecta*/ *Mesodinium* clade within the ciliates (55%) and alveolates (68%) (Fig. 4). In the ML-tree the ciliates, dinoflagellates, and apicomplexans all form monophyletic groups within the alveolates. The *Myrionecta*/*Mesodinium* clade appears with the Karyorelictids and the Heterotrichs as the sibling group to all other ciliates. The other ciliate groups formed a clade that received considerably higher bootstrap support (96%) then these basal taxa, and removal of the *Myrionecta* /*Mesodinium* sequences resulted in substantially higher maximum likelihood bootstrap support for the ciliates as a whole (97%; data not shown). The basal placement of *Myrionecta*/ *Mesodinium* was surprising given the traditional placement of *Myrionecta* and *Mesodinium* within the Litostomatea, raising the possibility that the model-based methods did not fully compensate for branch-length effects.

Discussion

Myrionecta and *Mesodinium* (Mesodiniidae) belong to the Litostomatea, subclass Haptorida (Lynn and Small 2000). Krainer and Foissner (1990) reclassified the order Cyclotrichida Jankowski 1980, family Mesodiniidae Jankowski 1980, as having the genera *Askenasia*, *Rhabdoaskenasia*, *Mesodinium*, and *Myrionecta*. However, Lynn (1991) remarked that the somatic ciliature of *Mesodinium* are so dramatically different from any other litostomes that, if it is a litostome, it "has diverged significantly from the ancestral stock". Based on our SSU rDNA sequences of *Myrionecta* and *Mesodinium* it is clear that either these ciliates do not belong to the Litostomatea, or their SSU rDNA genes have diverged so greatly as to make them a poor phylogenetic marker. Litostomes generally form a well-supported monophyletic clade within the ciliates and share the somewhat diagnostic deletions of helix E23-5 and portions of variable region 4 in the SSU rRNA gene (Wright et al. 1997). While *M. rubra* and *M. pulex* clearly have more extensive deletions and much higher substitution rates, an accurate placement of these taxa within the ciliates will require additional sequence data from other genes.

Due to the highly divergent nature of these sequences, we checked their validity using fluorescent *in situ* hybridization (FISH) and confocal laser microscopy (CLSM). FISH probes have been used successfully to differentiate closely related species of *Euplotes* ciliates that appear morphologically similar (Petroni et al. 2003). We found that the use of CLSM with FISH adds an additional level of confidence in determining that a probe is binding to a target genome, with the ability to localize binding of the probe to RNA within the nucleolus. The FISH/CLSM results clearly show that the probes designed for *Myrionecta* and *Mesodinium,* from highly variable and therefore taxon-specific regions hybridize to rRNA both within the cytoplasm of the cells and the ciliate macronucleus (Figs 1D3, 2D3).

The sequences determined in this study for *M. rubra* and *M. pulex* are highly divergent and of great phylogenetic interest. Only after removing nearly all ambiguously aligned regions of the rDNA alignment were we able to find support for these sequences within the ciliate clade. The initial affiliation of the *Myrionecta*/*Mesodinium* sequences with other divergent taxa using certain distance and maximum parsimony methods in our analysis was alleviated by more robust phylogenetic methods. This suggests that branch length may have been an important factor in previous analyses of similar environmental sequences that resulted in an affiliation to amoeboid taxa (i.e. Lòpez-Garcia et al. 2001; Savin et al. 2004; Stoeck and Epstein 2003). Long branch attraction (LBA) has been used to explain the phenomena of seemingly unrelated but fast-evolving taxa being drawn to one another in a tree (Philippe and Laurent 1998).

