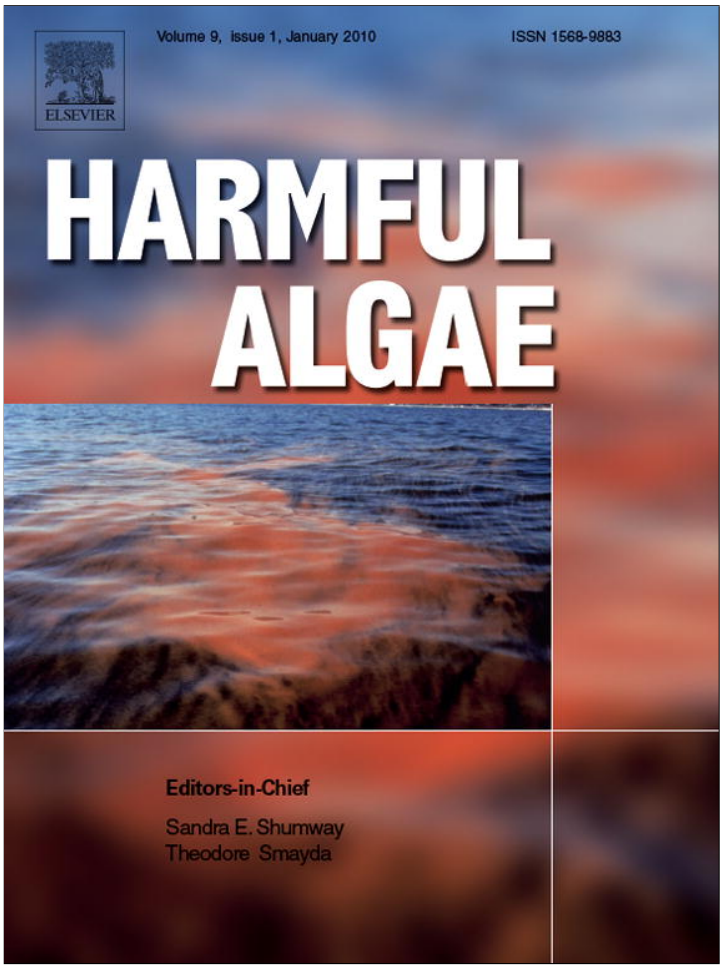

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The toxic dinoflagellate *Dinophysis acuminata* harbors permanent chloroplasts of cryptomonad origin, not kleptochloroplasts

Lydia Garcia-Cuetos [a,*], Øjvind Moestrup [a], Per Juel Hansen [b], Niels Daugbjerg [a]

[a] Phycology Laboratory, Department of Biology, University of Copenhagen, Øster Farimagsgade 2D, 1353 Copenhagen K, Denmark
[b] Marine Biological Laboratory, Department of Biology, University of Copenhagen, Strandpromenaden 5, 3000 Helsingør, Denmark

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ABSTRACT

Most species belonging to the toxigenic genus *Dinophysis* have chloroplasts of cryptophyte origin. Whether these chloroplasts are temporarily sequestered from the prey, or permanently established under the control of the dinoflagellate is currently disputed. To investigate this, a culture of *Dinophysis acuminata* was established by feeding it the phototrophic ciliate *Mesodinium rubrum* (= *Myrionecta rubra*), which again was fed the cryptophyte *Teleaulax amphioxeia*. Molecular analysis comprising the nucleomorph LSU and two chloroplast markers (tufA gene and a fragment from the end of 16S rDNA to the beginning of 23S rDNA) resulted in identical sequences for the three organisms. Yet, transmission electron microscopy of the three organisms revealed that several chloroplast features separated *D. acuminata* from both *T. amphioxeia* and *M. rubrum*. The thylakoid arrangement, the number of membranes around the chloroplast as well as the position and the arrangement of the pyrenoids were strikingly different. Considering both molecular and ultrastructural evidence, our data indicated that the chloroplasts in *D. acuminata* are permanent chloroplasts originating within *Teleaulax* or another closely related cryptophyte genus. Electron microscopy also provided new information on the peduncle of *D. acuminata*, which is used in food uptake.

1. Introduction

Dinophysis Ehrenberg is a genus of toxin-producing marine dinoflagellates, for which research has been challenged by difficulties in establishing it in culture (Schnepf and Elbrächter, 1999). The determination of the origin of the chloroplast in particular was problematic. Ultrastructural (Schnepf and Elbrächter, 1988, 1999; Lucas and Vesk, 1990) and pigment analyses (Hallegraeff and Lucas, 1988; Geider and Gunter, 1989; Vesk et al., 1996; Hewes et al., 1998) carried out on wild samples suggested that the chloroplasts were of cryptomonad origin. The impossibility of culturing and the rare cryptomonad origin of chloroplasts in dinoflagellates led to the assumption that the chloroplasts might be kleptochloroplasts (Melkonian, 1996). The term refers to a chloroplast which is sequestered from the prey by the predator and is kept as a functioning unit for a limited period of time. Yet, the presence of two membranes around the chloroplasts, described in the ultrastructural studies, pointed to a long established, i.e. permanent organelle. It was not until recently that the kleptochloroplast theory was taken up again based on molecular studies. The sequencing of several chloroplast genes suggested that the chloroplast derived from a free-living cryptophyte (Takishita et al., 2002; Hackett et al., 2003), more precisely *Teleaulax amphioxeia* (Conrad) Hill (Janson, 2004; Takahashi et al., 2005; Minnhagen and Janson, 2006).

When Park et al. (2006) succeeded in cultivating *Dinophysis acuminata* Claparède et Lachmann, experimental research on the origin of the chloroplast of *Dinophysis* became possible. *D. acuminata* was found to grow when fed the ciliate *Mesodinium rubrum* Lohmann (synonym *Myrionecta rubra* Jankowski). This ciliate, which is unusual in being photosynthetically active, was maintained in culture on a diet of the autotrophic cryptomonad flagellate *T. amphioxeia* (Gustafson et al., 2000). Cells of *M. rubrum* harbored endosymbionts resembling *Teleaulax*, but there is disagreement whether the endosymbionts are permanent (Hansen and Fenchel, 2006) or temporary (Gustafson et al., 2000; Yih et al., 2004; Johnson and Stoecker, 2005; Johnson et al., 2006). The cryptophyte endosymbiont consists of many chloroplasts, mitochondria, nucleomorphs, endoplasmic reticulum and a single so-called symbiont nucleus (Taylor et al., 1971; Hibberd, 1977; Hansen and Fenchel, 2006). If transfer of chloroplasts from the cryptophyte to *Dinophysis* via *M. rubrum* can be proven, it would mean that *Dinophysis* is the third organism to utilize the chloroplast of *Teleaulax* for photosynthesis.

The present study aims to examine the origin of the chloroplasts in *D. acuminata*. For the first time, the chloroplasts of the three organisms involved in the food chain will be characterized from established cultures using molecular as well as ultrastructural tools.

* Corresponding author. Tel.: +45 35 32 22 93; fax: +45 35 32 23 21.
E-mail address: lgarcia@bio.ku.dk (L. Garcia-Cuetos).

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2. Materials and methods

2.1. Cultures

A culture of the food organism for *Dinophysis*, the photosynthetic ciliate *M. rubrum* (Mr-DK2007), was established from single cells isolated from surface seawater samples collected near Frederiksund, Denmark, during a bloom event on 17 April 2007. The cryptophyte *T. amphioxeia* (SCCAP K-0434) was used as prey for *M. rubrum*. It was established from seawater samples collected from the Øresund in March 1990, Denmark, and provided by the Scandinavian Culture Collection of Algae and Protozoa at the University of Copenhagen. Both cultures were grown in f/2 medium at 32 PSU, 15 °C or f/20 medium at 16 PSU, 20 °C and a photon flux of ca. 100 μmol m^{-2} s^{-1} in a L:D cycle of 14:10. *M. rubrum* was fed as previously described (Hansen and Fenchel, 2006).

D. acuminata (Da-DK2007) was established by isolating single cells from surface water samples (18 °C, 22 PSU) taken during a bloom event in Hvalpsund, Denmark, 16 June 2007 (7000 cells l^{-1}). For further information, see Riisgaard and Hansen (2009). The cells were cultured in 65-ml tissue culture flasks with sterile-filtered 16 PSU f/20 medium. Cultures were incubated on a glass shelf. Illumination was provided from beneath by cool white fluorescent lamps of 100 μmol photons m^{-2} s^{-1} following a L:D cycle of 14:10 h. The temperature was kept at 20 ± 1 °C. All cultures were non-axenic.

2.2. Light microscopy

The fixed cells were observed using an Olympus Provis AX70 microscope (Olympus, Tokyo, Japan) equipped with DIC. Digital micrographs were taken with an AxioCam (Zeiss, Oberkochen, Germany).

2.3. Scanning electron microscopy (SEM)

Cells were fixed in acid Lugol solution, dehydrated in a graded ethanol series, critical point dried and coated with platinum. The microscope used was a Jeol JSM-6335F operated at 12 kV (Jeol, Tokyo, Japan).

