J. Phycol. **38,** 1236–1244 (2002)

CRYPTOMONAD EVOLUTION: NUCLEAR 18S rDNA PHYLOGENY VERSUS CELL MORPHOLOGY AND PIGMENTATION[1]

James A. Deane, Isabelle M. Strachan, Gary W. Saunders,[2] David R. A. Hill, and Geoffrey I. McFadden[3]

School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia

A nuclear18S rDNA phylogeny for cryptomonad algae is presented, including 11 species yet to be investigated by molecular means. The phylogenetic positions of the cryptomonad genera *Campylomonas* and *Plagioselmis* are assessed for the first time. *Campylomonas* groups most closely with morphologically similar species with the same accessory pigment from the genus *Cryptomonas*. *Plagioselmis* groups with the genera *Teleaulax* and *Geminigera* forming a clade whose members are united by unusual thylakoid arrangement. Nuclear 18S rDNA phylogeny divides cryptomonads into seven major lineages, two of which consist of the monospecific genera *Proteomonas* and *Falcomonas*. Analysis of nuclear18S rDNA sequence supports suggestions that a *Falcomonas*-like cryptomonad gave rise to all other blue-green cryptomonads. New sequence from the plastid-lacking cryptomonad genus *Goniomonas* is also included, and the order of divergence of the major cryptomonad lineages is discussed. The morphology, number, and pigmentation of the cryptomonad plastidial complex are congruent with nuclear 18S rDNA phylogenies. Host cell features, such as periplast type, furrow/gullet system, and cell shape, can be more variable and may be markedly different in species that are closely related by nuclear 18S rDNA phylogeny. Conversely, some species that are not closely related by molecular phylogeny may display a very similar, possibly primitive, periplast and furrow morphology.

Key index words: **cryptomonad; evolution; furrow; gullet; molecular phylogeny; periplast; plastid**

Plastid-containing cryptomonads (Class Cryptophyceae Clay et al.) are a group of freshwater and marine unicellular algae easily recognized by their distinctive furrow/gullet system and unique plastidial complex. Cryptomonad algae are a chimera of two eukaryotic organisms, a motile host cell, and a photosynthetic endosymbiont of red algal origin referred to as the plastidial complex (Douglas et al. 1991, McFadden et al. 1994a). The cryptomonad endosymbiont contains a highly reduced nucleus known as the nucleomorph. Traditionally, cryptomonads have been classified on the basis of phycobilin accessory pigment content, size, and shape (Butcher 1967, Bourelly 1970), as well as internal organization and cell surface (periplast) detail (Novarino and Lucas 1993). Recently, DNA sequence data have been used to test hypotheses concerning cryptomonad evolution. Molecular phylogenies constructed from cryptomonad nuclear (host cell nucleus) 18S rDNA genes suggest that plastid-lacking flagellates from the genus *Goniomonas* are basal cryptomonads that diverged before the cryptomonad plastidial complex was acquired (McFadden et al. 1994b, Marin et al. 1998). However, the freshwater species *Goniomonas truncata* is presently the only representative of its genus for which 18S rDNA sequence has been determined. The most detailed phylogenies to date demonstrate the existence of a number of well-supported cryptomonad lineages and suggest that a clade consisting of freshwater species with two plastids per cell (*Cryptomonas* and *Chilomonas*) is the earliest diverging lineage among plastid-containing cryptomonads (Marin et al. 1998, Clay and Kugrens 1999). The findings of these molecular studies are incorporated into a revised classification scheme for cryptomonads by Clay et al. (1999).

We expand on previous molecular studies of cryptomonad phylogeny by including 10 new 18S rDNA sequences for species representing a broad range of cryptomonad morphological and phycobilin pigment diversity. The phylogenetic positions of the cryptomonad genera *Campylomonas* and *Plagioselmis* are determined for the first time, and the status of several other cryptomonad genera is investigated more thoroughly. We also consider sequence from the plastid-lacking marine species *Goniomonas pacifica* to ensure better outgroup representation for plastid-containing species. The branching order of the major cryptomonad lineages and the evolution of cryptomonad morphology are discussed.

MATERIALS AND METHODS

Strains for which nuclear18S rDNA sequence were determined are shown in Table 1. *Storeatula* sp. was collected from Parakeet Bay, Rottnest Island, Western Australia by Ross Waller. *Goniomonas pacifica* (Larsen and Patterson 1990) and *Rhinomonas* sp. were isolated from Port Phillip Bay, Melbourne, Victoria, Australia. Photosynthetic cryptomonad species were grown in "K" marine medium (Keller 1987) or diatom medium. *Goniomonas pacifica* was grown in filter sterilized seawater supplemented with barley grains.

Cryptomonad cells were harvested by centrifugation and lysed in 50–100 μL 1.0 M Tris base, pH 8.0, 0.05 M EDTA, 0.2 M NaCl, 1% SDS, and 0.2 $\mu g \cdot mL^{-1}$ Proteinase K. Samples were incubated at 60° C for 1.5 h and centrifuged at 1400 rpm in a microfuge to pellet cellular debris. A 1/9 volume of 5 M potassium acetate was added to samples; these sample were then chilled on ice for 15 min and centrifuged for 15 min at 1400 rpm. The supernatant was extracted twice with phenol/

[1]Received 12 December 2001. Accepted 27 August 2002.

[2]Present address: University of New Brunswick, Department of Biology, Fredericton, NB E3B 5A3, Canada.

[3]Author for correspondence: e-mail gim@unimelb.edu.au.

chloroform (1:1) and twice with chloroform. DNA was precipitated by the addition of two volumes of ethanol, chilled on ice for 5 min, and centrifuged for 5 min at 1400 rpm. DNA was washed with 70% ethanol and air dried. For cryptomonads other than *Storeatula* sp. and *Goniomonas pacifica*, nuclear 18S rDNA genes were PCR amplified from genomic DNA as two subfragments using the universal 18S rDNA primers G01 and G07 (Saunders and Kraft 1994) in conjunction with internal primers C-1 (5′-TGGAGTCGCAAATTGACATCC-3′), C-A (5′-TGA GGTCCTAAATTGACACTC-3′), and C-2 (5′-TGTTCAAAGCAG GCGTACGC-3′) designed to specifically amplify the host nuclear 18S gene. PCR products were purified using Wizard™ PCR Preps (Promega, Madison, WI, USA) and sequenced using 18S rDNA primers G01, G02, G03, G06, G08, G10, G11, and G16 (Saunders and Kraft 1994). *Storeatula* sp. and *Goniomonas pacifica* 18S rDNA was amplified in a single piece using the general 18S rDNA primers FAD3 (5′-TACCTGGTGGATCCTGC CAG-3′) and FAD4 (5′-TGATCCTCCTGCAGGTTCACCTAC-3′). These products were cloned into pGEM T-vector (Promega) and sequenced using vector primers and 18S primers (as for other species). All sequencing was done using dye-labeled dideoxy terminators and an ABI 373a Automated Sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were edited using Sequencher 3.0 (Gene Codes, Ann Arbor, MI, USA).