These results are by no means a decisive characterization of the phylogenetic position of this group. It is possible that the rDNA gene may not be useful for interpreting the phylogeny of *Myrionecta* and *Mesodinium* due to their highly accelerated substitution and deletion rates. Therefore the grouping of *M. rubra* and *M. pulex* with Karyorelictids and Heterotrichs has to be treated with some caution. While rDNA genes of ciliates generally have typical eukaryotic substitution rates (e.g. Van de Peer and Wachter 1997), other ciliate genes have been shown to have high rates of sequence divergence, such as the elongation factor 1α (EF-1α) (Moreira et al. 1999), actin (Villalobo et al. 2001), and histone (H4) (Berhard and Schlegel 1998; Katz et al 2004) genes. The resulting long branch lengths of the rDNA gene for *Myrionecta* and *Mesodinium* are one of the more dramatic found thus far in the alveolates, a group already known for high genetic diversity and long branches. In other phylogenetic clades, particularly those of symbiotic or parasitic organisms, long branches have been explained by asexuality and population bottlenecks enhancing rDNA substitution rates, relaxed selection on rDNA structure, or positive selection for sequence change (Stiller and Hall 1999). In the free-living heterotrophic dinoflagellate *O. marina*, accelerated evolutionary rates are also found in the rDNA gene, yet several protein-encoding genes (actin, α-tubulin and β-tubulin) appear to be equally as divergent as other dinoflagellate homologues (Saldarriaga et al. 2003). The protein-encoding phylogenies of *O. marina* lend support to several plesiomorphic cellular characteristics and place it at the base of the dinoflagellates near the Perkinsids, while the SSU rRNA gene groups it with *Gonyaulax* as a more recent branch in the dinoflagellate tree (Saldarriaga et al. 2003). While our data suggest that the *Myrionecta*/*Mesodinium* clade may also be an early and divergent branch of its phylum, like *O. marina*, the highly divergent rRNA gene phylogeny of these ciliates could contradict future protein phylogenies. However, additional support for an alternative taxonomic classification for *Myrionecta* and *Mesodinium* may also stem from several unusual phenotypic characteristics. These include the presence of feeding tentacles with a unique 14-microtubule structure (Lindholm et al. 1988), the complete absence of alveoli, an unusual somatic ciliature arrangement, and an unusual nuclear arrangement (two macronuclei and one micronuclei) (Taylor et al. 1971), all of which are synapomorphic within the Litostomatea. Currently we are working towards determining the sequence of several protein-encoding genes for these taxa and accumulating phenotypic data, in order to test these hypotheses.

Recent studies of amplified and sequenced rDNA from ocean samples have revealed a great deal of uncharacterized genetic diversity (e.g. Dawson and Pace 2002; López-Garcia et al. 2001; van der Staay et al. 2001,). Within this recently discovered diversity, alveolates have been among the most frequently recovered sequences (Moreira and López-Garcia 2002). These studies have been valuable in identifying new branches of genetic diversity on familiar lineages of organisms. However, not all of these novel sequences may represent novel organisms. We found several sequences within Genbank purporting to represent novel eukaryotic diversity, which are closely affiliated or nearly identical to our *Myrionecta* and *Mesodinium* sequences. These environmental sequences have been determined from diverse habitats, including deep Antarctic water (López-Garcia et al. 2001), surface water from the Bay of Fundy, CAN (Savin et al. 2004), and microaerobic water samples from Cape Cod, USA, (Stoeck and Epstein 2003). We have determined nearly identical partial sequences from cells of *M. rubra* isolated from Chesapeake Bay. *Myrionecta* and *Mesodinium* have a cosmopolitan oceanic, estuarine, and fresh water (for *Mesodinium*) distribution (Foissner et al. 1999; Taylor et al. 1971). Therefore the wide geographical and ecological diversity of sites with matching environmental clones is not surprising. The presence of *M. rubra* in microaerobic water is also not unusual, as *M. rubra* has been observed to congregate near anoxic boundary layers in stratified waters (Lindholm and Mörk 1990). In all of the above studies, clones matching our *Myrionecta* sequence are described as having uncertain phylogenetic ascription, and are weakly affiliated with various amoeboid organisms such a *Mastigamoeba* (Lòpez-Garcia et al. 2001; Savin et al. 2004; Stoeck and Epstein 2003). We believe that these results were due primarily to branch length effects that were alleviated in the present study by removal of ambiguously aligned regions and using maximum likelihood methods.

In using sequence analysis alone, the artificial phylogenetic affiliation of *Myrionecta* and *Mesodinium* with other divergent taxa is perhaps unavoidable, due to the extremely divergent nature of their SSU rDNA. Only by working with cultures and employing FISH probes was it apparent that these sequences belong to the respective ciliates and not to parasites or contaminants, as we initially suspected. While it is unclear if our analysis of the SSU rRNA genes of these ciliates has succeeded in determining their true phylogenetic position, it has revealed a striking example of divergent sequence evolution within the ciliates.