2.4. Transmission electron microscopy (TEM)

Culture materials were mixed 1:1 with 4% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.4 and containing 0.4 M sucrose, or with 4% glutaraldehyde in f/2 culture medium. After 1 h at 4 °C, the cells were concentrated by centrifugation. Subsequently, they were rinsed 3 times in cold cacodylate buffer of decreasing sucrose content, or f/2 medium. Once rinsed, the material was post-fixed overnight in 2% osmium tetroxide in 0.2 M cacodylate buffer at pH 7.4 at 4 °C. Before dehydration, the material was rinsed briefly in buffer. Each step of the dehydration lasted 20 min at 4 °C in the following ethanol concentrations: 30%, 50%, 70%, 90% and 96%. The material was transferred to room temperature while in 96% ethanol and dehydration completed in two changes of absolute ethanol, 20 min in each change. Following two brief rinses in propylene oxide, the material was transferred to a 1:1 mixture of Spurr's embedding mixture (Spurr) and propylene oxide and left uncovered overnight, followed by 5 h in a fresh mixture of Spurr. The material was then moved to a new recipient and Spurr was added. Finally, it was polymerized at 70 °C overnight. Sectioning was carried out on a Reichert Ultracut E ultramicrotome using a diamond knife. The sections were collected on slot grids (Rowley and Moran, 1975) and stained for 15 min with 2% uranyl acetate in methanol, followed by Reynold's lead citrate. The grids were examined in a JEM-1010 electron microscope (JEOL, Tokyo, Japan).

2.5. DNA extraction, PCR amplification and sequencing

The extractions were performed as previously described in Hansen et al. (2003) on the three species of interest *T. amphioxeia*, *M. rubrum* from different locations (Denmark, Korea and Antarctica) and *D. acuminata*, and also on several other cryptophyte species to be included in the phylogenetic analyses: *Geminigera cryophila* Taylor et Lee (CCMP2564), *Hanusia phi* Deane (CCMP325), *Proteomonas sulcata* Hill and Wetherbee (CCMP321), *Hemiselmis rufescens* Hill (CCMP440), *Hemiselmis tepida* Lane and Archibald (CCMP442), *Chroomonas vectensis* Carter (SCCAP K-0432). For *D. acuminata* (Da-DK2007) as well as for *M. rubrum* (Mr-DK2007), cultures were starved prior to extraction for three and two weeks, respectively, based on prior growth experiments (Hansen and Fenchel, 2006; Riisgaard and Hansen, 2009). Cells were checked under the light microscope for presence or absence of food vacuoles and prey in the culture.

PCR were carried out in 50 μl volume. PCR amplifications of the nucleomorph LSU rDNA (nmLSU rDNA) were performed with the primer combination nmLSUCr3F (5′-GTT GCT TGG GAG TGC AGC-3′) and D3B (Nunn et al., 1996). Amplification profile consisted of one initial cycle of denaturation at 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 62 °C, and 3 min at 72 °C finalized by 10 min at 72 °C for final extension for the nucleomorph LSU rDNA.

PCR amplifications of the chloroplast fragment containing the partial 16S rDNA, tRNA-Ile gene, the tRNA-Ala gene, the Intergenic Transcribed Spacer (ITS) and the partial 23S rDNA were performed with the primer combination CRY-I and ITS-II (Minnhagen and Janson, 2006). This long fragment will be referred to as the rDNA block. Amplification profile consisted of one initial cycle of denaturation at 94 °C for 3 min followed by 35 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1.5 min at 72 °C, finalized by 10 min at 72 °C for final extension.

PCR amplifications of the elongation factor Tu (tufA) were carried out with the primers and the settings published in Famà et al. (2002). All PCR were carried out on a MJ Research PTC-200 Peltier Thermal Cycler (MJ Research Inc, Waltham, MA, USA).

To discriminate between possible copies of the genes present in *D. acuminata*, all *D. acuminata* gene amplifications were cloned with the TOPO TA Cloning Kit (Catalogue nr. K4500-01) from Invitrogen (Carlsbad, CA). Moreover, rDNA block of the species of importance were cloned to obtain both copies of the gene cluster, if present. Following plating, transformed clones were selected and the respective genes amplified, as described above.

All DNA fragments were purified using Nucleofast, following the manufacturer's recommendations (Macherry Inc., town, state, USA). 500 ng PCR product was air-dried over night and sent to the sequencing service at Macrogen (Seoul, Korea) for determination in both directions using the same primers used for amplification.

2.6. Alignments and phylogenetic analyses

Three data sets of sequences were analysed. All three sets were first aligned using MAFFT 6.624 (Katoh and Toh, 2008) and then improved manually using BioEdit 7.0.5 sequence alignment software (Hall, 1999). The first set was composed of 20 partial nucleomorph LSU rDNA sequences including 8 cryptophytes, 3 *M. rubrum* and 10 red algae sequences of nuclear 28S rDNA. The second set contained 25 chloroplast partial 16S rDNA, tRNA-Ile gene, the tRNA-Ala gene, the ITS and the partial 23S rDNA (rDNA block) sequences of *D. acuminata*, 2 different *M. rubrum*, 8 cryptophytes and 5 red algae. The third set comprised 16 sequences of elongation factor Tu (tufA), including 1 sequence from *D. acuminata*, 2 from *M. rubrum*, 8 sequences from cryptophytes and 5 from red algae. In all alignments, members

of the Florideophyceae were used as outgroup based on Hoef-Emden et al. (2002).

A Bayesian method was used to infer phylogeny, using the program MrBayes v.3.2 (Huelsenbeck and Ronquist, 2001). Two simultaneous Monte Carlo Markov chains (MCMC; Yang and Rannala, 1997) were run from random trees for a total of 2,000,000 generations (metropolis-coupled MCMC). One of every 50 trees was sampled. AWTY (Wilgenbusch et al., 2004) was used to graphically evaluate the extent of the MCMC analysis. After excluding the first sampled trees categorized as the "burn-in period", a consensus tree was constructed using PAUP* 4.0.b10 software (Swofford, 2002) based on 39.840 trees. Then, Modeltest (Posada and Crandall, 1998), implemented in the PAUP* 4.0.b10 software (Swofford, 2002), identified as the best model the TrN+I+G model for the nucleomorph LSU rDNA alignment, the GTR+I+G model (Lanave et al., 1984) for the tufA alignment, and the GTR+G model for the rDNA block alignment. Using these settings, a tree was reconstructed with the online version of the PhyML software (Guidon and Gascuel, 2003) available on the Montpellier bioinformatics platform at http://www.atgc-montpellier.fr/phyml

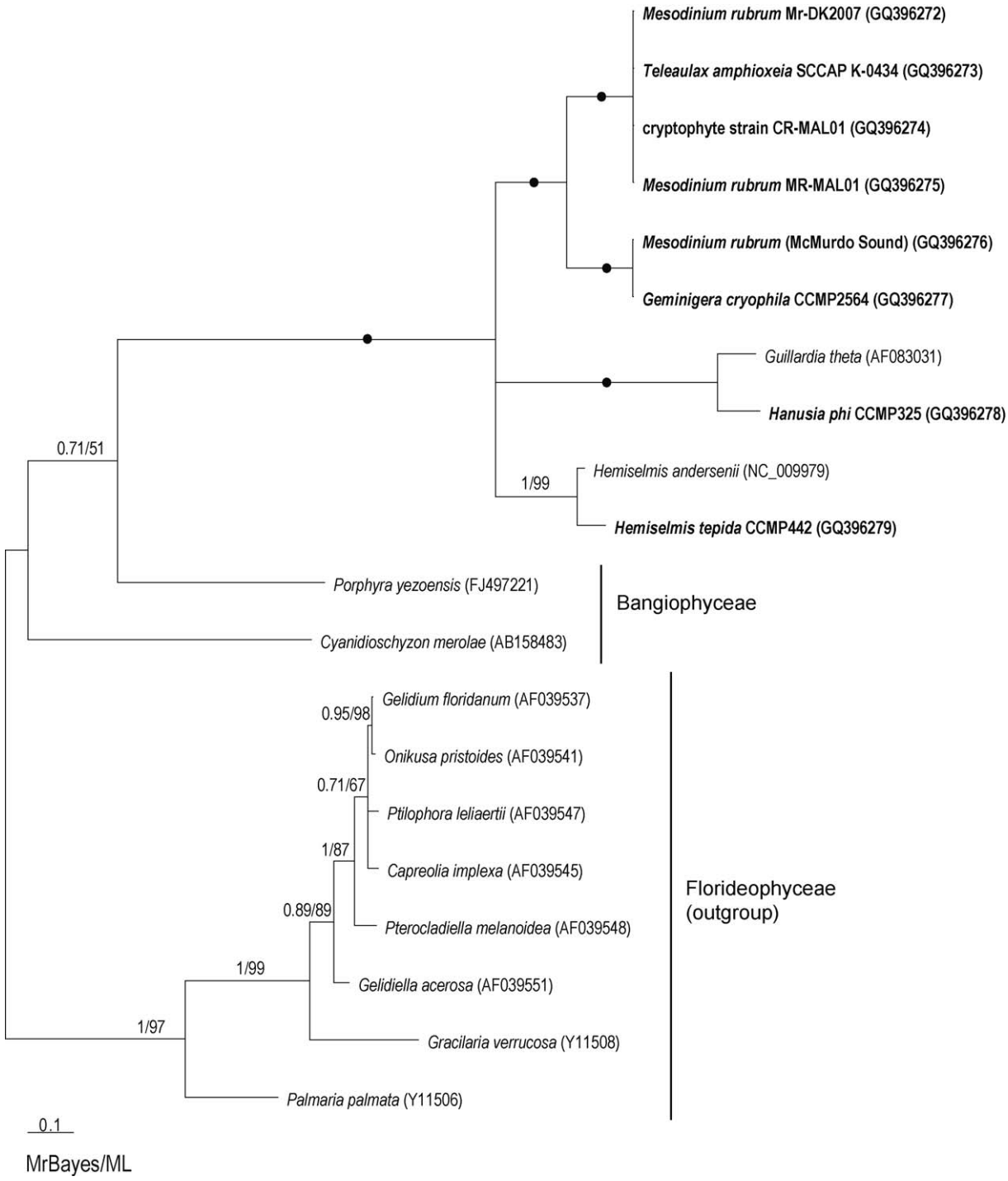

Fig. 1. Phylogeny based on nucleomorph-encoded LSU rDNA sequences including domains D1–D3 (942 bp) and inferred from Bayesian analysis. Eight species of red algae belonging to the Florideophyceae constituted the outgroup. Branch support was obtained from Bayesian posterior probabilities and bootstrap (100 replicates) in maximum likelihood analyses. At internodes, posterior probabilities (≤1) are written first followed by bootstrap values (in percentage) from ML. (●) The highest possible posterior probability (1.0) and bootstrap value (100%). Species in bold face were sequenced in this study.

using the maximum likelihood (ML) method (Felsenstein, 1981). The reliability of internal branches was assessed using the bootstrap method with 100 replicates (Felsenstein, 1985).