Nuclear 18S rDNA sequences obtained (Table 1) were aligned with cryptomonad and outgroup species sequences obtained from GenBank. Details of cryptomonad and glaucocystophyte sequences obtained from GenBank are as in Marin et al. (1998) and Clay and Kugrens (1999). Two data sets were used to construct the phylogenies presented here. One consisted of cryptomonads and selected glaucocystophyte sequence (1740 alignable positions) and the other of just cryptomonad sequences with the exclusion of the highly divergent sequence from *Goniomonas truncata* (1752 alignable positions). To check the choice of the glaucocystophytes as an outgroup to cryptomonads, analyses were also conducted on data sets including sequence from chromophytes, dinoflagellates, haptophytes, and an apicomplexan. Clustal X (Thompson et al. 1994) was used for an initial alignment of sequences that was then refined manually using SeqApp (Gilbert 1992). A number of unique inserts in the *Goniomonas truncata* 18S rDNA sequence were omitted. Phylogenetic trees were constructed using Phylip 3.5 (Felsenstein 1993), FastDNAml 1.0.6 (Olsen et al. 1994), and PAUP 3.1.1 (Swofford 1993). Neighbor-joining distance analysis used the Kimura two-parameter model of nucleotide change. Maximum parsimony analysis used the heuristic search option and branch swapping. Fast DNAml used jumbled addition of sequences and the global search option. Canvas 7.0.2 (Deneba Systems, Inc., Miami, FL, USA) was used to generate Figure 3.

RESULTS

Two new cryptomonad isolates (Table 1) were designated *Rhinomonas* sp. and *Storeatula* sp., respectively, on the basis of their cell morphology as observed by SEM and LM and pigment content (data not shown). Both strains contain the expected cryptomonad phycoerythrin with an absorption maximum of 545 nm (CrPE 545) and conform to the respective external morphological descriptions of *Rhinomonas* (Hill and Wetherbee 1988) and *Storeatula* (Hill 1991b, Kugrens et al. 1999).

Plastid containing cryptomonads yielded a nuclear 18S rDNA PCR product of about 1750 bp. *Goniomonas pacifica* gave a single product of 1764 bp, smaller than the 1987-bp 18S rRNA gene amplified from *Goniomonas truncata* by McFadden et al. (1994b). The extra length of the *G. truncata* gene is due to a number of unique inserts not shared by *G. pacifica* or any other species investigated. These inserts were not included in analyses.

Table 1. Species used, strain source, and accession numbers.

Species	Strain source	GenBank
Campylomonas reflexa	CCMP 152, MUCC 067	AF508267
Chroomonas mesostigmatica	CCMP 1168	AF508268
Chroomonas nordstedtii	MUCC 231	AF508269
Cryptomonas ovata	UTEX 358	AF508270
Cryptomonas platyuris	MUCC 073	AF508271
Plagioselmis prolonga	MUCC 012	AF508272
Rhinomonas sp.	Undesignated	AF508273
Rhodomonas maculata	MUCC 053	AF508274
Teleaulax acuta	MUCC 088	AF508275
Storeatula sp.	CCMP1868	AF508276
Goniomonas pacifica	CCMP1869	AF508277

CCMP, Provasoli-Guillard Center for Culture of Marine Phytoplankton; MUCC, Melbourne University Culture Collection; UTEX, University of Texas.

Cryptomonads are possibly part of a lineage that includes heterokont algae, haptophytes, dinoflagellates, and apicomplexans (Fast et al. 2001), and it has also been suggested that cryptomonads are most closely related to glaucocystophytes (Bhattacharya et al. 1995). Phylogenies constructed from a data set including cryptomonads and representatives of all possible related groups listed above, using neighbor-joining distance methods and maximum parsimony methods (data not shown), show cryptomonads forming a clade with glaucocystophytes (as shown by Bhattacharya et al. 1995). Tree topology within the cryptomonad lineage is essentially the same as for a cryptomonad + glaucocystophyte data set (Fig. 1). Thus, for the purposes of the present study, and in the interest of keeping the data set small enough to allow maximum likelihood analysis, glaucocystophytes were selected as the most appropriate outgroup for cryptomonads.

In nuclear 18S rDNA phylogenies constructed from the cryptomonad + glaucocystophyte data set, *G. pacifica* and *G. truncata* branch below a clade that exclusively contains all plastid-containing cryptomonads considered (Fig. 1). The 18S rDNA sequence of *G. pacifica* is more similar to that of plastid-containing cryptomonads than that of the previously investigated *G. truncata*, and because of this bootstrap support for *G. truncata* and *G. pacifica* forming a clade is moderate (Fig. 1). Because *G. pacifica* 18S rDNA sequence is close to that of photosynthetic cryptomonads and more alignable than that of *G. truncata*, phylogenies were created for plastid-containing cryptomonads using only *G. pacifica* as an outgroup (Fig. 2).

Nuclear 18S rDNA phylogeny divides most plastid-containing cryptomonads into five well-supported clades (A–E, Figs. 1 and 2). *Proteomonas sulcata* and *Falcomonas daucoides* are not included in the clades outlined above. There is moderate support for the placement of *F. daucoides* at the base of clade D (Figs. 1 and 2). The position of *P. sulcata* is unclear (Figs. 1 and 2).

The relative branching order of clades A–E is not well resolved. Unlike previous nuclear 18S rDNA studies (Marin et al. 1998, Clay and Kugrens 1999), there