Methods

Culture conditions and cell isolations: *Myrionecta rubra* was isolated from a nutrient enrichment of water collected in McMurdo Sound, Antarctica in 1996 as described previously (Gustafson et al. 2000). Cultures were maintained in 33 psu f/2 (-Si) culture media (Guillard 1976) and periodically fed the cryptomonad, *Teleaulax acuta*. *Mesodinium pulex* was isolated from an estuarine portion of the Choptank River, Cambridge, MD, USA, after enriching river water with flagellate prey for several days. The culture was maintained at 15 ºC in 15 psu seawater, made from diluting full-strength seawater. Nutrients were not added directly to the *M. pulex* culture, except when carried over from adding its prey, *Rhodomonas* sp. *M. rubra* cells were also isolated from the Choptank River, but all efforts to culture them failed. Therefore multiple (10–50) *M. rubra* cells were isolated from water samples, washed several times with clean media, added directly to 1× TE buffer (0.1 M tris-HCl, 0.01 M EDTA) and frozen (–20 ºC) for later PCR. Cultures of *M. rubra* and *M. pulex* are available upon request.

DNA extraction, PCR amplification and DNA sequencing: Cultures of *M. rubra* (~3×10^4 cells ml^{-1}) and *M. pulex* (~1000 cells ml^{-1}) were centrifuged in 50 ml centrifuge tubes at 4 ºC and 4000 g for 10 min. The Plant DNA Extraction Kit (Qiagen) was used and the manufacturers protocol was followed. PCR was conducted using 1× PCR buffer (TaqPro, Denville), 0.2 µM nucleotides, 0.25 mg/ml bovine serum albumin (BSA), 3 mM $MgCl_2$, 0.4 µM primers, and 0.6 u Taq DNA polymerase, and were combined with 10–20 ng of genomic DNA from cultures in a volume of 25 µl. To amplify the SSU rDNA gene of isolated *M. rubra* cells from Chesapeake Bay, the cells were heated at 95 ºC for 2 min and 10 µl of the TE suspension (described above) was then added to the PCR mix. The following general eukaryotic primers for small subunit (SSU) rRNA were used to amplify the gene from conserved regions: 4616, 4618 (Medlin et al. 1988; Oldach et al. 2000), 516 (CACATCTAAGGA AGGCAGCA), and 1416 (GAGTATGGTCGCAAGGC TGAA). PCR conditions were as follows: an initial 3 min 95 ºC melting step, 40 cycles of 30 sec at 95 ºC (melting), 30 s at 55 ºC (hybridization), and 70 s at 72 ºC (elongation), followed by a final 10 m 72 ºC elongation step. Products were then cloned using an Invitrogen TOPO TA cloning kit, following manufacturers instructions. Colonies were then isolated and gene products were reamplified with PCR using the same gene-specific primers. Cloned PCR products were sequenced directly in both directions using the above gene-specific primers and the BigDye terminator kit (Perkin Elmer). All sequencing was conducted using an ABI 377. Species-specific SSU rDNA primers (Qiagen) were designed for all novel sequences identified from sequencing the SSU rDNA clone library, and all sequences were generated at least 10 times. The species-specific primers, UNIDEUK(670R) (TATGAAGACTTGGTCTACCTTGA), UNIDEUK(880F) (ACTGAAACTATGCCAACTTGG), and UNIDEUK(1416R) GTTTCAGACTTGTGTCCAT ACTA), were used to verify the sequence from our cultures and to amplify the SSU rRNA gene from environmentally isolated cells.

Phylogenetic analysis: Contig sequences were generated using Sequencher (Gene Codes Corp.) and added to an alignment of sequences obtained from Genbank. All alignments were created using the Clustal X algorithm (Thompson et al. 1997) and ambiguous regions of the alignment found in highly variable regions were removed by eye in MacClade 4.05 (Maddison and Maddison 1991). An alignment matrix was constructed of diverse alveolates and numerous other lineages of protists, and is available upon request.

The initial analysis of the data set was aimed at determining the relationship of our sequence data to a larger and more diverse group of eukaryotes, including most of the unidentified eukaryote environmental clones that shared high sequence similarity to our *M. rubra* and *M. pulex* cultures from Genbank. The analysis was performed using minimum evolution (ME) gamma (Γ)-corrected (4 category: 0.5576) distance maximum likelihood (DML) analysis, with proportion of invariable sites (pinvar: 0.1079), estimated base frequencies (A: 0.2568, C: 0.2131, G: 0.2856, T: 0.2445), and the general time-reversible (GTR) model for base substitutions (A-C: 1.2673, A-G: 2.4762, A-T: 1.4931, C-G: 0.9709, C-T: 34.2604, G-T: 1), selected using Modeltest version 3.04 (Posada and Crandall 1998). For this analysis 57 ingroup taxa and 8 outgroup taxa were used. Heuristic searches (25×) were performed using step-wise random addition and tree bisection-reconstruction (TBR) branch swapping.