3. Results

3.1. Phylogeny

The nucleomorph LSU rDNA alignment consisted of 942 bp. The molecular phylogeny based on this alignment and inferred from Bayesian analysis yielded the tree topology shown in Fig. 1. Florideophycean red algae rooted the tree. Bangiophycean red algae formed the basal group. The cryptophytes were divided in three well-supported groups. The first clade was composed of *Hemiselmis* species. The second clade included the genera *Guillardia* and *Hanusia*, and the third clade *T. amphioxeia*, *M. rubrum* and *G. cryophila*. Yet, the relationship between them was not resolved. Sequences of the nmLSU of both *M. rubrum* Mr-DK2007 and MR-MAL01, and of the two cryptophyte strains SCCAP K-0434 and CR-MAL01 were identical. Their sister group was formed by the two identical sequences of *G. cryophila* and *M. rubrum* from McMurdo Sound.

The rDNA block alignment consisted of 1090 bp. The molecular phylogeny based on this alignment and inferred from Bayesian analysis yielded the tree topology shown in Fig. 2. *Gracilaria tenuistipitata* rooted the tree, followed by the bangiophyceans forming the base of the tree. The cryptomonads formed a monophyletic group consisting of five clades. In this phylogeny,

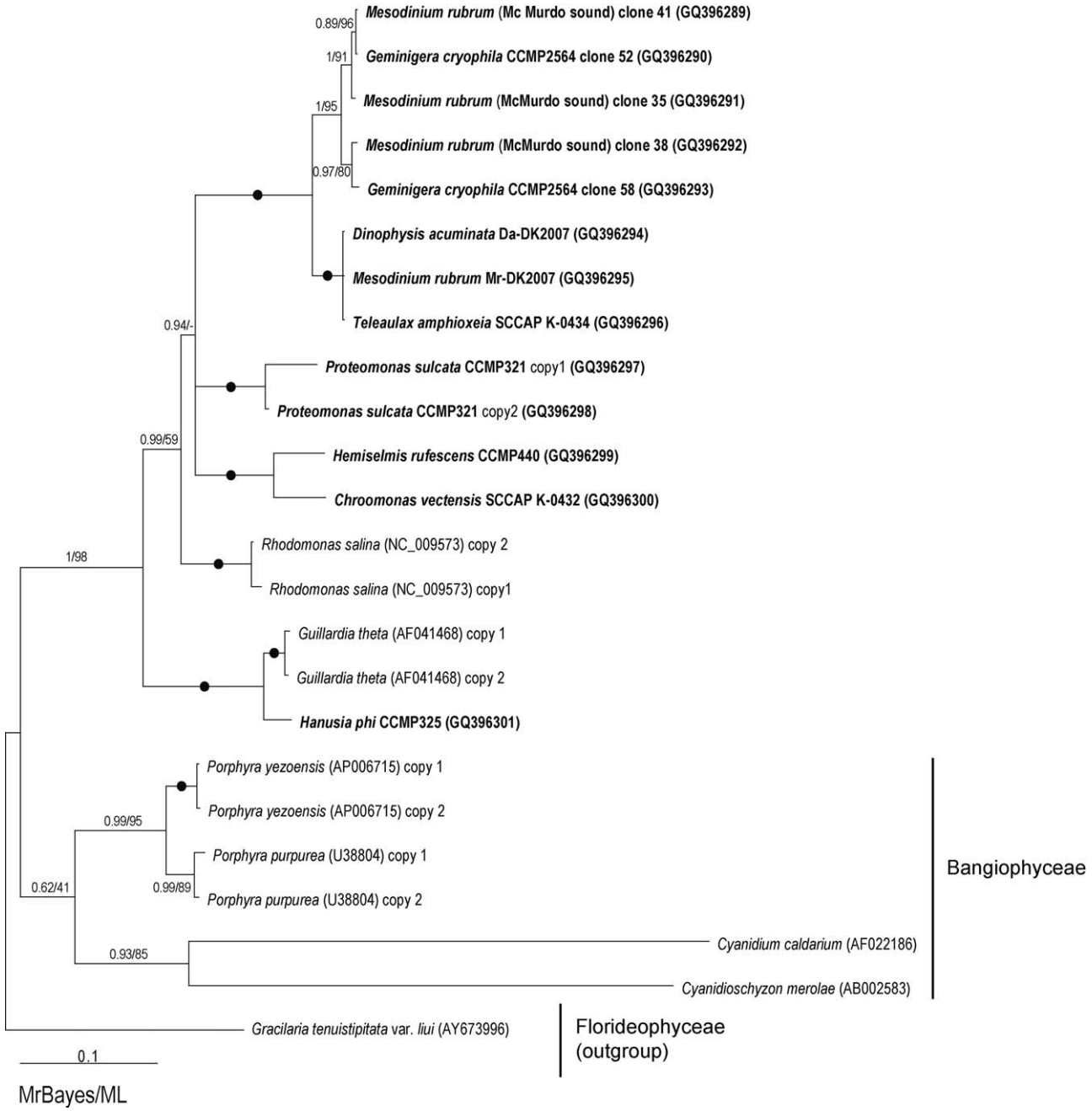

Fig. 2. Phylogeny based on chloroplast-encoded sequences of a fragment containing the partial 16S rDNA, tRNA-Ile gene, the tRNA-Ala gene, the Intergenic Transcribed Spacer (ITS) and the partial 23S rDNA (1090 bp) inferred from Bayesian analysis. *Gracilaria tenuistipitata* var. *liui* constituted the outgroup. Branch support was obtained from Bayesian posterior probabilities and bootstrap (100 replicates) in maximum likelihood analyses. At internodes, posterior probabilities ($\leq$1) are written first followed by bootstrap values (in percentage) from ML. (●) The highest possible posterior probability (1.0) and bootstrap value (100%). Species in bold face were sequenced in this study.

Guillardia theta and *H. phi* were at the base of the cryptomonads with a high support. However, the resolution between the five other clades was not well resolved. Yet, each clade (*Hemiselmis*/*Chroomonas*, *Rhodomonas salina*, *P. sulcata*, *Teleaulax*/*Geminigera*/*Mesodinium*/*Dinophysis*) was well supported. *T. amphioxeia*, *M. rubrum* and *D. acuminata* formed a sister group to *G. cryophila* and *M. rubrum* from McMurdo Sound. Sequences of *T. amphioxeia* SCCAP K-0434, *M. rubrum* Mr-DK2007 and *D. acuminata* were identical.

The tufA alignment consisted of 842 bp. The molecular phylogeny based on this alignment and inferred from Bayesian analysis yielded the tree topology shown in Fig. 3. As in the previous tree, *G. tenuistipitata* rooted the tree, and the bangiophycean sequences were at the base of the tree, followed by the cryptophytes. Yet, the cryptophyte clades were arranged differently. Despite this rearrangement of the clades, *T. amphioxeia*, *M. rubrum* and *D. acuminata* were still a sister group to *G. cryophila* and *M. rubrum* from McMurdo Sound. Again, sequences of *T. amphioxeia*, *M. rubrum* and *D. acuminata* were identical.