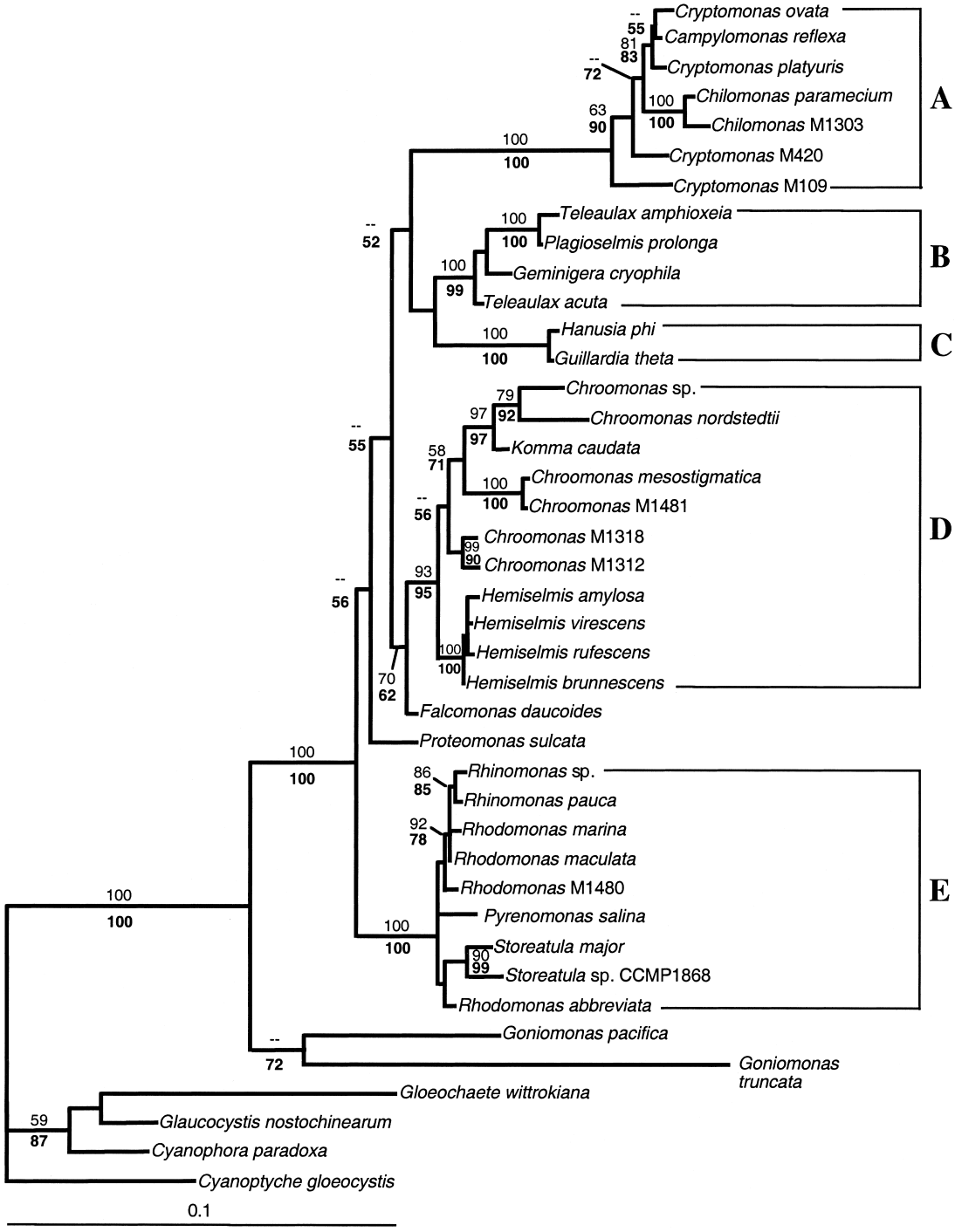

FIG. 1. A phylogeny of cryptomonad 18S rDNA sequences constructed from a cryptomonad + glaucocystophyte data set using the maximum likelihood method. Numbers at nodes are bootstrap support percentages from 1000 replicates using maximum parsimony (lower, bold) and neighbor-joining distance (upper, roman) methods. Where bootstrap support was less than 50%, no numbers are shown. Highly supported clades within plastid-containing cryptomonads are labeled A–E.

is no clear indication that clade A is the earliest diverging lineage among plastid-containing cryptomonads. This difference is attributable to the inclusion of sequence from *G. pacifica*. The removal of *G. pacifica* sequence from the data set gives trees that support group A as the earliest diverging plastid-containing cryptomonad lineage (data not shown) as seen in previous studies.

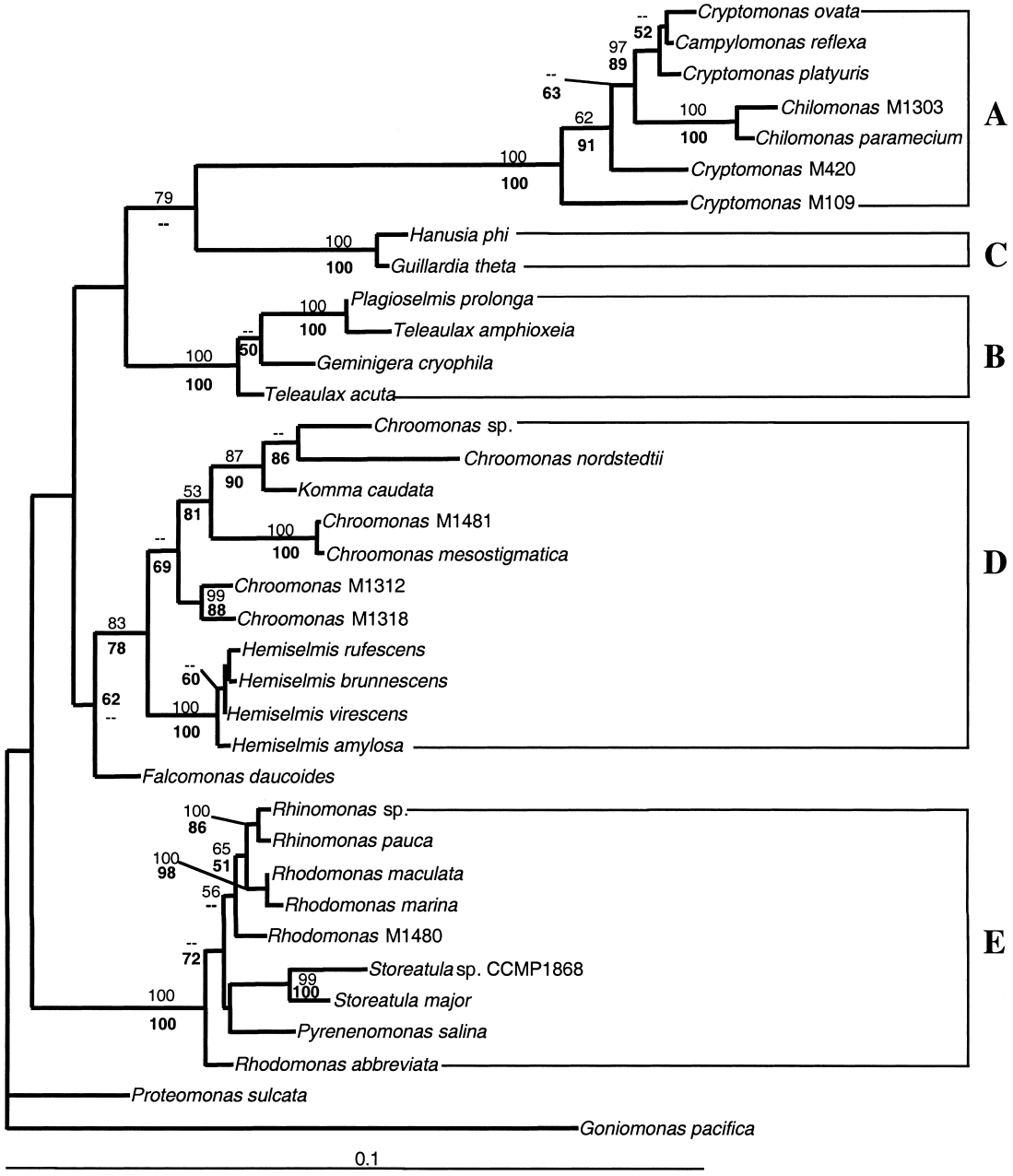

FIG. 2. A phylogeny of cryptomonad 18S rDNA sequences constructed from a cryptomonad data set exclusive of *Goniomonas truncata* using the maximum likelihood method. Numbers at nodes are bootstrap support percentages from 1000 replicates using maximum parsimony (upper, bold) and neighbor-joining distance (lower, roman) methods. Where bootstrap support was less than 50%, no numbers are shown. Highly supported clades within plastid-containing cryptomonads are labeled A–E.