For the maximum likelihood analyses we first ran a distance analysis to estimate base frequencies (A: 0.29258, C: 0.19152, G: 0.24914, T: 0.26676) and GTR substitution rates (A-C: 1.06649, A-G: 2.5439, A-T: 1.46564, C-G: 1.18343, C-T: 3.99694, G-T: 1) using stepwise addition and 25× random addition heuristic searches with TBR. These values were then used for a Γ-corrected (0.5444) maximum likelihood (ML) analysis with pinvar (0.14153), using stepwise addition and a 10× random addition heuristic search. This analysis included 26 ingroup taxa and 6 outgroup taxa. The ML tree was found

10 of 10 times with a score of 15334.84. Bootstrap analysis was performed on all trees, with the respective initial model, on one hundred resampled datasets using stepwise addition and a 2× random addition heuristic search. All phylogenetic analyses were conducted in PAUP* version 4.0b (Swofford 1999).

Fluorescence in situ hybridization and confocal microscopy: A fluorescence *in situ* hybridization (FISH) oligonucleotide probe, Myr2, labeled with 5-N-N′-diethyl-tetramethylindo*di*carbocyanine (Cy5) (see Table 1 for sequence) for *M. rubra* and *M. pulex* was designed by eye from DNA alignments using MacClade. All probes were ordered from Qiagen, and tested using Primer Express 1.0 (Applied Biosystems) for possible complications due to secondary structure. A positive control probe (uniC) labeled with fluoroscein isothiocyante (FITC) was used, capable of labeling all eukaryotic SSU rRNA present in cells. Negative control probes included an anti-sense (reverse) probe of Myr2, called Myr2-neg (Cy5), as well as the anti-sense probe of the universal probe uniC, called uniR (FITC). Both uniC and uniR were designed by Scholin et al. (1996) (see also Miller and Scholin 1998). To preserve *M. rubra* and *M. pulex* cells for hybridization they were added to 4% paraformaldehyde with 5× SET (0.75 M NaCl, 5 mM EDTA, 0.1 M Tris-HCl, pH 7.8). Cells were fixed for 12–24 hr prior to hybridization. The FISH protocol was adopted from Miller and Scholin (1998). Preserved cells were gently filtered onto a 2.0 µm nucleopore filter, using a 5 µm backing filter, and washed twice with hybridization buffer (final concentration: 5× SET, 1% IGEPAL-CA630, 31.25 µg/ml polyadenylic acid). Cells were resuspended in 0.5 ml of hybridization buffer and 5 ng/µl of probe was added. All hybridizations were conducted at 45 ºC (determined empirically) in a water bath, for 1–2 h. After hybridization, cells were filtered onto a new membrane and washed several times with 45 ºC 5× SET. Cells were then resuspended in 45 ºC 5× SET and incubated for 2–3 minutes, after which the cells were filtered and resuspended in 1 ml fresh 5× SET buffer and stored at 4 ºC in the dark until used for microscopy (<3 h).

A Zeiss LSM 510 confocal system attached to a Zeiss inverted microscope, fitted with a C-Apochromat 63×/1.2 W lens, was used for viewing the FISH labeled cells. Cells were added to a slide chamber and at least 50 cells for each treatment were observed by optically sectioning through the cell. Single scan or Z-stack imaging analysis was preformed through several representative cells for capturing images. Images were captured using the multichannel option at three wavelength settings: 1) blue light (for FITC) excitation (ex) 488 nm, 2) far red (for Cy5) ex 633 nm, and 3) green (for phycoerythrin in chloroplasts) ex 543 nm. Emission filters for each channel were as follows: blue: band pass 505–560 nm, far-red: long pass (LP) 650nm, and green: LP 560 nm.