3.2. Morphological and ultrastructural studies

The three organisms of the food chain were illustrated in a series of plates starting with the cryptophyte *T. amphioxeia* SCCAP K-0434 (Fig. 4), which served as food for the ciliate *M. rubrum* Mr-

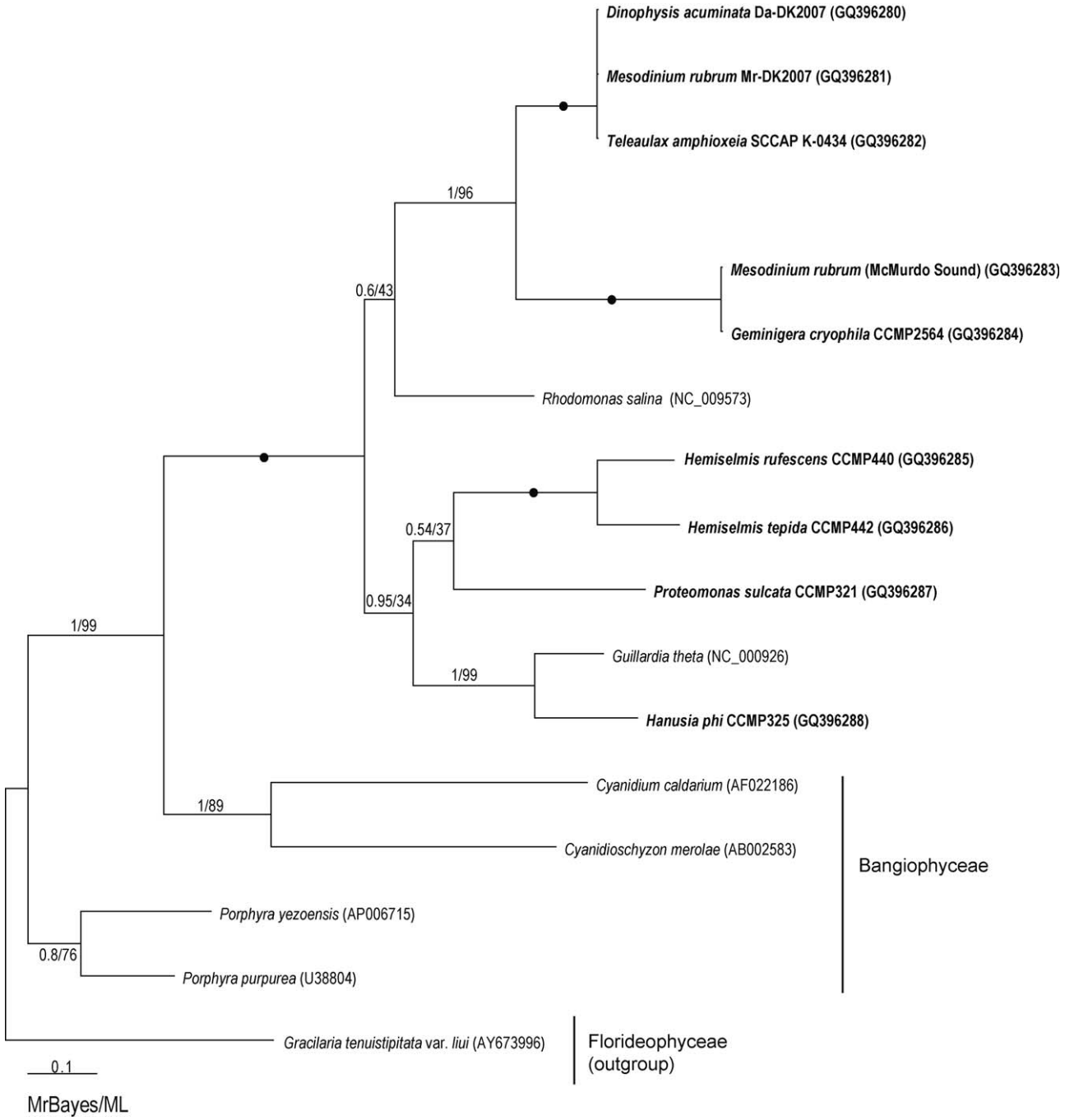

Fig. 3. Phylogeny based on chloroplast-encoded tufA sequences (842 bp) inferred from Bayesian analysis. *Gracilaria tenuistipitata* var. *liui* constituted the outgroup. Branch support was obtained from Bayesian posterior probabilities and bootstrap (100 replicates) in maximum likelihood analyses. At internodes, posterior probabilities ($\leq$1) are written first followed by bootstrap values (in percentage) from ML. (●) The highest possible posterior probability (1.0) and bootstrap value (100%). Species in bold face were sequenced in this study.

DK2007 (Fig. 5), which in turn served as food for *D. acuminata* Da-DK2007 (Figs. 6–8). In the following, we provide a brief description of each species.

3.2.1. *Teleaulax amphioxeia*

Live cells were ca. 10 μm long and 5 μm wide. Each cell possessed a longitudinal furrow on the ventral side, seen best in the SEM (Fig. 4D). The two flagella inserted near the anterior opening of the furrow (Fig. 4D). Each cell had a central nucleus (Fig. 4A, C and E) and a single parietal, cup-shaped chloroplast with a slit opposite the cell furrow: a conspicuous pyrenoid was located on the concave side of the chloroplast immediately behind the cell nucleus (Fig. 4A and C). The pyrenoid lacked thylakoids while the rest of the chloroplast was filled with thylakoids typically arranged in groups of three (Fig. 4B). Single or paired thylakoids occurred for short distances, especially as branches interconnecting the triplet lamellae. Large starch grains were located around the pyrenoid (Fig. 4A, C and E) and elsewhere in the chloroplast. A single nucleomorph was characteristically positioned between the nucleus and the pyrenoid (Fig. 4A and C). The cell further contained trichocysts of two size groups: large trichocysts (Fig. 4A) located near the cell furrow (not visible in the figures) and smaller trichocysts along the cell periphery (Fig. 4A). In nutrient-depleted cells, the arrangement of the thylakoids was

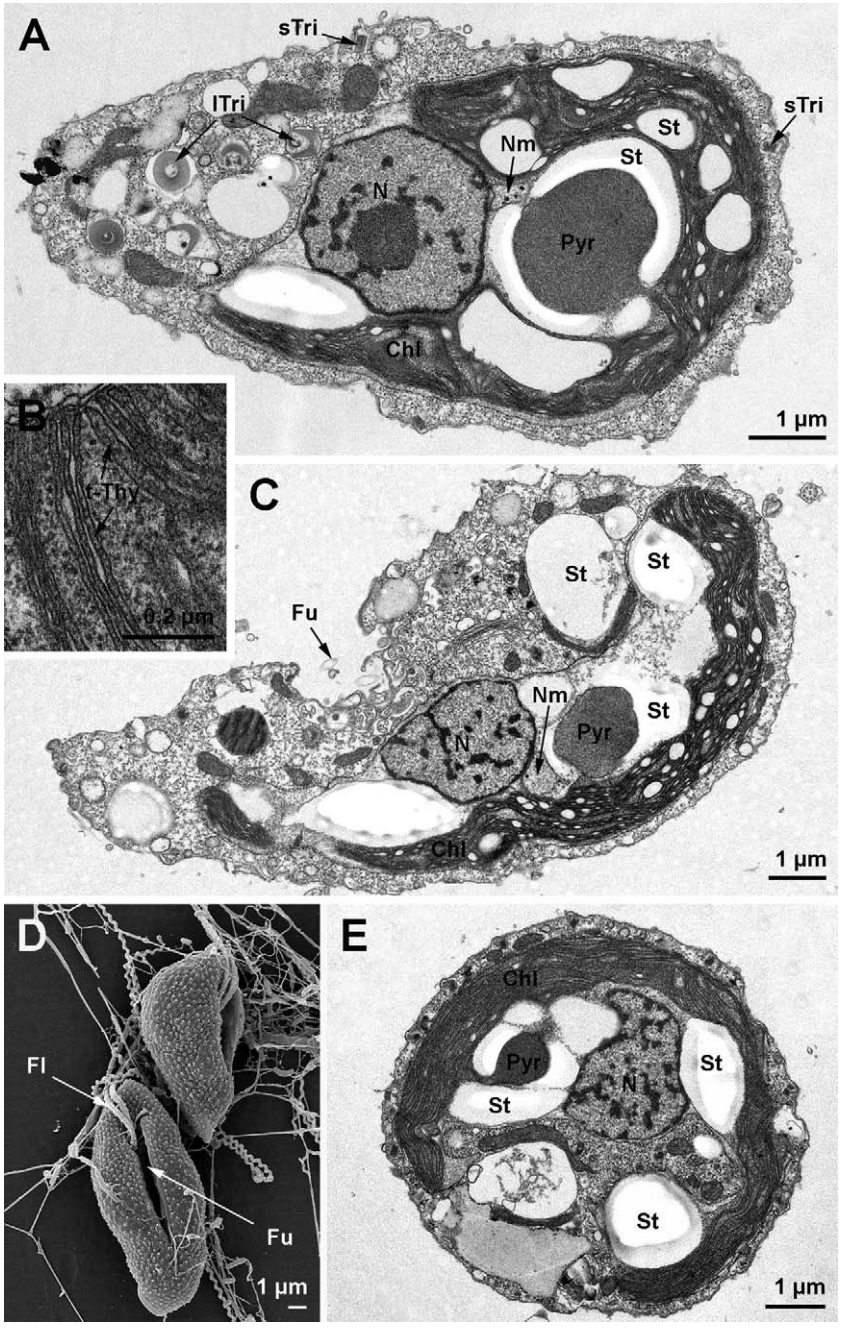

Fig. 4. *Teleaulax amphioxeia*, grown in culture. (A and C) Longitudinal sections at different orientation, the cell in C illustrating part of the longitudinal furrow (Fu). Other visible organelles are the nucleus (N), the pyrenoid (Pyr), the nucleomorph (Nm). Trichocysts of two sizes are also visible (sTri and lTri) as are the parietal chloroplast (Chl) and several starch grains. (B) Details of the chloroplast. The chloroplast lamellae comprise thylakoids in groups of three, sometimes very swollen (top), in the middle triplet less so. (D) SEM, illustrating the two flagella (Fl), inserted near the anterior end of the furrow (Fu), and the rugged surface of the cell. (E) Slightly oblique transverse section of a cell at the level of the nucleus and the pyrenoid. Labelling as in A and C.