DISCUSSION

Goniomonas *and its relationship to plastid-containing cryptomonads.* Phylogenetic reconstruction using nuclear 18S rDNA sequence shows that cryptomonads with plastids represent a monophyletic group and that the closest known relatives of the cryptomonad host cell are plastid-lacking heterotrophic flagellates from the genus *Goniomonas.* Previous studies also demonstrated the monophyly of plastid-containing cryptomonads using *G. truncata* as the solitary representative of the genus *Goniomonas.* In select analyses *Goniomonas* was sometimes found to branch with *Chilomonas* within the radiation of plastid-containing cryptomonads (Cavalier-Smith et al. 1996). Cavalier-Smith et al. (1996) proposed that *Goniomonas* might be secondarily lacking an endosymbiont on the basis of its grouping with *Chilomonas.* They also cautioned that the grouping of *G. truncata* and *Chilomonas paramecium* could be a long-branch-attraction artifact. We have added *G. pacifica* sequence to the data set and show that the plastid-lacking genus *Goniomonas* is clearly a sister group to plastid-containing cryptomonads and there-

fore unlikely to have secondarily lost a plastid. The large amount of sequence divergence between *G. pacifica* and *G. truncata* is somewhat surprising given their nearly identical morphology. In fact, the major feature that distinguishes these two species is that *G. truncata* frequents freshwater whereas *G. pacifica* is a marine species (Larsen and Patterson 1990). The *G. truncata* 18S rDNA gene contains a number of unique inserts and appears to be evolving very rapidly in comparison with the other 18S rDNA genes considered in this study. The less divergent *G. pacifica* 18S rDNA sequence is likely to give a more accurate indication of the true root for plastid-containing cryptomonads in nuclear 18S rDNA phylogenies.

Plastid-containing cryptomonad lineages. Within plastid-containing cryptomonads, the seven major lineages (Figs. 1 and 2, clades A–E, *Falcomonas daucoides* and *Proteomonas sulcata*) identified in two previous studies of cryptomonad 18S rDNA sequence (Marin et al. 1998, Clay and Kugrens 1999) are corroborated. These lineages and features of their members are discussed below. The cryptomonad cell surface (periplast) is an extraordinarily complex and variable structure that is only discussed here in terms of its gross morphology (for more detail, see Brett et al. 1994, Clay et al. 1999). Cryptomonad genera investigated are represented diagrammatically in Figure 3 to aid comparison and to provide a summary of diagnostic features for each genus.

Clade A: This clade (Fig. 3A) contains exclusively freshwater species with two plastidial complexes per cell, a medium length furrow, and a gullet. All other cryptomonads considered are believed to have only a single plastidial complex except immediately before cell division. Pigmented members of clade A, *Campylomonas reflexa* and *Cryptomonas*, contain the accessory pigment cryptomonad phycoerythrin (CrPE) 566 (Hill and Rowan 1989), whereas *Chilomonas*, a colorless genus, has two plastids lacking pigment (leucoplasts) per cell that appear to have evolved through pigment loss (Kugrens and Lee 1991). *Campylomonas reflexa* has a distinctive recurved cell posterior that is a diagnostic feature of this species (Hill 1991b). Hill (1991b) noted that the periplast, furrow/gullet, and flagellar apparatus of *C. reflexa* is very similar to that of *Chilomonas paramecium*. *Campylomonas reflexa* and *Chilomonas paramecium* also differ from *Cryptomonas ovata* (the type species for the genus *Cryptomonas*) in the ultrastructure of their periplast, furrow/gullet, and flagellar apparatus (Roberts et al. 1981, Roberts 1984, Kugrens et al. 1986, Kugrens and Lee 1987, Hill 1991b, Kugrens and Lee 1991). Thus, based on morphology, *Campylomonas* is an obvious candidate for the photosynthetic cryptomonad that gave rise to *Chilomonas* through pigment loss. Surprisingly, our nuclear 18S rDNA phylogenies show that *Campylomonas reflexa* is more closely related to *Cryptomonas ovata* than to *Chilomonas paramecium*. *Cryptomonas platyuris*, although assigned to *Cryptomonas*, is actually morphologically similar to *Campylomonas reflexa* and *Chilomonas* with regard to it periplast and furrow/gullet (Kugrens et al. 1986) but branches in a clade that includes *Cryptomonas ovata* and not *Chilomonas* species. It would be informative to know more of the morphology of the two undescribed strains of *Cryptomonas* (M1303 and M109) that branch at the base of clade A. These data may help determine whether the primitive cell morphology in clade A is of the type displayed by *Cryptomonas ovata* or the type displayed by *Campylomonas reflexa*. This in turn would show whether the similarities between *Campylomonas reflexa* and *Chilomonas paramecium* indicate a close relationship between these species or are merely primitive traits within clade A.

Clade B: This is a morphologically diverse clade (Fig. 3B) consisting of genera with CrPE 545, a furrow, and no gullet. *Plagioselmis prolonga* is the first member of its genus for which 18S rDNA sequence has been determined. Clay and Kugrens (1999) expressed the hope that molecular investigation of *Plagioselmis* species might help fill a gap in the cryptomonad phylogenetic tree and provide greater resolution. However, *P. prolonga* is closely related to *Teleaulax* species and *Geminigera cryophila* and not helpful in this respect. Surprisingly, *Teleaulax amphioxeia* is more closely related to *P. prolonga* than to *T. acuta*, a morphologically similar member of the same genus. This may indicate that the morphological features used to define *Teleaulax* represent a primitive state for clade B and are therefore phylogenetically uninformative within this clade.

One possible unique derived character of clade B genera is a modified thylakoid membrane arrangement. *Teleaulax* and *P. prolonga* have thylakoids arranged in loose groups of three, whereas *G. cryophila* has disorganized stacks consisting of a variable number of thylakoid membranes (Hill 1988, 1991b). In keeping with this theme, a disorganized thylakoid arrangement has also been reported for the freshwater cryptomonad *Rhodomonas lacustris* (Klaveness 1981). The overall cellular morphology of this species suggests that it should be assigned to the genus *Plagioselmis* (Novarino et al. 1994).