Genbank accession numbers: (U27500) *Alexandrium ostenfeldii*, (AF239260) *Amoebophrya* sp., (AF472555) *Amoebophrya* ex *Scrippsiella* sp., (AF274256) *Amphidinium semilunatum*, (AF283305) *Astasia longa*, (AF548006) *Babesia canis*, (AB049999) *Babesia rodhaini*, (AF029763) *Balantidium coli*, (AF317831) *Blepharisma americanum*, (33317834) *Bodo designis*, (33330170) *Cercomonas* sp., (AB062703) *Chaetomorpha moniligera*, (AB080308) *Chlorella vulgaris*, (CHU97109) *Coleps hirtus*, (M97908) *Colpoda inflata*, (AB022819) *Costaria costata*, (AJ007275) *Cyanoptyche gloeocystis*, (AF111184) *Cyclospora cercopitheci*, (L19080) *Cytauxzoon felis*, (AF488386) *Dasya sinicola*, (U57771) *Didinium nasutum*, (AB073117) *Dinophysis acuminata*, (4680238) *Diplonema papillatum*, (AF339490) *Eimeria dipodomysis*, (AF339492) *Eimeria peromysci*, (M87327) *Emiliania huxleyi*, (29466123) *Euglena pisciformis*, (AJ305255) *Euplotes crassus*, (U97110) *Frontonia vernalis*, (AY187925) *Geleia fossata*, (AF274258) *Gonyaulax cochlea*, (U17354) *Ichthyophthirius multifiliis*, (AF029762) *Isotricha prostoma*, (AF272046) *Karlodinium micrum* (= *Gyrodinium galatheanum*), (L31519) *Loxodes magnus*, (L26448) *Loxophyllum utricularіae*, (U73232) *Mallomonas striata*, (AF153206) *Mastigamoeba invertens*, (33309658) *Massisteria marina*, (AJ535164) *Nitzschia frustulum*, (AF022200) *Noctiluca scintillans*, (AF123294) *Ochromonas sphaerocystis*, (Y10570) *Odontella sinensis*, (AB033717) *Oxyrrhis marina*, (AF100314) *Paramecium bursaria*, (AJ310495) *Pattersoniella vitiphila*, (AB058362) *Pavlova lutheri*, (AY033488) *Pfiesteria piscicida*, (AJ277877) *Phacodinium metchnikoffi*, (U93235) *Plasmodium vivax*, (AF099183) *Polarella glacialis*, (AF342746) *Porphyra leucosticta*, (AJ421145) *Porphyridium aerugineum*, (PVU97111) *Prorodon viridis*, (AJ246269) *Prymnesium parvum*, (U53127) *Rhodomonas abbreviata* (nucleomorph), (U53128) *Rhodomonas abbreviata*, (AF176940) *Sarcocystis hirsuta*, (AJ428106) *Staurastrum lunatum*, (AF357913) *Stentor roeseli*, (AH009986) *Sarcocystis neurona*, (AF462060) *Skeletonema pseudocostatum*, (33309650) *Sponogomonas* sp., (U97112) *Strombidium purpureum*, (AJ511862) *Tetrahymena* sp., (L02366) *Theileria parva*, (L31520) *Trachelo-raphis* sp., (38304358) *Trypanosoma avium*, (AF290065) uncultured marine eukaryote DH145-EKD11, (27802617) Uncultured eukaryote clone

CCW100, (27802608) Uncultured eukaryote clone CCW75, (AY331783) Uncultured marine eukaryote clone m110, (AY331778) Uncultured marine eukaryote clone m43, (AY331777) Uncultured marine eukaryote clone m112.

Acknowledgements

We thank Tsetso Bachvaroff, Holly Bowers, Robert Brown, D. Wayne Coats, Dan Gustafson, Scott Heyward, and Wayne Litaker for their assistance and advice during this project. We would especially like to thank Chris Scholin for his generous guidance with our *in situ* hybridization probe work. This research was funded by NSF award IBN-0131847. UMCES contribution 3783.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215**: 403–410

Bernhard D, Schlegel M (1998) Evolution of histone H4 and H3 genes I different ciliate lineages. J Mol Evol **46**: 344–354

Darwin C (1839) Journal of researches into the geology and natural history of the various countries visited by the HMS Beagle under the command of Captain Fitzroy, R.N. from 1832–1836. 1st edition. Henry Colburn, London

Dawson SC, Pace NR (2002) Novel kingdom-level eukaryotic diversity in anoxic environments. Proc Natl Acad Sci USA **99**: 8324–8329

Dolan JR, Coats DW (1991) A study of feeding in predacious ciliates using prey ciliates labeled with fluorescent microspheres. J Plankton Res **13**: 609–627