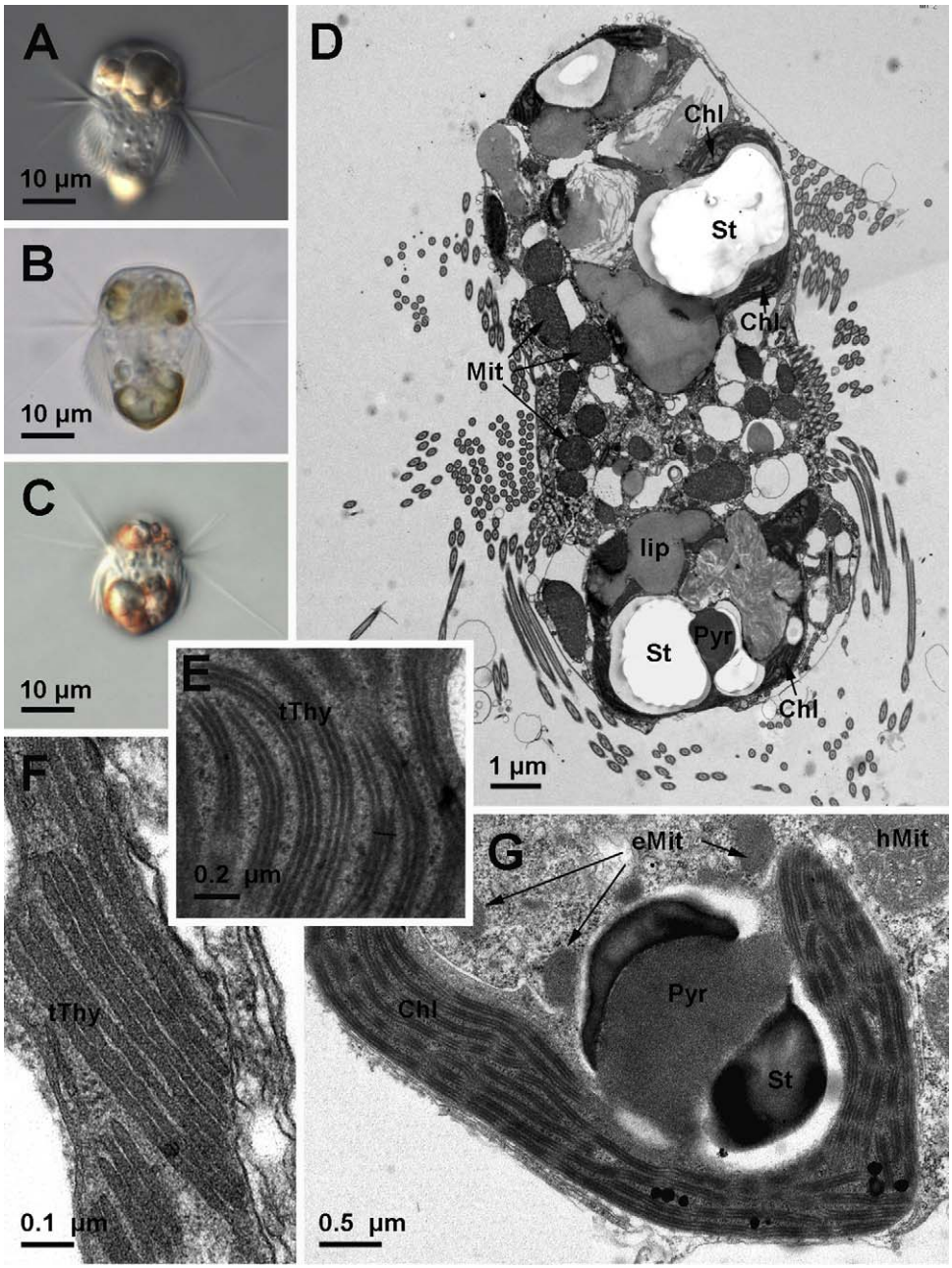

Fig. 5. *Mesodinium rubrum*, grown in culture. (A and B) Light micrograph of a starved cell of *M. rubrum* prior to extraction. (C) Light micrograph of a fed cell of *M. rubrum*. (D) Longitudinal section through the cell illustrating the cilia, the profiles of the chloroplast (Chl), a pyrenoid (Pyr) and starch grains (St). (E and F) Details of chloroplast thylakoids, which are always arranged in 3-thylakoid lamellae (tThy). (G) Chloroplast showing the lateral position of the pyrenoid. The pyrenoid is surrounded by a few starch grains. Mitochondria of two types are also visible in the figure, from the ciliate (hMit) and from the cryptophyte endosymbiont (eMit), respectively, but the cristae of the latter cannot be distinguished at this low magnification.

often irregular; the thylakoids were often more or less swollen and separated from each other. Many smaller swellings were visible in the cell in Fig. 4C, less numerous in Fig. 4A and nearly absent in Fig. 4E. The translucent area in the middle triplet (Fig. 4B) represented the space between two thylakoids while the lumen of each thylakoid was more or less opaque.

3.2.2. *Mesodinium rubrum*

Live cells were ca. 25 μm long and ca. 14 μm wide (Fig. 5A–C). The chloroplasts in starved cells were pale (Fig. 5A and B), while they fluoresced bright orange in well-fed cells (Fig. 5C). A longitudinal section through the cell showed the general appearance of the cell, including the insertion of the anterior and posterior cilia (Fig. 5D). The starch-containing chloroplasts were visible in the front part of the cell as well as in the posterior part. The posterior part also showed a pyrenoid of a chloroplast. Other opaque structures in the cell were mitochondria, and lipid droplets associated with the chloroplast. Nuclei of the ciliate as well as the so-called symbiont nucleus were present (data not shown). Details of a chloroplast (Fig. 5G) showed the pyrenoid, inserted in the cavity of the chloroplast and surrounded by starch grains. Thylakoids of the chloroplast were arranged in triplets, which differed somewhat in appearance, probably depending on the physiological state of the chloroplast. Thus, the thylakoids in Fig. 5F re swollen as typical of cryptomonad chloroplasts while in the chloroplast in Fig. 5E, the lumen of each thylakoid was strongly stained but not swollen. The lumen was always opaque.

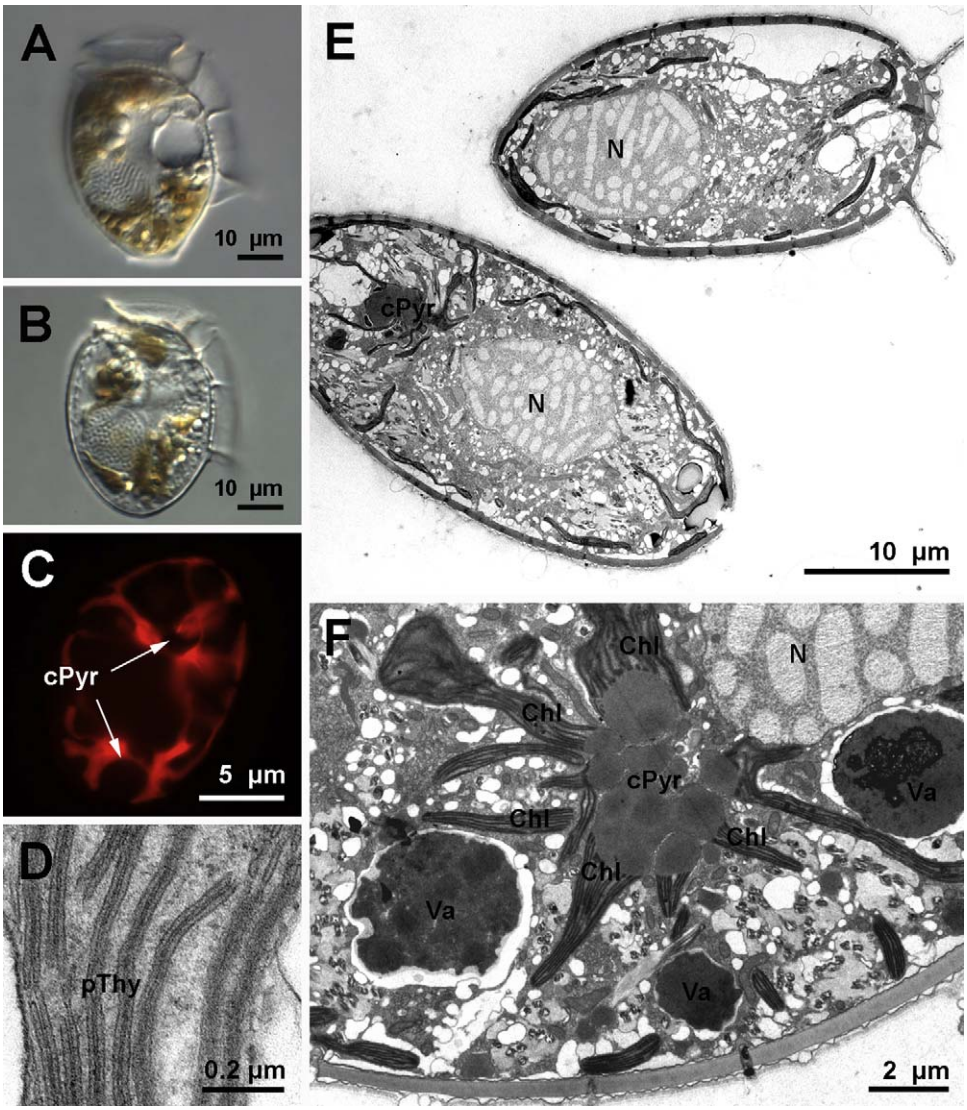

Fig. 6. *Dinophysis acuminata*, grown in culture. (A) Light micrograph of a starved cell of *D. acuminata* prior to extraction. (B) Light micrograph of a well-fed cell of *D. acuminata*. (C) Epifluorescence micrograph of a starved cell of *D. acuminata* illustrating the compound pyrenoids (cPyr) of both stellate chloroplasts. (D) Paired thylakoids within the chloroplasts. (E) Longitudinal sections through two cells, showing general appearance of the cell, nucleus (N) and, in the cell on the left, the posterior chloroplast complex with its compound pyrenoid (cPyr). (F) The posterior chloroplast complex, located immediately behind the nucleus (N), with many close-packed pyrenoids (cPyr), from which the individual chloroplasts extend. Three food vacuoles are also present (Va).