Clade C: This clade (Fig. 3C) consists of *Guillardia theta* and *Hanusia phi*, two species very closely related by nuclear 18S rDNA sequence. Nucleomorph (endosymbiont nucleus) 18S rDNA phylogeny and nucleomorph karyotype data from previous studies also suggest that these two species are very close relatives (Cavalier-Smith et al. 1996, Deane et al. 1998). Despite their apparent relatedness, *G. theta* and *H. phi* are morphologically dissimilar. *Guillardia theta* has a longitudinal periplast plates, a gullet, and no furrow (Hill and Wetherbee 1990), whereas *H. phi* lacks periplast plates, has a furrow, and lacks a gullet (Deane et al. 1998). Both species have a plastid that contains CrPE 545. We note that *H. phi* has appeared in previous studies as "*Cryptomonas* sp. Φ" (Gillott and Gibbs 1983) or "unidentified cryptomonad CCMP 325" (Cavalier-Smith et al. 1996, Marin et al. 1998) but has since been formally described (Deane et al. 1998).

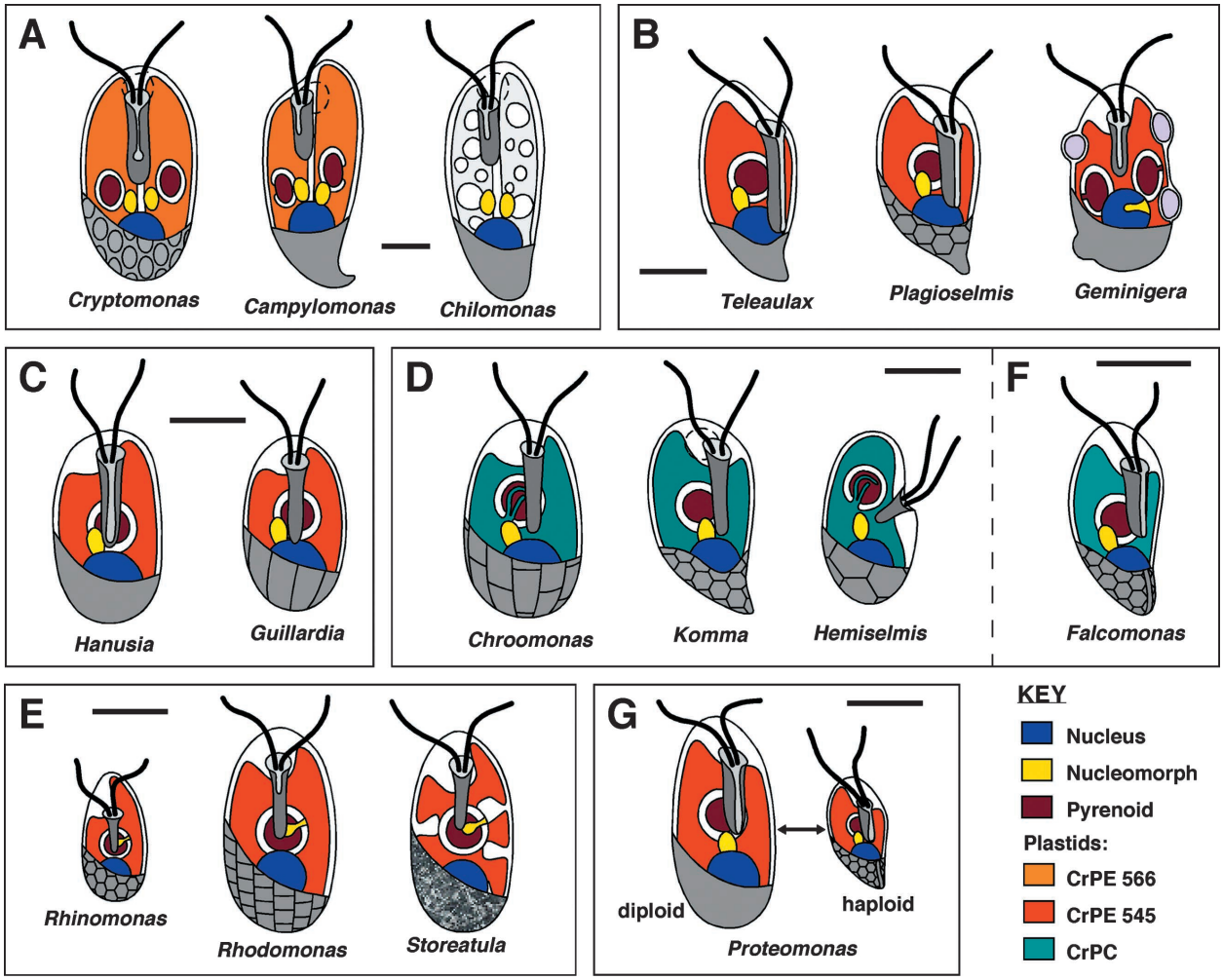

FIG. 3. Diagrammatic representations of the plastid-containing cryptomonad genera considered in this study. A–E correspond to clades A–E from Figures 1 and 2. Contractile vacuoles are depicted in freshwater genera with a broken line. The white space surrounding pyrenoids represents starch deposits. The leucoplasts of *Chilomonas* are shown in light gray with white starch granules (A). *Geminigera* (B) contains lipid accumulations (light purple). Thylakoids are depicted in the pyrenoid of *Chroomonas* and *Hemiselmis* (D). *Hemiselmis* is shown with a plastid containing cryptomonad phycocyanin (D), although some *Hemiselmis* species have a plastid containing a derived form of cryptomonad phycoerythrin. *Storeatula* has a coarse fibrous exterior periplast component (E). *Proteomonas* alternates between the haplomorph and diplomorph forms shown in G. Scale bars, 5 μm.

Clade D: This clade (Fig. 3D) consists predominantly of blue-green cryptomonads containing cryptomonad phycocyanin (CrPC). Genera in clade D have a gullet, no furrow, and periplast plates. With the exception of *Komma caudata*, clade D species have thylakoid membranes traversing the pyrenoid (Dodge 1969, Hill 1991a, Erata et al. 1995, Clay and Kugrens 1999). Eye spots have been described in some members of clade D such as *Hemiselmis amylosa* (Clay and Kugrens 1999) and *Chroomonas mesostigmatica* (Dodge 1969), but this feature is not restricted to any particular subclade within clade D. *Hemiselmis* species form a well-supported monophyletic lineage within clade D whose members are distinguished by their kidney-shaped cell morphology, lateral insertion of flagella, and large hexagonal periplast plates (Wetherbee et al. 1986, Clay and Kugrens 1999). *Hemiselmis* species are either blue-green due to CrPC 615, as in *H. virescens* and *H. amylosa*, or brown in color as in *H. brunnescens* and *H. rufescens*, a situation apparently resulting from the modification of blue-green CrPC 615 to brown CrPE 555 (Hill and Rowan 1989, Marin et al. 1998, Clay and Kugrens 1999). The small amount of sequence divergence between *Hemiselmis* species means that nuclear 18S rDNA phylogenies do not resolve relationships between the various members of this genus. The other lineage in clade D is more diverse in 18S rDNA sequence and consists of five *Chroomonas* species and *Komma caudata*, the single described representative of the genus *Komma*. *Chroomonas* species have a round or barrel-shaped cell outline, subapically inserted flagella, and offset rectangular periplast plates (Gantt 1971, Kugrens and Lee 1987, Hill 1991a) and are only known to possess blue-green