Foissner W, Berger H, Schaumburg J (1999) Identification of limnetic planktonic ciliates. Informationsberichte des Bayer. Landesamtes für Wasserwirtschaft, Heft 3/99

Guillard RRL (1975) Culture of Phytoplankton for Feeding Marine Invertebrates. In Smith WL, Chanley MH (eds) Culture of Marine Invertebrate Animals. Plenum Publishing Corp, New York, pp 29–60

Gustafson Jr DE, Stoecker DK, Johnson MD, Van Heukelem WF, Sneider K (2000) Cryptophyte algae are robbed of their organelles by the marine ciliate *Mesodinium rubrum*. Nature **405**: 1049–1052

Hibberd, DJ (1977) Observations on the ultrastructure of the cryptomonad endosymbiont of the red water ciliate *Mesodinium rubrum*. J Mar Biol Assoc UK **57**: 45–61

Jankowski AW (1976) Revision of the Classification of the Cyrtophorids. In Markevich AP, Yu I (eds) Materials of the II All-Union Conference of Protozoology, Part I, General Protozoology, Naukova Dumka, pp 167–168

Jiménez R, Intriago P (1987) Observations on blooms of *Mesodinium rubrum* in the upwelling area off Ecuador. Oceanol Acta Special issue No **6**: 145–154

Katz LA, Bornstein JG, Lasek-Nesselquist E, Muse SV (2004) Dramatic diversity of ciliate histone H4 genes revealed by comparisons of patterns of substitutions and paralog divergences among eukaryotes. Mol Biol Evol **21**: 555–562

Krainer KH, Foissner W (1990) Revision of the genus *Askenasia* Blochmann, 1895, with proposal of two new species, and description of *Rhabdoaskenasia minima* N G, N Sp (Ciliophora, Cyclotrichida). J Eukaryot Microbiol **37**: 414–427

Leander BS, Kuvardina ON, Aleshin VV, Mylnikov AP, Keeling PJ (2003) Molecular phylogeny and surphase morphology of *Copodella edax* (Alveolata): Insights into the phagotrophic ancestral of apicomplexans. J Eukaryot Microbiol **50**: 334–340

Lee SY (2001) Unalignable sequences and molecular evolution. Trends Ecol Evol **16**: 681–687

Lindholm T, Lindroos P, Mörk AC (1988) Ultrastructure of the photosynthetic ciliate *Mesodinium rubrum*. BioSystems **21**: 141–149

Lindholm T, Lindroos P, Mörk AC (1990) Depth maxima of *Mesodinium rubrum* (Lohmann) Hamburger and Buddenbrock- Examples from a stratified Baltic Sea inlet. Sarsia **75**: 53–64

López-Garcia P, Rodríguez-Valera F, Pedrós-Alió C, Moreira D (2001) Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature **409**: 371–656

Lynn DH (1991) The implications of recent descriptions of kinetid structure to the systematics of the ciliated protists. Protoplasma **164**: 123–142

Lynn DH, Small EB (2002) Phylum Ciliophora. In Lee JJ, Leedale GF, Bradbury P (eds) An Illustrated Guide to the Protozoa. Society of Protozoologists, Lawrence, pp 477–478

Maddison WP, Maddison DR (1992) MacClade-Analysis of Phylogeny and Character Evolution. Sinauer, Sunderland, MA

Medlin L, Elwood HJ, Stickel S, Sogin ML (1988) The characterization of enzymatically amplified eukaryotic 16S-like rRNA-coding regions. Gene **71**: 491–499

Miller PE, Scholin CA (1998) Identification and enumeration of cultured and wild *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. J Phycol **34**: 371–382

Moreira D, López-Garcia P (2002) The molecular ecology of microbial eukaryotes unveils a hidden world. Trends Microbiol **10**: 31–38

Moreira D, Guyader HL, Philippe H (1999) Unusually high evolutionary rate of the elongation factor 1 genes from the Ciliophora and its impact on the phylogeny of eukaryotes. Mol Biol Evol **16**: 234–245

Oakley BR, Taylor FJR (1978) Evidence for a new type of endosymbiotic organization in a population of the ciliate *Mesodinium rubrum* from British Columbia. BioSystems **10**: 361–369