3.2.3. *Dinophysis acuminata*

Live cells were ca. 34 µm long and ca. 25 µm wide (Fig. 6A and B). The chloroplasts in starved cells were reduced to the poles of the cell (Fig. 6A), while they were larger in well-fed cells (Fig. 6B). In *D. acuminata*, the single dinoflagellate nucleus was located in the central–posterior part of the cell (Fig. 6A and B). The chloroplasts were arranged in two axial clusters (Fig. 6C), one in the anterior part of the cell, the other immediately behind the nucleus (Fig. 6A–E). Single chloroplast branches were visible in both cells (Fig. 6E), some in the area in front of the nucleus, others behind or along the sides of the nucleus. Each chloroplast had a terminal pyrenoid, and all pyrenoids congregated in a complex, compound pyrenoid (Fig. 6F, illustrating the posterior chloroplast complex). The number of pyrenoids appeared to be rather high, perhaps ca. 10 per cluster, and long, thin chloroplast branches extended from the pyrenoid into the cell or towards the cell periphery. Some of the branches were seen to merge distally (not illustrated). Thylakoids were absent in the pyrenoids but elsewhere in the chloroplasts were typically arranged in pairs (Fig. 6D). The lumen of each thylakoid in this figure was translucent, as opposed to the opaque lumen in the thylakoid in *M. rubrum* (Fig. 5E and F) and *T. amphioxeia* (Fig. 4B). Details of the chloroplast envelope and the pyrenoids (Fig. 7) were represented by the anterior pyrenoid complex (Fig. 7A). Each chloroplast was separated from the cytoplasm or from other chloroplasts by two membranes, but incomplete remains of a third membrane were sometimes visible in the space between the two complete membranes, especially in the area between the individual pyrenoids. In cases where three membranes were visible, the innermost two membranes were situated close together (Fig. 7B). The terminal position of each pyrenoid was visible in both Fig. 7B and C. Occasionally two chloroplast branches were seen to extend from the same pyrenoid.

Food vacuoles were commonly seen in the sections, thus three food vacuoles were visible in Fig. 6E. The contents of the vacuoles could not be identified. The internal parts of the peduncle (Fig. 8A–C) served in food uptake. The peduncle was large and comprised a band of ca. 100 microtubules (Fig. 8B) lined by vesicles with electron opaque content clearly visible in the transverse sections (Fig. 8B and C). The microtubular strand extended through a considerable part of the cell, and the anterior tip of the peduncle in

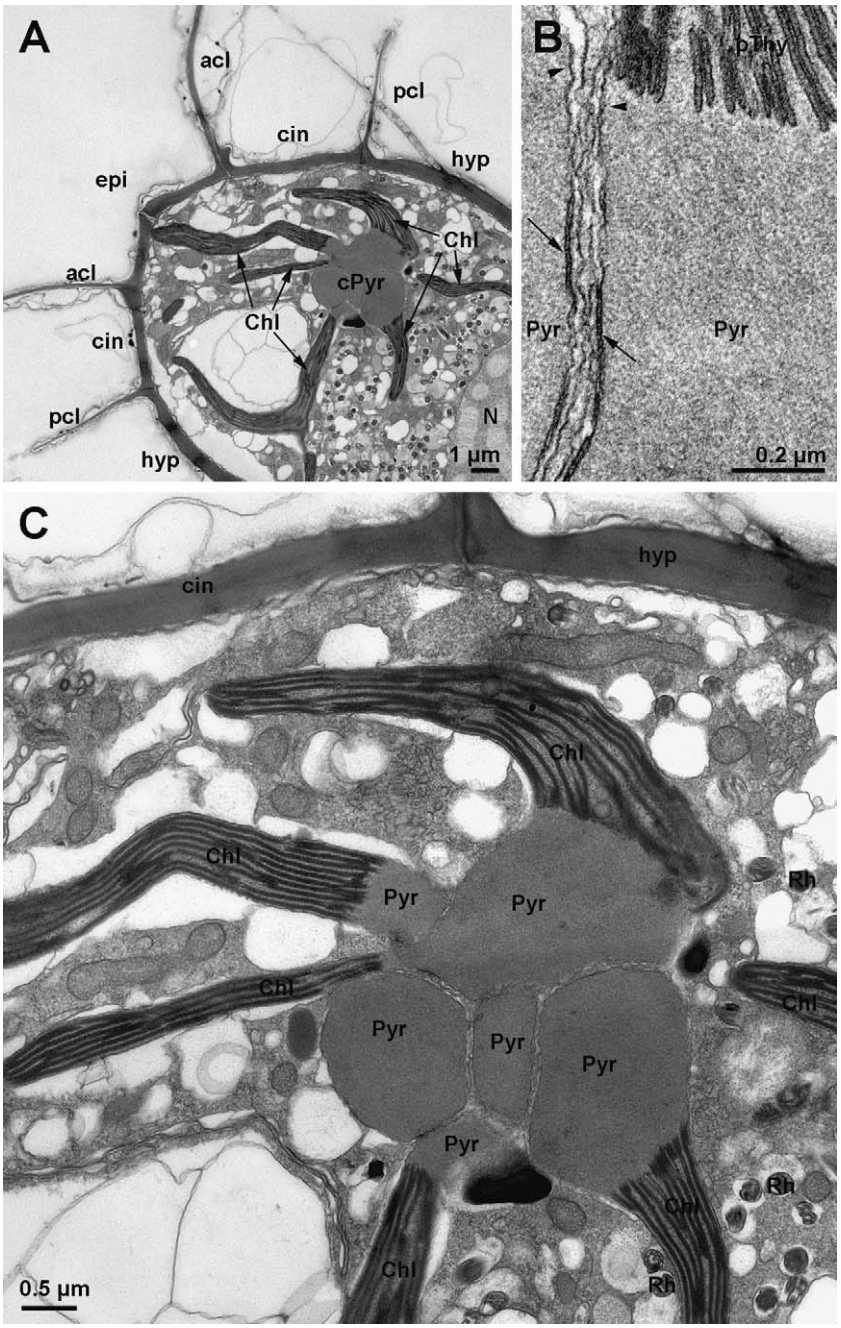

Fig. 7. *Dinophysis acuminata*, grown in culture. (A) The anterior end of the cell, illustrating part of the epicone (epi), cingulum (cin), anterior (acl), posterior cingular list (pcl) and hypocone (hyp). The anterior chloroplast complex is also visible (Chl) with its compound pyrenoid (cPyr) located between the epicone area and the nucleus (N). (B) Details of two pyrenoids (Pyr) illustrating the two or sometimes three membranes lining each chloroplast (arrow). Notice also the thylakoids in pairs (pThy). (C) The anterior chloroplast complex of micrograph (A) at higher magnification. Each pyrenoid (Pyr) has a terminal position within its chloroplast (Chl), in other words, the thylakoids terminate at the pyrenoid. The many opaque bodies are rhabdosomes (Rh).

Fig. 8A was retracted to a position just beneath the cell exterior. In the oblique section, one opaque vesicle extended alongside the microtubular ribbon (Fig. 8A).

4. Discussion

4.1. Dinoflagellate chloroplasts of cryptomonad origin

It has been documented that cryptophytes obtained their chloroplast by ingestion of a red alga in a secondary endosymbiosis (Douglas et al., 1991). The chloroplasts are quite unique from an evolutionary point of view by possessing the remnant of the red algal nucleus, the nucleomorph (Greenwood, 1974; Greenwood et al., 1977). The chloroplast of *T. amphioxeia* has a typical cryptomonad ultrastructure. It is delimited by four membranes, the two outer membranes surrounding the nucleomorph and the chloroplast, and the two inner ones surrounding the chloroplast. However, the thylakoid arrangement is unusual. Most cryptophytes have thylakoids in pairs while in the genus *Teleaulax*, these are assembled in triplets (Hill, 1991). The accessory photosynthetic pigments are located in the lumen of each thylakoid, which therefore appears more or less swollen.