CrPC (Hill and Rowan 1989, Hill 1991a, Erata et al. 1995). *Komma caudata* is distinguished from *Chroomonas* by virtue of its small hexagonal periplast plates, elongate cell posterior, and the lack of thylakoid membranes traversing the pyrenoid (Hill 1991a). The branching position of *K. caudata* within the *Chroomonas* lineage suggests that *K. caudata* represents a nontypical modification of the basic *Chroomonas* cell type. 18S rDNA data shows that the closest well-characterized relative of *K. caudata* is *Chroomonas nordstedtii*, a species that has a cell morphology typical of *Chroomonas* (Erata et al. 1995). The *Chroomonas/Komma* lineage is divided into two sister clades, each of which has a characteristic type of CrPC. *Chroomonas* M1318 and *Chroomonas* M1312 contain CrPC 630 (Marin et al. 1998) and form a sister group to other *Chroomonas* species and *K. caudata* that contain CrPC 645 (Hill and Rowan 1989, Erata et al. 1995).

Clade E: A unique feature of this clade (Fig. 3E) is a nucleomorph embedded in the pyrenoid. All investigated species with nucleomorphs embedded in the pyrenoid have a plastid containing CrPE 545 (Hill and Rowan 1989). *Rhinomonas* species are small (6–10 μm in length), have hexagonal periplast plates and a rhinote (highly prominent) anterior, and completely lack a flagellar furrow (Hill and Wetherbee 1988). This allows *Rhinomonas* to be distinguished from *Rhodomonas* (= *Pyrenomonas*), whose members are larger (15–20 μm) and have rectangular periplast plates, a rounder anterior, and a small flagellar furrow (Hill and Wetherbee 1989, Kugrens et al. 1999). *Storeatula* is characterized by the lack of a furrow, a periplast that lacks plates and has a coarse fibrillar exterior, and a multilobed plastid (Hill 1991b). In a study that included single representatives of *Rhinomonas* and *Storeatula*, Marin et al. (1998) noted that these two genera are very similar to each other and *Rhodomonas* species in nuclear 18S rDNA sequence and suggested that the importance of morphological features used to define *Rhinomonas* and *Storeatula* may have been overestimated. We have obtained two additional strains that conform to the description of *Rhinomonas* (*Rhinomonas* sp.) and *Storeatula* (*Storeatula* sp.). Although these strains have not been formally described to the species level, nuclear 18S rDNA phylogeny confirms they are new taxa distinct from *Rhinomonas pauca* and *Storeatula major*. Phylogenies also show that *Rhinomonas* sp. and *Rhinomonas pauca* branch together with strong support, as do *Storeatula* sp. and *Storeatula major*. Thus, 18S rDNA phylogeny validates the morphological features used by Hill (1991b) to define the genera *Rhinomonas* and *Storeatula*. Kugrens et al. (1999) described the freshwater *Storeatula* species, *Storeatula rhinosa*. *Storeatula rhinosa* lacks the multilobed plastid that is a distinctive feature of *Storeatula major* and *Storeatula* sp., and it will be interesting to see where it branches in molecular phylogenies. The fact that *Rhodomonas* species do not form a monophyletic group suggests that the features used to define this genus represent the primitive state for clade E.

Falcomonas daucoides: This cryptomonad (Fig. 3F) is the only known blue-green (containing CrPC) cryptomonad with a furrow rather than a gullet (Hill 1991a) and also contains a unique type of phycocyanin, CrPC 569 (Hill and Rowan 1989). *Falcomonas daucoides* has an acute cell posterior, small hexagonal periplast plates, and a midventral band (Hill 1991a). We find support, albeit with modest bootstrap values, for *F. daucoides* branching at the base of clade D. Clade D contains all other blue-green species considered, and the basal position of *F. daucoides* supports the suggestion that a *Falcomonas*-like cell gave rise to the blue-green cryptomonad lineage (Clay and Kugrens 1999).

Proteomonas sulcata: This species is the only cryptomonad known to have two ploidy-dependent morphotypes (Fig. 3G) (Hill and Wetherbee 1986). Both morphotypes have a furrow, no gullet, and a plastid containing CrPE 545. The haploid form (haplomorph) of *P. sulcata* has small hexagonal periplast plates, a midventral band, and an acute cell posterior, whereas the diploid form (diplomorph) lacks periplast plates and a midventral band and has a rounded cell profile. At 9–14 μm in length, the diplomorph is approximately twice the size of the 7.5–10.5 μm long haplomorph. The phylogenetic position of *P. sulcata* is not clear because the two data sets used and various methods of phylogenetic reconstruction give conflicting results. Between its two morphotypes, *P. sulcata* displays features seen in a number of other cryptomonads. The midventral band seen in the *P. sulcata* haplomorph is also seen in blue-green species *F. daucoides* (Hill 1991a) as well as *Rhodomonas stigmatica*, a species with it nucleomorph embedded in the pyrenoid as for species in clade E (Hill and Wetherbee 1989). On the other hand, the diplomorph of *P. sulcata* is comparable in morphology with *H. phi* (Deane et al. 1998) and is also quite similar to *Teleaulax* species (Hill 1991b).

Given that *P. sulcata* is such a unique cryptomonad, both in its variable morphology and its phylogenetic position according to nuclear 18S rDNA, it would be useful to obtain sequence for additional *Proteomonas* species. Novarino (1991) described *Proteomonas pseudobaltica* based on specimens believed to represent a diplomorph life phase. No haplomorph stage has been observed for *P. pseudobaltica*, and it has a gullet instead of a furrow as in *P. sulcata*. *Proteomonas pseudobaltica* has yet to be investigated using molecular techniques, but based on morphology it appears incorrect to refer *P. pseudobaltica* to the genus *Proteomonas*. The search for another cryptomonad that alternates between morphotypes like *P. sulcata* continues.

Relationships between cryptomonad lineages. The relationships between the seven plastid-containing cryptomonad lineages detailed above are not well resolved in nuclear 18S rDNA phylogenies. We have included sequence from a greater number of plastid-containing cryptomonads than previous studies as well as an addi-

tional plastid-lacking species in *G. pacifica* that is possibly the closest known relative to the host cell component of plastid-containing cryptomonads. In contrast to previous phylogenetic studies (Marin et al. 1998, Clay and Kugrens 1999), our results do not support the notion that clade A (*Cryptomonas*, *Campylomonas*, and *Chilomonas*) represents the earliest diverging plastid-containing cryptomonad lineage. This tree topology may well have been a long-branch attraction artifact between the relatively divergent cryptomonad group A and highly divergent sequence from *G. truncata*. We have circumvented this problem by using less divergent sequence from *G. pacifica* as an outgroup for plastid-containing cryptomonads. *Proteomonas sulcata* and cryptomonads with a nucleomorph embedded in the pyrenoid (group E) are candidates for the earliest diverging cryptomonad (Figs. 1 and 2), but in reality this remains an open question. Resolution of this issue is important with regard to proposals for natural higher order classification of cryptomonads. Novarino et al. (1994) separated cryptomonads with nucleomorphs embedded in the pyrenoid (the Pyrenomonadales *sensu* Novarino et al.) from species with free nucleomorphs (the Cryptomonadales *sensu* Novarino et al.). Clay et al. (1999) revised this scheme to reflect an apparent sister group relationship between *Cryptomonas* as defined by Hill (1991) and its close relatives (the Cryptomonadales *sensu* Clay et al.) and all other cryptomonads (the Pyrenomonadales *sensu* Clay et al.) (Marin et al. 1998). As outlined above, we now believe this apparent sister group relationship is likely to be an artifact.