Oldach DW, Delwiche CF, Jakobsen KS, Tengs T, Brown, EG, Kempton JW, Schaefer EF, Bowers HA, Glasgow Jr HB, Burkholder JM, Steidinger KA, Rublee PA (2000) Heterodouplex mobility assay-guided sequence discovery: elucidation of the small subunit (18s) rDNA sequences of *Pfiesteria piscicida* and related dinoflagellates from complex algal cultures and environmental sample DNA pools. Proc Natl Acad Sci USA **97**: 4303–4308

Petroni G, Rosati G, Vannini C, Modeo L, Dini F, Verni F (2003) In situ identification by fluorescently labeled oligonucleotide probes of morphologically similar, closely related ciliate species. Microbiol Ecol **45**: 156–162

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinformatics **14**: 817–818

Ryther JH (1967) Occurrence of red water off Peru. Nature **214**: 1318–1319

Saldarriaga JF, McEwan ML, Fast NM, Taylor FJR, Keeling PJ (2003). Multiple protein phylogenies show that *Oxyrrhis marina* and *Perkinsus marinus* are early branches of the dinoflagellate lineage. Int J Syst Evol Microbiol **53**: 355–365

Savin MC, Martin JL, LeGresley M, Giewat M, Rooney-Varga J (in press) Planktonic diversity in the Bay of Fundy as measured by morphological and molecular methods. Microbiol Ecol

Scholin CA, Buck KR, Britschgi T, Cangelosi, Chavez FP (1996) Identification of *Pseudo-nitzschia australis* (Bacillariophyceae) using rRNA-targeted probes in whole cell and sandwich hybridization formats. Phycologia **35**: 190–197

Snoeyenbos-West OL, Salcedo T, McManus GB, Katz LA (2002) Insights into the diversity of choreotrich and oligotrich ciliates (Class: Spirotrichea) based on genealogical analyses of multiple loci. Int J Syst Evol Microbiol **52**: 1901–1913

Stiller JW, Hall BD (1999) Long-branch attraction and the rDNA model of early eukaryotic evolution. Mol Biol Evol **16**: 1270–1279

Stoeck T, Epstein S (2003) Novel eukaryotic lineages inferred from small-subunit rRNA analysis of oxygen-depleted marine environments. Appl Eviron Microbiol **69**: 2657–2663

Strüder-Kypke MC, Lynn DH (2003) Sequence analysis of the small subunit rRNA gene conform the paraphyly of oligotrich ciliates sensu lato and support the monophyly of the subclass Oligotrichia and Choreotrichia (Ciliophora, Spirotrichea). J Zool **260**: 87–97

Swofford DL (1999) PAUP*: Phylogenetic Analysis Using Parsimony. Sinauer, Sunderland, MA

Taylor FJR, Blackbourn DJ, Blackbourn J (1969) Ultrastructure of the chloroplasts and associated structures within the marine ciliate *Mesodinium rubrum* (Lohmann). Nature **224**: 819–821

Taylor FJR, Blackbourn DJ, Blackbourn J (1971) The red-water ciliate *Mesodinium rubrum* and its "incomplete symbionts": a review including new ultrastructural observations. J Fish Res Board Canada **28**: 391–407

Tengs T, Dahlberg OJ, Shalchian-Tabrizi K, Klaveness D, Rudi K, Delwiche C, Jakobsen KS (2000) Phylogenetic analyses indicate that the 19'Hexanoyloxy-fucoxanthin-containing dinoflagellates have tertiary plastids of haptophyte origin. Mol Biol Evol **17**: 718–729

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res **25**: 4876–4882

Van de Peer Y, De Wachter R (1997) Evolutionary relationships among the eukaryotic crown taxa taking into account site-to-site rate variation in 18s rRNA. J Mol Evol **45**: 619–630

Villalobo E, Perez-Romero P, Sanchez-Silva R, Torres A (2001) Unusual characteristics of ciliate actins. Int Microbiol **4**: 167–174

Wright A-DG, Dehority BA, Lynn DH (1997) Phylogeny of the rumen ciliates *Entodinium*, *Epidinium* and *Polyplastron* (Litostomatea: Entodiniomorphida) inferred from small subunit ribosomal RNA sequences. J Eukaryot Microbiol **44**: 61–67

Wuyts J, De Rijk P, Van de Peer Y, Pison G, Rousseeuw P, De Wachter R (2000) Comparative analysis of more than 3000 sequences reveals the existence of pseudoknots in area V4 of eukaryotic small subunit ribosomal RNA. Nucleic Acids Res **28**: 4698–4708