During their evolutionary history, several groups of dinoflagellates independently ingested cryptomonads from which

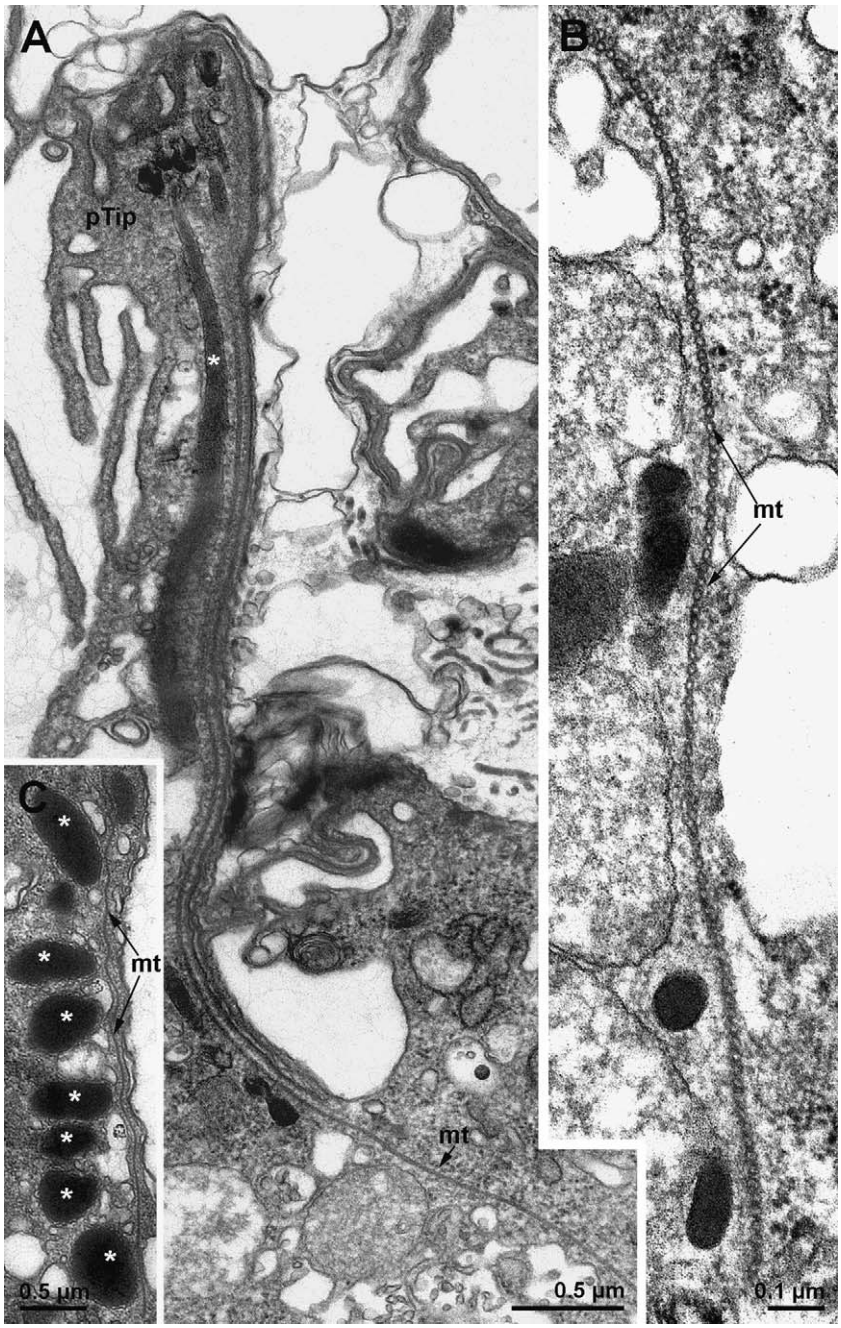

Fig. 8. *Dinophysis acuminata*, grown in culture. (A) The peduncle apparatus comprising a band of microtubules (mt), sectioned obliquely, and a electron-opaque vesicle (asterisk). The tip of the peduncle (pTip) is retracted to a position immediately beneath the amphiesma. (B) Detail of the peduncle apparatus comprising a band of ca 100 microtubules (mt) in transverse section. (C) Series of electron-opaque vesicles (asterisks) lining the microtubular band.

they obtained their chloroplast, establishing a tertiary endosymbiosis (Table 1). In most cases, it is not possible at this stage to judge the exact relationship between the host and the symbiont. However, several degrees of enslavement have been observed in three species whose ultrastructure has been studied. For instance, species such as *Amphidinium poecilochroum* Larsen and *Gymnodinium acidotum* Nygaard are examples of phagotrophic dinoflagellates with a transient cryptophyte symbiont in which little transformation has taken place (Wilcox and Wedemayer, 1984, 1985; Larsen, 1988). *G. acidotum* has been shown to ingest, retain and utilize a cryptophyte endosymbiont for up to ten days (Fields and Rhodes, 1991). The chloroplasts in both species are present within an endosymbiont together with the nucleus, the mitochondria and the nucleomorph of the

Table 1
Dinoflagellates with permanent or transient chloroplasts of cryptomonad origin, except *Dinophysis*.

Species	Reference
Amphidinium latum	Horiguchi and Pienaar (1992)
A. poecilochroum	Larsen (1988)
A. wigrense	Wilcox and Wedemayer (1985)
Amylax buxus	Koike and Takishita (2008)
A. triacantha	Koike and Takishita (2008)
Cryptoperidiniopsis sp.	Eriksen et al. (2002)
Gymnodinium acidotum	Wilcox and Wedemayer (1984, 1985), Fields and Rhodes (1991)
G. gracilentum	Skovgaard (1998)
G. eucyaneum	Hu et al. (1980)
Pfiesteria piscicida	Lewitus et al. (1999)

Table 2
List of publications on the chloroplast of *Dinophysis*.

Taxon	Reference
Dinophysis spp.[a,b]	Hallegraeff and Lucas (1988)
D. acuminata, *D. acuta*[a]	Schnepf and Elbrächter (1988)
Dinophysis norvegica[a]	Geider and Gunter (1989)
D. acuminata, *D. fortii*[a]	Lucas and Vesk (1990)
D. acuminata, *D. fortii*[a]	Vesk et al. (1996)
D.acuminata, *D. caudata*, *D. fortii*[a]	Hewes et al. (1998)
D. norvegica[a]	Meyer-Harms and Pollehne (1998)
Dinophysis sp.[a]	Schnepf and Elbrächter (1999)
D. acuminata, *D. fortii*, *D. norvegica*[a]	Takishita et al. (2002)
D. acuminata, *D. acuta*, *D. norvegica*[a]	Hackett et al. (2003)
Dinophysis spp.[a]	Janson (2004)
D. mitra[b]	Koike et al. (2005)
D. acuminata, *D. fortii*, *D. norvegica*, *D. tripos*[a]	Takahashi et al. (2005)
D. acuminata, *D. norvegica*, *Dinophysis* sp.[a]	Minnhagen and Janson (2006)
D. norvegica[a]	Minnhagen et al. (2008)
D. fortii[a]	Nagai et al. (2008)
Dinophysis infundibulus[a]	Nishitani et al. (2008b)
D. caudata[a]	Park et al. (2008)

[a] Cryptomonad origin.
[b] Non-cryptomonad origin.

cryptophyte, and are lined by five membranes, of which the innermost pair is close together (Wilcox and Wedemayer, 1985, Fig. 1; Larsen, 1988, Figs. 25 and 26). In the dinoflagellate *Amphidinium wigrense* Wołoszyńska, on the other hand, the original cryptomonad has become reduced almost beyond recognition (Wilcox and Wedemayer, 1985). It is surrounded by only three membranes, and a nucleomorph is absent. This is identical to the structure of the most common chloroplast among dinoflagellates, which contains peridinin as the main accessory (photosynthetically active) pigment. The peridinin chloroplasts are thought to have arisen by ingestion of a red alga to form an established (permanent) secondary endosymbiosis (Schnepf and Elbrächter, 1999). The red alga was transformed radically in the dinoflagellate ancestor. Thus, the chloroplasts are lined by three membranes, of which the innermost two are often located close or very close together. They are thought to originate from the cyanobacterium that was enslaved by the red alga in the primary endosymbiosis. The origin of the outermost, third, membrane is less certain as it may represent the food vacuole membrane of the host, or the plasmalemma of the red alga. It is somewhat separated from the inner pair of membranes. Therefore, it is likely that the chloroplast of *A. wigrense* is also a well-established permanent chloroplast.

4.2. The chloroplasts of Dinophysis

Most phototrophic *Dinophysis* species studied so far have chloroplasts of cryptophyte origin (Table 2). Since Park et al. (2006) established the first successful culture of *D. acuminata*, three other species have been cultured using *M. rubrum* as prey: *D. caudata* Kent (Nishitani et al., 2008a), *D. infundibulus* Schiller (Nishitani et al., 2008b) and *D. fortii* Pavillard (Nagai et al., 2008). These studies all lean towards the hypothesis that the chloroplast is transient and acquired from *M. rubrum*. The molecular data shown in this study do not contradict the previous published results (Takishita et al., 2002; Hackett et al., 2003; Janson, 2004; Takahashi et al., 2005; Minnhagen and Janson, 2006; Nagai et al., 2008; Nishitani et al., 2008b; Park et al., 2008). For the chloroplast-encoded tufA gene and the rDNA block, the sequences of the *D. acuminata* (Da-DK2007), *M. rubrum* (Mr-DK2007) and *T. amphioxeia* (SCCAP K-0434) are identical and group with other cryptophyte species. Even in the nmLSU rDNA, the temperate *M. rubrum* from both Korea and Denmark share the same sequence identity with *T. amphioxeia* from Denmark and the cryptophyte strain Cr-MAL01 from Korea. This shows a certain degree of conservation at the nucleomorph level between strains from different localities. Yet, we did not use the Korean strains for the studies of the chloroplast gene since it was important to establish the cell content before DNA extraction.