Complete resolution of relationships between the major cryptomonad lineages is likely to require the use of genes that contain more phylogenetic information than nuclear 18S rDNA. Cryptomonad nucleomorph 18S rDNA, which has been diverging faster than nuclear 18S rDNA, may be appropriate and has already been used to some extent for cryptomonad phylogeny (Cavalier-Smith et al. 1996). However, a shortcoming of using nucleomorph sequence is that the evolutionary distance between the cryptomonad endosymbiont and the red algae, from which it apparently arose, is large and will make outgroup rooting of the cryptomonad clade difficult (Cavalier-Smith et al. 1996, Van der Auwera et al. 1998). Another possibility would be to use a combined 18S/28S nuclear rDNA data set to give greater phylogenetic resolution. An advantage of using nuclear rDNA sequence is that it is possible to use sequence from *Goniomonas* as a closely related outgroup for plastid-containing cryptomonads. Whatever approach is chosen, the framework provided by nuclear 18S rDNA phylogenies will allow strategic sampling of species representing each of the major cryptomonad lineages.

Nuclear 18S rDNA phylogeny with regard to cell morphology and pigmentation. Cryptomonad endosymbiont characters such as nucleomorph position (free or within pyrenoid), plastidial complex number (one or two), and the presence of thylakoid membranes in the pyrenoid are consistent with nuclear 18S rDNA phylogenies. For example, nucleomorphs embedded in the pyrenoid (clade E), two plastidial complexes per cell (clade A), and thylakoid membranes in the pyrenoid (clade D) are each restricted to single well-supported clades. Plastid pigmentation is also in agreement with nuclear 18S phylogeny if *F. daucoides* is included at the base of clade D, and it is taken into consideration that the CrPE 555 in brown *Hemiselmis* species is a modified form of CrPC (Marin et al. 1998). CrPE 566 is restricted to clade A, whereas CrPC and its derivative CrPE 555 are restricted to *F. daucoides* and clade D. The distribution of various types of CrPC within clade D is also in agreement with 18S rDNA results. Thus, nuclear 18S rDNA phylogeny vindicates the use of nucleomorph position, plastidial complex number, the presence of thylakoids in the pyrenoid, and pigment type as phylogenetic markers.

Other features such as furrow/gullet structure, periplast type, and cell shape are often more variable, even between closely related taxa. For example, using molecular criteria, *H. phi* and *G. theta* are very closely related but have different periplast and furrow/gullet types. *Plagioselmis prolonga* and *T. amphioxeia* are closely related by nuclear 18S rDNA but have different periplast types. Moreover, a single species, *Proteomonas sulcata*, even displays two ploidy-dependent cell types with different shapes and periplasts types.

Having noted that morphology may be very variable between closely related species, we also observe that the converse is possible. The morphology of *Teleaulax* species, *H. phi*, and the diplomorph of *P. sulcata* are very similar (long furrow, no gullet, a periplast devoid of plates, a free nucleomorph, and a single plastid with CrPE 545), even though there is no evidence of a close link between these genera in nuclear 18S rDNA phylogenies. On the contrary, *Teleaulax* species and *H. phi* are both most closely related to species with a different type of cell morphology to their own, and *P. sulcata* does not appear to be closely related to any other cryptomonad species considered. The morphology displayed by *Teleaulax*, *H. phi*, and the *P. sulcata* diplomorph is possibly of an ancestral type for cryptomonads with a free nucleomorph and indicates a lack of derived features rather than a close relationship between species that posses it. This argument is strengthened by the fact that morphologically similar *Teleaulax acuta* and *Teleaulax amphioxeia* fail to form a monophyletic group within clade B.

A large gap in our knowledge concerns whether ploidy-dependent changes in morphology occur in cryptomonads other than *P. sulcata*. Presently, *P. sulcata* is the only cryptomonad known to have distinct haploid and diploid morphotypes. The ploidy status of other cryptomonads is unknown, and none has been observed to alternate between two distinctly different cell types. Clearly, there is a need to explore how ploidy influences morphology in cryptomonads and how such morphotype manifestations relate to the underlying genetic content of a species.

Bhattacharya, D., Helmchen, T., Bibeau, C. & Melkonion, M. 1995. Comparisons of nuclear-encoded small-subunit ribosomal RNAs reveal the evolutionary position of the Glaucocystophyta. *Mol. Biol. Evol.* 12:415–20.

Bourelly, P. 1970. *Les algues d'eau douce: initiation à la systematique. Tome III: Les algues blues et rouges. Les Euglèniens, Peridiniens et Cryptomonadines.* Editions N. Boubée & Cie, Paris, 512 pp.

Brett, S. J., Perasso, L. & Wetherbee, R. 1994. StructuAre and development of the cryptomonad periplast—a review. *Protoplasma* 181:106–22.

Butcher, R. W. 1967. An introductory account of the smaller algae of British coastal waters. Part IV. Cryptophyceae. *Fish. Invest. Ser. IV* : 1–54.

Cavalier-Smith, T., Couch, J. A., Thorsteinsen, K. E., Gilson, P., Deane, J. A., Hill, D. R. A. & McFadden, G. I. 1996. Cryptomonad nuclear and nucleomorph 18S rRNA phylogeny. *Eur. J. Phycol.* 31:315–28.

Clay, B. L. & Kugrens, P. 1999. Characterization of *Hemiselmis amylosa* sp. nov. & phylogenetic placement of the blue-green cryptomonads *H. amylosa* and *Falcomonas daucoides*. *Protist* 150:297–310.

Clay, B. L., Kugrens, P. & Lee, R. E. 1999. A revised classification of Cryptophyta. *Bot. J. Linn. Soc.* 131:131–51.

Deane, J. A., Hill, D. R. A., Brett, S. J. & McFadden, G. I. 1998. *Hanusia phi* gen. et sp. nov. (Cryptophyceae): characterization of "*Cryptomonas* sp. F." *Eur. J. Phycol.* 33:149–54.

Dodge, J. D. 1969. The ultrastructure of *Chroomonas mesostigmatica* Butcher (Cryptophyceae). *Arch. Mikrobiol.* 69:266–80.

