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

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**A B S T R A C T**

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 of *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.

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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 Veski, 1990) and pigment analyses (Hallegraeff and Lucas, 1988; Geider and Gunter, 1989; Veski 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 klepto-chloroplast 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; Minnihan 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.
2. Materials and methods

2.1. Cultures

A culture of the food organism for Dinophysis, the photosynthetic ciliate M. rubrum (Mr-DK2007), established from single cells isolated from surface seawater samples collected near Frederikshund, Denmark, during a bloom event on 17 April 2007. The cryptophyte T. amphioxea (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 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/2 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, Japan). 500 ng PCR product was air-dried overnight and sent to the manufacturer’s recommendations (Macherry Inc., town, state, USA). 500 ng PCR product was air-dried overnight and sent to the manufac

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 100%. The material was transferred to room temperature while in 96% ethanol and dehydration continued 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 Reynolds’ 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. amphioxea, M. rubrum from different locations (Denmark, Korea and Antarctic) and D. acuminata, and also on several other cryptophyte species to be included in the phylogenetic analyses: Cemignera cryphila Taylor et Lee (CCMP2564), Hansaia phi Deane (CCMP325), Proteomonas sulcata Hill and Wetherbee (CCMP331), Hemiselmis rufescens Hill (CCMP440), Hemiselmis tepida Lane and Archibald (CCMP4442), Chromonema vescsants 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 nmlSU3F (5′-GTG CCT 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, IDNA 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

![Phylogenetic tree](Fig. 1)

### 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.

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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,

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**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 (≤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/Dinophysys) 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 bangio-phycean 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-

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**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 (≥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...
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 \( \mu m \) long and ca. 14 \( \mu m \) 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.
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 congegated 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. 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...
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

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**Table 1**

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

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>Amphidinium latum</em></td>
<td>Horiguchi and Pienaar (1992)</td>
</tr>
<tr>
<td><em>A. poecilochroum</em></td>
<td>Larsen (1988)</td>
</tr>
<tr>
<td><em>A. wigrense</em></td>
<td>Wilcox and Wedemayer (1985)</td>
</tr>
<tr>
<td><em>Amylax buxus</em></td>
<td>Knoke and Takishita (2008)</td>
</tr>
<tr>
<td><em>A. triacantha</em></td>
<td>Knoke and Takishita (2008)</td>
</tr>
<tr>
<td>Cryptoperidiniopsis sp.</td>
<td>Eriksen et al. (2002)</td>
</tr>
<tr>
<td><em>G. gracilentum</em></td>
<td>Skovgaard (1998)</td>
</tr>
<tr>
<td><em>G. esoyameum</em></td>
<td>Hu et al. (1980)</td>
</tr>
<tr>
<td><em>Pfiesteria piscicida</em></td>
<td>Lewitus et al. (1999)</td>
</tr>
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</table>
likely that the chloroplast of separated from the inner pair of membranes. Therefore, it is less certain as it may represent the food vacuole membrane of the endosymbiosis. The origin of the outermost, third, membrane is cyanobacterium that was enslaved by the red alga in the primary or very close together. They are thought to originate from the membranes, of which the innermost two are often located close dinoflagellate ancestor. Thus, the chloroplasts are lined by three membranes (Schnepf and Elbrächter, 1999). In our case, only two membranes remain, suggesting even the pyrenoid.

The idea of the chloroplasts of 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 Dinophysis 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. wige rense chloroplasts are lined by three membranes (Schnepf and Elbrächter, 1999). In our case, only two membranes remain, suggesting even

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 Woloszyńska, 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 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 Dinophysis in 1999, the chloroplasts of Dinophysis have been cultured using established (permanent) secondary endosymbiosis (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>
<thead>
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<th>Taxon</th>
<th>Reference</th>
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<tbody>
<tr>
<td>D. acuminata, D. acuta</td>
<td>Schnepf and Elbrächter (1988)</td>
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<td>Dinophysis norvegica</td>
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<td>Hews et al. (1998)</td>
</tr>
<tr>
<td>Dinophysis sp.</td>
<td>Schnepf and Elbrächter (1999)</td>
</tr>
<tr>
<td>D. acuminata, D. fortii, D. norvegica</td>
<td>Takishita et al. (2002)</td>
</tr>
<tr>
<td>D. mitre</td>
<td>Koike et al. (2005)</td>
</tr>
<tr>
<td>D. acuminata, D. fortii</td>
<td>Takahashi et al. (2005)</td>
</tr>
<tr>
<td>D. norvegica, D. tripus</td>
<td>Minnhagen and Janson (2006)</td>
</tr>
<tr>
<td>D. acuminata, D. norvegica, Dinophysis sp.</td>
<td>Minnhagen et al. (2008)</td>
</tr>
<tr>
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<td>Nagai et al. (2008)</td>
</tr>
<tr>
<td>Dinophysis infundibularis</td>
<td>Nishitani et al. (2008b)</td>
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<tr>
<td>D. caudata</td>
<td>Park et al. (2008)</td>
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<table>
<thead>
<tr>
<th>Species</th>
<th>Pyrenoid structure</th>
<th>Pyrenoid position</th>
<th>Thylakoids</th>
<th>Nm</th>
<th>Plastid membranes</th>
<th>Plastid genes</th>
<th>Nm LSU genes</th>
</tr>
</thead>
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<tr>
<td>T. amphioxea</td>
<td>Single</td>
<td>Lateral</td>
<td>In triplets</td>
<td>Present</td>
<td>Four</td>
<td>Identical</td>
<td>Identical</td>
</tr>
<tr>
<td>M. rubrum</td>
<td>Single</td>
<td>Lateral</td>
<td>In triplets</td>
<td>Present</td>
<td>Four</td>
<td>Identical</td>
<td>Identical</td>
</tr>
<tr>
<td>D. acuminata</td>
<td>Compound</td>
<td>Terminal</td>
<td>In pairs</td>
<td>Absent</td>
<td>Two</td>
<td>Identical</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Nm = nucleomorph; plastid genes = tufA and rDNA block.
further transformation of these chloroplasts. The close proximity of the inner membrane to the incomplete ones 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 *Dinophysium* 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 rectilatum* (Claparède and Lachmann) Bütschli: Hansen et al., 1997; *Alexandrium catenella* (Whedon and Kofoid) Balech: Hansen and Moestrup, 1998, *Toxella sanguinea* Moestrup et al.: Lindberg et al., 2005, *Balantium 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 euplancygea* 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 *Dinophysium* 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 dinophysiods. 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 *Dinophysium* 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 *Dinophysium* (work in progress).

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