Another interesting aspect of these phylogenies is that *M. rubrum* from McMurdo Sound, Antarctica has the same sequence as *G. cryophila* from the same location. In 2006, Minnhagen and Janson published 16S rDNA sequences of *D. acuminata* from Greenland and another *Dinophysis* type 1 closely related to the sequences of *G. cryophila*. They suggested that these could have taken up their plastid from another source. Yet, since the cells were collected from a water sample, it is possible that they amplified chloroplast DNA located in a food vacuole and not the plastid. This case has been shown to happen in wild samples (Hackett et al., 2003). To avoid confusion, all *D. acuminata* amplifications should be cloned to discriminate between copies of a gene that could be located in different cell compartments.

Despite the fact that the three new markers point towards the same conclusion as previous ones, the ultrastructural evidence does not support the idea that the chloroplasts of *D. acuminata* are a result of kleptoplastidy (Table 3). Instead, it supports the conclusion reached early on by both Lucas and Vesk (1990) and Schnepf and Elbrächter (1988) that the chloroplasts are permanent organelles of cryptophyte origin. Moreover, our results show that the chloroplasts of *D. acuminata* differ from those of *M. rubrum* not only in having chloroplast thylakoids arranged in pairs as opposed to the triplets in both *M. rubrum* and *T. amphioxeia* but also in the polar position of the pyrenoid in each chloroplast. In other words, the thylakoids within each chloroplast terminate at the pyrenoid while in *T. amphioxeia* and *M. rubrum* the pyrenoids are located laterally, and the thylakoids usually bypass the pyrenoid.

The idea of the chloroplasts of *D. acuminata* being a recent acquisition is invalidated by the fact that each chloroplast is lined by only two complete membranes with remains of a third membrane. The latter is sometimes visible in the space between the two main ones as illustrated in the present paper (Fig. 7B). Such a partial third membrane was also observed in *D. acuta* Ehrenberg by Schnepf and Elbrächter (1999). It makes the chloroplasts unique among dinoflagellates. Moreover, it provides insight into the state of transformation of the chloroplasts. As mentioned earlier, all peridinin-containing chloroplasts as well as *A. wigrense* chloroplasts are lined by three membranes (Schnepf and Elbrächter, 1999). In our case, only two membranes remain, suggesting even

Table 3
Plastid characteristics of the chloroplast in study species.

Species	Pyrenoid structure	Pyrenoid position	Thylakoids	Nm	Plastid membranes	Plastid genes	Nm LSU genes
T. amphioxeia	Single	Lateral	In triplets	Present	Four	Identical	Identical
M. rubrum	Single	Lateral	In triplets	Present	Four (five)	Identical	Identical
D. acuminata	Compound	Terminal	In pairs	Absent	Two	Identical	Absent

Nm = nucleomorph; plastid genes = tufA and rDNA block.

further transformation of these chloroplasts. The close proximity of the inner membrane to the incomplete one indicates that these represent the original pair of cyanobacterial membranes. If the chloroplasts of *D. acuminata* were to be kleptochloroplasts, the chloroplast structure described above for *G. acidotum* and *A. poecilochroum* would be more probable.

Of the four *Dinophysis* cultures established so far, only Nagai et al. (2008) provided transmission micrographs of their isolates. The micrographs illustrate both 2- and 3-thylakoid lamellae in cells of *D. fortii*. In one figure (Fig. 3B in Nagai et al., 2008) the chloroplast illustrated is almost certainly the functional chloroplast of *D. fortii*, judged by the polar position of the pyrenoid. The thylakoids, said to be arranged in 3-thylakoid lamellae, may be interpreted as 2-thylakoid lamellae, although the very low magnification of the figure prevents any definite conclusions. This apparent confusion regarding the number of thylakoids can be explained by the differential swelling and staining of the lumen in thylakoids of cryptophytes depending on the fixation and the physiological state of the cell. As mentioned above, the swelling is thought to be due to the presence of phycobilins in the lumen. Fig. 3C in Nagai et al. (2008), on the other hand, resembles a chloroplast of the prey organism, *M. rubrum*, by having thylakoids arranged in groups of three. The pyrenoid is not visible but this chloroplast is most probably the result of a recent food uptake, prior to being digested by the host.

4.3. Some comments on the stellate chloroplasts in D. acuminata

One of the most striking differences between the chloroplasts of *D. acuminata* and *M. rubrum* is the arrangement of the pyrenoids. As mentioned previously, all terminally positioned pyrenoids group together forming two stellate compound chloroplasts in *D. acuminata*. This structure is found in many species of dinoflagellates (*Protoceratium reticulatum* (Claparède and Lachmann) Bütschli: Hansen et al., 1997; *Alexandrium catenella* (Whedon and Kofoid) Balech: Hansen and Moestrup, 1998, *Tovellia sanguinea* Moestrup et al.: Lindberg et al., 2005, *Baldinia anauniensis* G. Hansen and Daugbjerg: Hansen et al., 2007; *Hemidinium nasutum* Stein and *Cystodinium* sp.: Moestrup, unpublished observation) and also in other algal groups. Two chloroplast clusters, with pyrenoids identical to those of *D. acuminata*, occur in cells of the euglenoid flagellate *Eutreptiella eupharyngea* Moestrup and Norris (Walne et al., 1986). In nutrient-stressed cells of *Eutreptiella*, the stellate clusters of chloroplasts separate into single chloroplasts. It is not presently known whether this occurs also in nutrient-starved dinoflagellates. Yet if it is the case, it could explain the few reduced chloroplasts recorded in starved cells of *D. fortii* (Nagai et al., 2008).

The presence of the two stellate chloroplast complexes can also provide an alternative hypothesis to the conclusion reached by Minnhagen et al. (2008), who studied DNA replication in the nucleus and chloroplast of dividing (G2) and non-dividing (G1) cells of *D. norvegica* Claparède et Lachmann. Based on the absence of replication of the chloroplast DNA in dividing cells, Minnhagen et al. (2008) concluded that the chloroplast did not undertake division and had therefore to be taken from the environment. Yet, if each daughter cell receives one chloroplast cluster after division and replicates it immediately after, the chloroplast DNA content would not be significantly different.

4.4. Food vacuole content and peduncle

Despite the fact that the cells of *D. acuminata* were recently fed before fixation, no identifiable content was found in the food vacuoles (Fig. 6F), indicating that food is digested rapidly after uptake. Jacobson and Andersen (1994), however, illustrate and describe food vacuoles with visible, identifiable contents in *D. acuminata* and *D. norvegica*. Thus many food vacuoles contained cup-shaped starch-like grains resembling the starch grains surrounding the pyrenoid in cryptomonads, these were never accompanied by a chloroplast, showing a differential rate of digestion between starch and the chloroplast of the prey (*M. rubrum*).

Reconstruction of the peduncle was beyond the scope of the present paper and only a few comments will be given. The type of peduncle found in *Dinophysis* belongs to the most common type known in dinoflagellates (Hansen, 2001), comprising a microtubular ribbon that appears to provide structural support to the peduncle, and a large number of vesicles with electron-opaque contents, most likely containing material used during capturing or handling of prey. Jacobson and Andersen (1994) provided the first details of the peduncular system in dinophysioids. They found 95–165 microtubules in different species, lined on one side by an inconspicuous sheet of material. The presence of opaque vesicles was not mentioned, but a few opaque droplets are visible in some of the micrographs published. We have not observed the supporting sheet in *D. acuminata*. A full reconstruction of the path of the peduncle within the cell would be interesting for comparison with other dinoflagellates, including its position relative to the flagellar basal bodies and flagellar root system, which has never been examined.

4.5. Conclusion and future perspectives

There is a clear contradiction between ultrastructural data and molecular data. How can we explain the discrepancy? If we bring our attention to the two phylogenies obtained from chloroplast-encoded genes, no convincing resolution of the cryptophyte species was obtained. This illustrates the limitation of the chloroplast genes to infer phylogenies of closely related species. It is not unlikely that closely related species of cryptophytes may have very identical chloroplast genomes. Moreover, it seems unlikely that so much structural transformation of the chloroplasts would occur if these were only temporary. If indeed this happened, intermediate chloroplast clusters being formed or destroyed should be observed in some of the sections, which we never found. However, there must be a reason why *D. acuminata* cannot maintain growth in culture in the long run without being fed *M. rubrum* (Park et al., 2006; Kim et al., 2008; Nagai et al., 2008; Nishitani et al., 2008a,b; Riisgaard and Hansen, 2009). One of the possible explanations is the need for a growth factor or some other compound synthesized by the prey that *Dinophysis* needs to sustain growth. To resolve the opposition between ultrastructure and molecular data, several approaches are possible. It would be useful to find a more informative chloroplast marker or look at gene expression of the nucleomorph and chloroplast genes in both *T. amphioxeia* and *M. rubrum* that could have an effect on the growth and maintenance of *D. acuminata* chloroplasts. Moreover, it seems important to elucidate the origin of the symbiont in *Mesodinium* before extrapolating to *Dinophysis* (work in progress).

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