Douglas, S. E., Murphy, C. A., Spencer, D. F. & Gray, M. W. 1991. Cryptomonad algae are evolutionary chimeras of two phylogenetically distinct unicellular eukaryotes. *Nature* 350:148–51.

Erata, M., Kubota, M., Takahashi, T., Inouye, I. & Watanabe, M. 1995. Ultrastructure and phototactic action spectra of two genera of cryptophyte flagellate algae, *Cryptomonas* and *Chroomonas*. *Protoplasma* 188:258–66.

Fast, N. M., Kissinger, J. C., Roos, D. S. & Keeling, P. J. 2001. Nuclear-encoded, plastid-targeted genes suggest a single common origin for apicomplexan and dinoflagellate plastids. *Mol. Biol. Evol.* 18:418–26.

Felsenstein, J. 1993. *PHYLIP (Phylogeny Inference Package)*. Department of Genetics, University of Washington, Seattle.

Gantt, E. 1971. Microanatomy of the periplast of *Chroomonas* sp. (Cryptophyceae). *J. Phycol.* 7:177–84.

Gilbert, D.G. 1992. Seq App - A biosequence editor and analysis application. Bloomington, Indiana, USA.

Gillott, M. A. & Gibbs, S. P. 1983. Comparison of the flagellar rootlets and the periplast in two marine cryptomonads. *Can. J. Bot.* 61: 1964–78.

Hill, D. R. A. & Wetherbee, R. 1986. *Proteomonas sulcata* gen. et sp. nov. (Cryptophyceae), a cryptomonad with two morphologically distinct and alternating forms. *Phycologia* 25:521–43.

Hill, D. R. A. 1988. *The Cryptophyceae: Ultrastructure and Taxonomy*. PhD thesis. University of Melbourne, Australia.

Hill, D. R. A. & Wetherbee, R. 1988. The structure and taxonomy of *Rhinomonas pauca* gen. et sp. nov. (Cryptophyceae). *Phycologia* 27:355–65.

Hill, D. R. A. & Rowan, K. S. 1989. The biliproteins of the Cryptophyceae. *Phycologia* 28:455–63.

Hill, D. R. A. & Wetherbee, R. 1989. A reappraisal of the genus *Rhodomonas* (Cryptophyceae). *Phycologia* 28:143–58.

Hill, D. R. A. & Wetherbee, R. 1990. *Guillardia theta* gen. et sp. nov. (Cryptophyceae). *Can. J. Bot.* 68:1873–6.

Hill, D. R. A. 1991a. *Chroomonas* and other blue-green cryptomonads. *J. Phycol.* 27:133–45.

Hill, D. R. A. 1991b. A revised circumscription of *Cryptomonas* (Cryptophyceae) based on examination of Australian strains. *Phycologia* 30:170–88.

Keller, M. D. 1987. Media for the culture of oceanic ultraphytoplankton. *J. Phycol.* 23:633–8.

Klaveness, D. 1981. *Rhodomonas lacustris* (Pascher & Ruttner) Javornicky (Cryptomonadida): ultrastructure of the vegetative cell. *J. Protozool.* 28:83–90.

Kugrens, P., Lee, R. E. & Anderson, R. A. 1986. Cell form and surface patterns in *Chroomonas* and *Cryptomonas* cells (Cryptophyta) as revealed by scanning electron microscopy. *J. Phycol.* 22:512–22.

Kugrens, P. & Lee, R. E. 1987. An ultrastructural survey of cryptomonad periplasts using quick-freezing freeze-fracture techniques. *J. Phycol.* 23:365–76.

Kugrens, P. & Lee, R. E. 1991. *Organization of Cryptomonads*. Clarendon Press, Oxford, pp. 219–233.

Kugrens, P., Clay, B. L. & Lee, R. E. 1999. Ultrastructure and systematics of two new freshwater red cryptomonads, *Storeatula rhinosa*, sp nov and *Pyrenomonas ovalis*, sp nov. *J. Phycol.* 35:1079–98.

Larsen, J. & Patterson, D. J. 1990. Some flagellates (Protista) from tropical marine sediments. *J. Nat. Hist.* 24:801–937.

Marin, B., Klinberg, M. & Melkonian, M. 1998. Phylogenetic relationships among the cryptophyta: analyses of nuclear-encoded SSU rRNA sequences support the monophyly of extant plastid-containing lineages. *Protist* 149:265–76.

McFadden, G. I., Gilson, P. R. & Douglas, S. E. 1994a. The photosynthetic endosymbiont in cryptomonad cells produces both chloroplast and cytoplasmic-type ribosomes. *J. Cell Sci.* 102:649–57.

McFadden, G. I., Gilson, P. R. & Hill, D. R. A. 1994b. *Goniomonas*—rRNA sequences indicate that this phagotrophic flagellate is a close relative of the host component of cryptomonads. *Eur. J. Phycol.* 29:29–32.

Novarino, G. 1991. Observations on some new and interesting Cryptophyceae. *Nord. J. Bot.* 11:599–611.

Novarino, G. & Lucas, I. A. N. 1993. Some proposals for a new classification system of the Cryptophyceae. *Bot. J. Lin.* 111:3–21.

Novarino, G., Lucas, I. & Morrall, S. 1994. Observations on the genus *Plagioselmis* (Cryptophyceae). *Cryptogamie Algol.* 15:87–107.

Olsen, G. L., Matsuda, H., Hagstrom, R. & Ovenbeek, R. 1994. FastDNAml: a tool for the construction of phylogenetic trees of DNA sequences using maximum likelihood. *Comp. Appl. Bios.* 10:41–8.

Roberts, K. R., Stewart, K. D. & Mattox, K. R. 1981. The flagellar apparatus of *Chilomonas paramecium* (Cryptophyceae) and its comparison with certain zooflagellates. *J. Phycol.* 17:159–67.

Roberts, K. R. 1984. Structure and significance of the cryptomonad flagellar apparatus. I. *Cryptomonas ovata* (Cryptophyta). *J. Phycol.* 20:590–9.

Saunders, G. W. & Kraft, G. T. 1994. Small-subunit rRNA gene sequences from representatives of selected families of the Gigartinales and Rhodymeniales (Rhodophyta).1. Evidence for the Plocamiales ord. nov. *Can. J. Bot.* 72:1250–63.

Swofford, D. L. 1993. *Phylogenetic Analysis Using Parsimony (PAUP) 3.1.1*. Smithsonian Institute, Washington DC.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–80.

Van der Auwera, G., Hofmann, C. J. B., De Rijk, P. & De Wachter, R. 1998. The origin of red algae and cryptomonad nucleomorphs: a comparative phylogeny based on small and large subunit rRNA sequences of *Palmaria palmata*, *Gracilaria verrucosa*, and the *Guillardia theta* nucleomorph. *Mol. Pylogenet. Evol.* 10: 333–42.

Wetherbee, R., Hill, D. R. A. & McFadden, G. I. 1986. Periplast structure of the cryptomonad flagellate *Hemiselmis brunnescens*. *Protoplasma* 131:11–22.